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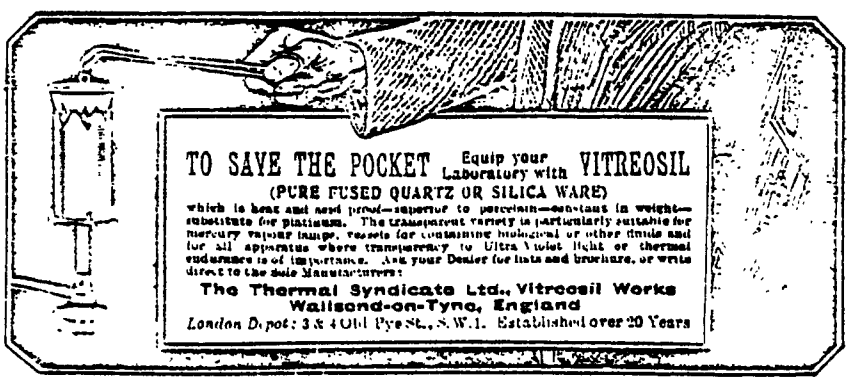
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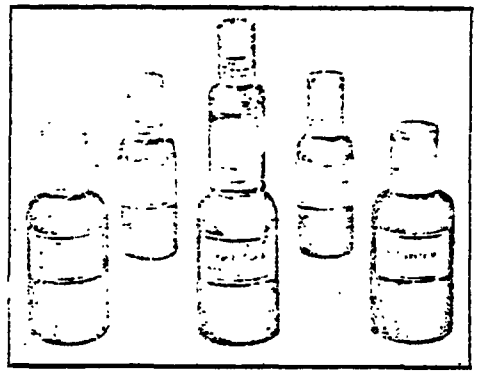
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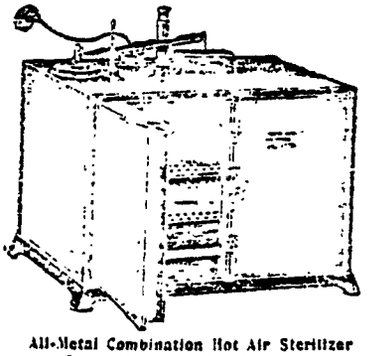
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FOCAL NECROSIS OF THE LIVER.

J. W. EDINGTON.
The University, Sheffield.

(PLATES I. AND II.)

WHILST investigating an endemic amongst guinea-pigs, which was due to *B. aertrycke* (*Mutton*), one of the chief pathological lesions noted was focal necrosis of the liver, showing as yellow opaque spots, usually about 0·5 mm. in diameter but varying from microscopic size to 5 to 6 mm. in diameter. The chief views regarding the causation of these foci are as follows:—

(1) The action of some toxic substance which kills the liver cells: the cellular infiltration and proliferation are of later occurrence (Frænkel and Simmonds, 1886; Reed, 1895).

(2) Vascular thrombosis:—

(*a*) The thrombosis is formed by an accumulation of large macrophages which by their degeneration produce fibrin thrombi (Mallory, 1898).

(*b*) The thrombi are hyaline and are formed from fused red blood corpuscles, the fusion being due to hæmagglutinins of bacterial origin (Pearce, 1904 and 1906; Pearce and Winne, 1904; Pease and Pearce, 1906).

(3) An accumulation of large macrophages which undergo early necrosis, the necrosis of the liver cells being a secondary phenomenon (MacCallum, 1921). Others explain the formation of these foci by a combination of some of the above views, *e.g.*, M'Crae and Klotz (1908) believe the lesions are due to the action of a diffusible chemical toxin combined with a vascular thrombosis.

In commencing the investigation of this condition, serial sections were cut of livers showing well-marked focal necrosis, so that the

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histological architecture of some of these areas could be carefully studied. The most suitable stain was found to be Leishman's, and the staining was so effected that the cytoplasm of the healthy liver cells was coloured a faint purple tint. In contrast to this, the cytoplasm of the dead or dying liver cells was stained a delicate pink. This change was an early indication of necrosis. Another early sign was that the nuclei of the affected cells stained but faintly and appeared as ghost-like forms. In all the focal necrotic areas examined from acute and subacute cases, necrotic liver cells were observed. In some, from very severe and rapidly fatal cases, this was the only change present. In the majority however of animals suffering from this disease, these focal areas contained numerous other cellular elements, chiefly large macrophages, though there were always a varying number of polymorphonuclear and small mononuclear cells to be found. These cells also showed necrosis, but they appeared much more resistant than the more highly specialised parenchymatous liver cells. In the more chronic cases the necrotic liver cells were entirely removed or absorbed and the other cellular elements left, a histological picture which is apt to give rise to the false impression that the infiltrating cells are of primary importance in the production of the focal areas. Later still, these infiltrating cells also disappeared, and there was sometimes a small amount of regeneration of the parenchymatous liver cells. Usually however the area originally occupied by the destroyed cells was filled by a little fibrous tissue, and by the encroachment of the adjacent liver tissue. The blood capillaries in the region of the focal areas often contained large numbers of white corpuscles which were chiefly of the large macrophage type. In some, definite thrombosis could be seen, but after a full histological examination the amount of vascular thrombosis appeared to be quite insufficient to account for the necrosis. Some proliferation of the capillary endothelial cells was also found. Organisms were always found scattered throughout the lesions, but never in any greater number than could be found in the adjacent liver tissue. Large clumps of the bacilli could occasionally be detected, but showed no special relation to the necrotic areas and produced no local cellular reaction.

In the portal spaces, the blood vessels and bile ducts usually appeared quite healthy. In some however the bile ducts, especially when small and near their origin and more particularly when draining an area of necrosis, showed catarrhal changes with proliferation and swelling of the lining epithelial cells. In the connective tissue around the bile ducts, and especially around those showing catarrh, marked cellular infiltration and proliferation were occasionally observed. These cells were similar to those found infiltrating the focal areas, but rarely showed any necrotic changes. They were intimately related to the lymph channels, the lining endothelium of which usually demonstrated well-marked proliferation. This type of lesion was noticed by Mallory

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FOCAL NECROSIS OF LIVER.

FIGS. 1–8 show the similarity between natural and experimental necrosis.

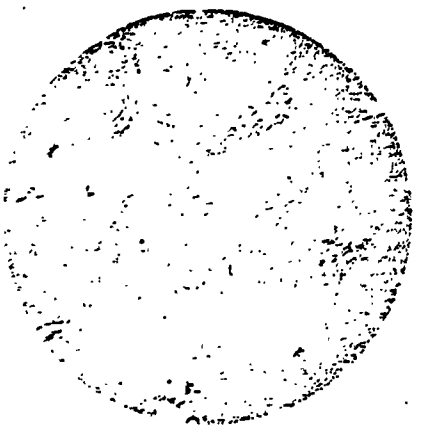


FIG. 1.—Early stage: natural infection. × 20.

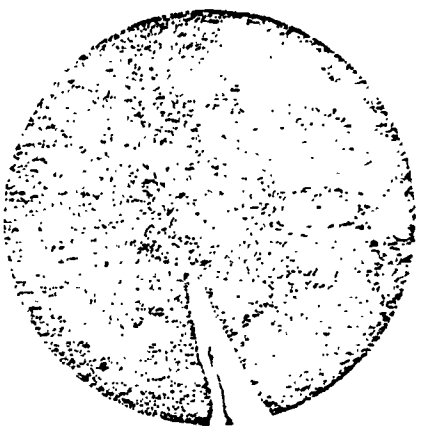


FIG. 2.—Early stage: experimental ligature of bile duct, *B. aertrycke* a week later, death in 18 hours. × 20.

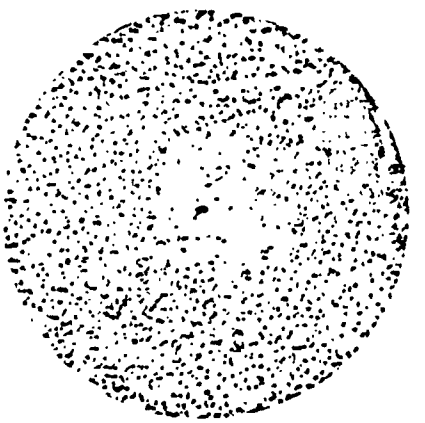


FIG. 3.—Same as Fig. 1: no infiltration. × 100.

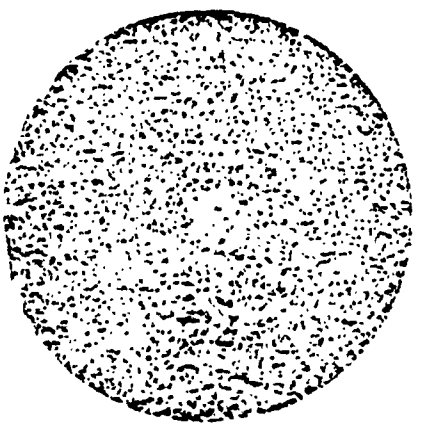


FIG. 4.—Same as Fig. 2. × 100.

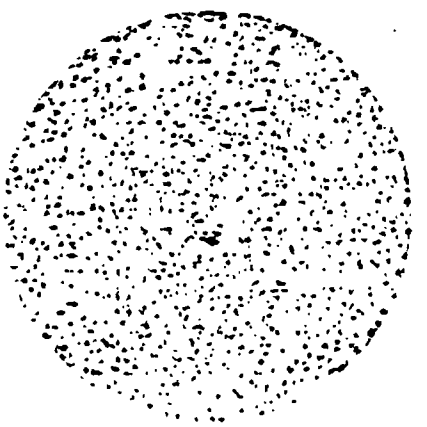


FIG. 5.—Commencing infiltration of necrotic focus: natural infection. × 100.



FIG. 6.—Commencing infiltration of necrotic focus: experimental ligature of bile duct, *B. diphtheriæ* subcut., death in 24 hours. × 100.

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(1898) in cases of human typhoid fever, and was considered by him to be due to the action of a toxin: according to MacCallum (1921) it is by no means a common feature in that disease.

In the smaller focal areas the various cells were thoroughly intermingled. In the larger areas the central portion consisted almost entirely of dead liver cells, surrounded by a wall of cellular elements similar in type to those found throughout the smaller foci. In some of these larger areas, especially when abutting on the peritoneal surface of the liver, abscess formation had taken place. This was characterised by softening of the necrotic centre and the presence in this of numerous disintegrating cellular elements, chiefly polymorphonuclear cells, and bacilli.

From these observations it appeared that the various vascular thrombosis theories concerning the production of focal necrosis in the liver were not tenable. The experiments of MacCallum (1921) in which he attempted and failed to produce similar lesions by the intravenous injection of corn starch into the portal vein are also against them. The remarkably efficient vascular circulation in the liver makes the production of such small necrotic areas by vascular thrombosis extremely unlikely. That thrombosed vessels are to be found in these foci is not to be denied nor indeed to be wondered at; though I do not think they are of primary importance in the production of these areas, there is no doubt that by deranging the circulation they interfere to a certain extent with the normal metabolism of the adjacent liver cells.

In all necrotic foci examined from acute and subacute cases, necrotic liver cells have always been found: in some, these were the only cells present. These observations are contrary to those of MacCallum (1921) on an early case of human typhoid fever. The histological findings thus tended to support the theory that the primary factor in the production of these focal areas was the destruction of the parenchymatous liver cells by some toxic substance, and that the cellular infiltration and proliferation occurred later. The question how the toxin picked out certain cells and not others, still remained to be answered.

The histological examination suggested the possibility that some change of, or interference with, the biliary secretion might be a localising factor. The idea was strengthened by the observation that the bile in the majority of cases of *B. acertryeke* infection in guinea-pigs was altered in consistence, being of a more viscid character than that found in the healthy animal. The following experiments were carried out on guinea-pigs in an attempt to elucidate this question.

Guinea-pigs were used which had never been infected with any organism of the *Salmonella* group, and were as nearly as possible of equal weight.

The animals to be operated on were anæsthetised by the open ether method. The hair was removed from the abdomen first by roughly clipping it and then applying a mixture of equal quantities of barium sulphide and starch powder to which sufficient water had been added to bring the whole to the consistence of a thick cream. The denuded area, after being washed with water, was swabbed first with methylated spirit and then with ether. Exercising rigid aseptic precautions, the abdomen was opened in the middle line for a distance of about two inches starting from the ensiform cartilage and proceeding in a caudal direction. The liver and stomach were gently separated, and a ligature was then carefully placed around the common bile duct about midway between its junction with the cystic duct and its entrance into the duodenum. Care was taken not to include any of the vessels in this ligature. The hepatic artery is in special danger of being accidentally ligated.

The greatest gentleness was also necessary when separating the liver from the stomach as the blood vessels in the gastro-hepatic omentum were found to be very fragile and easily ruptured.

When it was desired to drain the gall bladder externally, a guinea-pig in which that organ was distended with bile was selected. After carefully packing off the gall bladder from the rest of the abdominal cavity a fine rubber tube was introduced into it, and fixed there with a double purse-string suture. After removing the packing, any adjacent omentum was arranged around the point of entrance of the tube into the gall bladder. The tube was also fixed by means of sutures to the parietal peritoneum and to the skin.

The wound was closed with two layers of suturing; the first, including the peritoneum and muscle layers, being interrupted, and the second, taking in the skin alone, being continuous.

Fine linen thread or silk was used to ligate the common bile duct and to fix the tube in the gall bladder, No. 00 catgut for the interrupted suturing of the muscle and peritoneal layers, and No. 1 catgut for suturing the skin. The wound was dressed with a thin layer of cotton wool which was fixed to the skin with collodion. When the gall bladder was drained, the outer end of the tube was embedded in absorbent cotton wool which was fixed to the animal with strapping and frequently changed.

After the operation the animal was kept warm, and allowed to drink as much as it desired. Soft farinaceous food mixed with milk was also placed in its cage.

The dose of *B. aertrycke* had to be carefully determined by preliminary experiments. It was such that it killed guinea-pigs of about 300 grammes weight in from five to seven days. It was a saline emulsion of a 24-hours agar subculture of a recently isolated strain of *B. aertrycke*. The cultures were grown at 37° C.

The results were as follows:—

Animal 1: common bile duct ligated. Killed 24 hours after; its liver showed no areas of necrosis. The gall bladder and bile ducts were distended.

Animal 2: operation as in 1 but bile duct not ligated; subcutaneous dose of *B. aertrycke*. Killed after 24 hours; showed very little pathological change in its liver, the only thing abnormal being slight cloudy swelling of the parenchymatous liver cells.

Animal 3: subcutaneous *B. aertrycke* as in 2. Killed after 24 hours; its liver was similar to that of animal 2.

Animal 4: common bile duct ligated as in 1, subcutaneous *B. aertrycke* as in 2 and 3. Died 18 hours after its operation.

B. aertrycke was isolated from its heart blood. A definite acute enteritis and peritonitis were present. In numerous such experiments, ligation of the common bile duct always accelerated the course of the infection and hastened the death of the animal. On section, the liver showed numerous areas of focal necrosis, the contrast to what was found in the control animals being very striking. The liver cells elsewhere showed cloudy swelling. In the necrotic areas these cells were dead, and there was practically no cellular infiltration.

Animal 5: as 4 but gall bladder drained externally. Killed after 24 hours, and its liver showed the same histological picture as in animals 2 and 3.

A similar series of experiments was carried out using a virulent strain of *B. diphtheriae* instead of *B. aertrycke*. The dose was such that it killed guinea-pigs of about 300 grammes weight in from 4 to 7 days. A similar definite result was obtained. In this series guinea-pig 4 lived for 48 hours after the operation and showed numerous necrotic foci in its liver. These foci demonstrated commencing cellular infiltration. Diphtheria toxin was also used instead of the living cultures and similar results obtained with a dose which would kill guinea-pigs of about 300 grammes weight, in from 4 to 7 days. Satisfactory experiments could also be performed even when the dose of toxin was sublethal, provided that it was sufficient to produce definite symptoms of toxæmia. This was the most satisfactory method of demonstrating the more advanced types of focal necrosis, as by careful adjustment of the dose the focal areas could be produced and yet the animal would survive for many days. *B. enteritidis* (Gärtner) and streptococci have also been used in similar experiments, with similar results.

Amongst the controls with ligation of the common bile duct, a female animal well advanced in pregnancy was inadvertently used. It died four days after the operation and its liver showed well-marked focal necrosis. Careful cultural investigations failed to show any sign of infection in this animal.

Many of the above experiments were repeated, using animals which had had the ligation performed one or two weeks previous to the injection of the organism or toxin. Similar focal areas were produced though the longer period of ligation had in itself produced definite histological changes. These consisted of great dilatation, increased tortuosity and proliferation of the bile ducts, whilst, some weeks after ligation, proliferation of the fibrous tissue around the ducts became apparent. The liver cells at the periphery of the lobule showed atrophy, but actual necrosis was rarely observed. This necrosis, when present, was probably due to rupture of some of the small bile ducts with extravasation of the bile into the surrounding tissue.

In cases in which the dose of toxin or organismal emulsion was too

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great, huge areas of necrosis were found in the livers of those guinea-pigs in which the common bile duct had been ligated. The necrotic areas showed bile staining and the whole picture resembled the condition which has been described as *hepar necroticum cum ictero* (Curschmann 1899, Oertel 1904 and 1906, Weber 1909). Parkes Weber suggested in his paper that this disease is in all probability due firstly to some toxæmic condition producing the necrosis and secondly to increased bile pressure favouring the extravasation of bile into the necrosed areas.

From the above results it will be seen that, with appropriate dosage and time limit, neither organisms nor toxin nor the operation nor ligature of the common bile duct produced any necrotic foci in the liver. Animals treated like 4 however showed the marked effect of ligation of the common bile duct combined with a subcutaneous dose of bacillary emulsion or toxin: here necrotic foci in the liver were very numerous. When the gall bladder was drained externally, no such foci developed: the fact that the bile did not enter the intestine was thus not a factor in the production of the focal areas.

Recognising that the organisms in the *B. aertrycke* experiments were eliminated to a certain extent in the bile, it might have been thought that the prevention of their excretion was the cause of the necrosis. The facts that toxin alone (*e.g.* diphtheria toxin) produced similar results, and that in the histological examinations no special aggregation of organisms in the necrotic areas could be seen are much against this interpretation.

The question of the elimination of the toxins in the bile was next considered. The bile from about a dozen guinea-pigs dying from an infection produced by the subcutaneous inoculation of a virulent strain of *B. diphtheriae* was taken. An attempt was made to concentrate any toxin present by the pressure dialysis method of Walpole (1915) and Glenny and Walpole (1915). The precipitate obtained after acidulation was dissolved in a minimum amount of weak alkali, and then tested for toxicity by the guinea-pig intracutaneous test, suitable controls with antitoxin being always made. (The intracutaneous injection of normal bile produces definite necrosis; a similar injection of bile which had been treated by the pressure dialysis method, had no such effect.) No free toxin could ever be detected. This did not prove that the toxin was not eliminated in the bile, as it might have been present in some atoxic form due to a preceding chemical or physical change in, say, the liver cells. Indeed, according to Brunton and Bokenham (1905), the liver not only destroys the toxin of *B. diphtheriae* but excretes some antitoxin in the bile. The experimental work in connection with the last statement is unfortunately not at all convincing and is open to serious criticism. A more recent paper by E. B. Perry (1927) points out that it is exceedingly doubtful if antibodies are excreted in the bile, and that in any case the amounts excreted are minute.

The primary factor in the production of these focal necrotic areas appears to be the death of the parenchymatous liver cell. In toxic conditions it is obviously working at high pressure and is in a very

precarious and unstable state, as is shown by the frequent cloudy swelling and fatty change of its cytoplasm. Any factor therefore which interferes with its already strained metabolic activities will hinder its efforts to deal with the toxin, and by allowing this to accumulate will in the end indirectly cause its death. This interference with the cell metabolism is to be found in the slowing down, or complete cessation, of the bile excretion from certain areas of the liver. This may be assigned to three factors:—

(*a*) blocking of the intercellular bile capillaries by swelling of the adjacent liver cells (cloudy swelling, etc.):

(*b*) blocking of the small bile ducts due to catarrhal changes: this is characterised by swelling and proliferation of their lining epithelial cells:

(*c*) in some conditions, *e.g.* infection of guinea-pigs with *B. aertrycke*, the increased viscosity of the bile without doubt hinders its excretion.

The first is probably the chief cause, and accounts best for the production of the smaller necrotic foci. The second best explains the larger lesions such as *hepar necroticum cum ictero*.

Other factors which, though they appear of minor importance, may hinder cellular metabolism and thus indirectly help to produce cell death, are:—

(*a*) Thrombosis of the vascular capillaries; unimportant as a cause of focal necrosis.

(*b*) Interference with the lymph drainage. The lymph channels in the portal spaces were sometimes blocked with large macrophages, whilst their lining endothelium was swollen and undergoing active proliferation. Round these, there were focal accumulations of cells, and the whole pathological picture of the lesion was said by Mallory (1898) to be due to a toxin. This appears quite a feasible explanation of the condition as, when there is interference with the excretion of bile, other paths are sought for its removal. Thus it was shown by Vaughan Harley (1892) that when the bile ducts are ligated the bile does not pass directly into the blood stream, but does so indirectly *via* the lymphatics of the liver and the thoracic duct. These lesions, in relation to the lymph channels, were noticed in the histological examination to be most prominent in and around those vessels which drained necrotic areas. It is probable that the lymphatic vessels are here utilised to their full capacity to rid the liver of toxins and other products of cell death and cell metabolism.

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FOCAL NECROSIS OF LIVER.

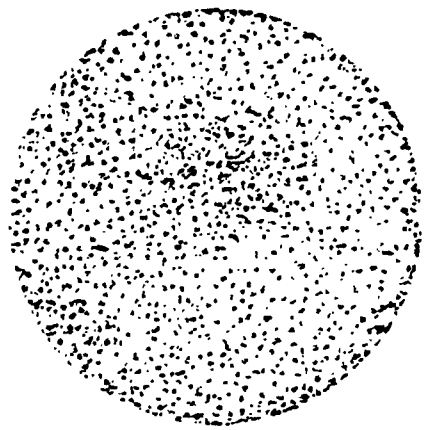


FIG. 7.—Advanced infiltration of necrotic focus: natural infection. × 100.

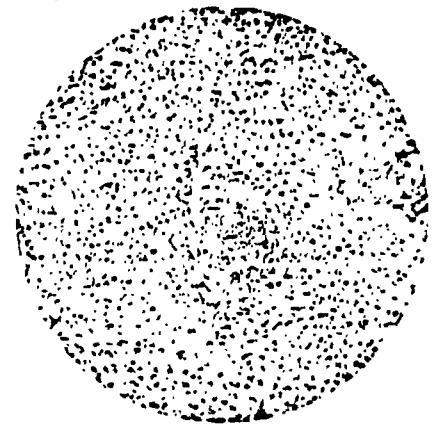


FIG. 8.—Advanced infiltration: experimental ligature of bile duct, diphtheria toxin subcut., death in 4 days. × 100.

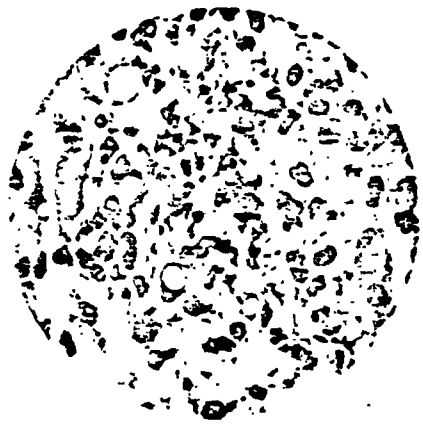


FIG. 9.—Necrosis of liver cells and infiltration with cells which also show early necrosis. × 500.

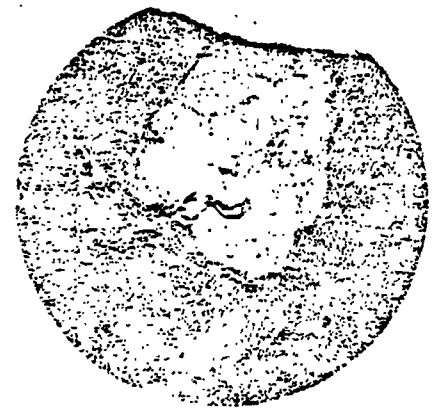


FIG. 10.—Large necrotic area: dead liver cells in centre, cellular infiltration round edge. × 20.



FIG. 11.—Abscess with commencing fibrous wall. × 20.

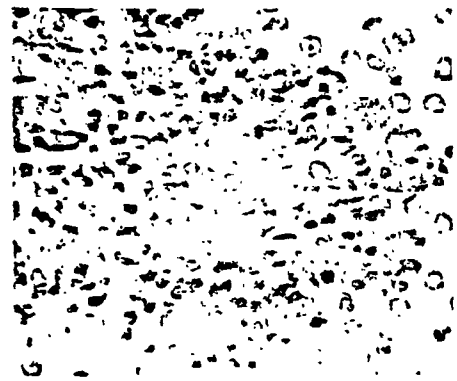


FIG. 12.—Small bile duct: epithelium swollen and proliferated, cellular infiltration around, specially related to lymphatics. × 450.

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SUMMARY.

1. The primary lesion in focal necrosis of the liver in infection with *B. aertrycke* is death of liver cells: the infiltration with macrophages is secondary.

2. Bile stasis is an important factor in localising the lesions.

I am greatly indebted to Dr J. H. Blakelock for the administration of numerous anæsthetics, and to Mr A. W. Collins for taking the photomicrographs.

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CHRONIC PEPTIC ULCER OF THE ŒSOPHAGUS.

M. J. Stewart and S. J. Hartfall.

From the Department of Pathology and Bacteriology, The University of Leeds.

(Plate III.)

As in the stomach and duodenum, peptic ulceration of the œsophagus may be met with in either the acute or chronic stage, the former not very infrequently, the latter only as a great rarity. Pringle Stewart and Teacher (1921) encountered 16 cases of acute peptic ulceration in the post-mortem room in the course of 7 years, the incidence being about 0·6 per cent. of all autopsies. The total number of reported cases of chronic ulcer, on the other hand, must be very small indeed. In Tileston's (1906) collected series of 44 cases of ulceration of the œsophagus only 9 can be regarded as definitely chronic. In an extensive though by no means complete survey of the literature we have found reports of 10 additional cases. The one here described is the first which we have encountered in over 10,000 autopsies performed during the last 18 years.

Morbid anatomy.

Chronic peptic ulcers of the œsophagus are limited to the lower half of the tube and most of them occur immediately above the cardiac orifice. Usually single, they vary in size from a tiny lesion a few millimetres in diameter to a large annular ulcer which may reach as high as the bifurcation of the trachea. Those of smaller size are round or oval, the larger tend to be irregular in form. The wall is penetrated to a variable depth, with exposure or actual perforation of the muscular coat, while the margins are clean cut and sometimes undermined. The chronicity of the lesion is chiefly indicated by the density of the fibrosis in the floor and margins of the ulcer, the fibrotic process radiating outwards into the surrounding mediastinal tissues. As in the more usual sites, peptic ulcers of the œsophagus may heal completely, or they may cause death by perforating into the pleura or by laying open a large blood vessel. In healing they are particularly liable to cause cicatricial stenosis, especially if the ulcer has been large and very chronic. In the case which we describe there was a well-healed scar in addition to the active ulcer, but it had apparently produced little if any deformity.

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Of 20 cases coming to autopsy, the ulcer was the actual cause of death in 14. In 2 there was fatal hæmorrhage from erosion of the aorta and in 1 from erosion of a small artery. In 4 there was perforation into the pleural sac and in 2 an ulcer situated in the abdominal part of the œsophagus opened into the lesser sac of the peritoneum. In 1 each perforation into bronchus, lung and pericardium had occurred, and in two cases death was due to inanition caused by œsophageal obstruction.

The development of carcinoma of the œsophagus in the scar of an old peptic ulcer has been described by Ortmann (1901), and Tileston (1906) states that he has seen a similar specimen in Vienna.

Pathogenesis.

The pathogenic factor of prime importance in acute ulcer is the digestive action of regurgitated stomach contents, aided in many cases by lowered tissue vitality (Pringle *et al.*). In practice there is no dividing line between acute peptic ulceration and so-called antemortem digestion of the œsophagus; such differences as exist are probably dependent merely on the time factor. The association with diseases which are accompanied by vomiting, and especially with gastric and duodenal ulcer, is remarkable. In Tileston's series of 44 cases, which includes both acute and chronic ulcers, there were 13 with accompanying lesions, ulcers or scars, in one or other of these viscera, and in 10 of the cases in this group there was pyloric or duodenal stenosis. Cases seen in the post-mortem room have usually died of the primary disease, but occasionally an acute ulcer of the œsophagus is the proximate cause of death, whether from perforation or from erosion of a large vessel. It is a reasonable conclusion from Pringle Stewart and Teacher's findings that a certain number of acute cases recover and heal completely, and it is obvious that only a very small proportion of the whole ever enter upon the chronic phase. The factors chiefly responsible for this latter development are the continued regurgitation of stomach contents, the local devitalising effect of muscular spasm and the irritation by food of the surface of the ulcer.

A very interesting feature of the case here reported was the presence of two large patches of gastric mucous membrane in the upper œsophagus just below the level of the cricoid cartilage. These consisted of fundal type mucous membrane, with abundant oxyntic cells, and there can be little doubt that they were capable of secreting acid gastric juice. Gastric heterotopia in the upper œsophagus is by no means rare (Schridde 1904, Taylor 1927), but the misplaced islets of mucous membrane are usually small. Ordinarily the secretion from those glands must be rapidly diluted and neutralised by saliva and carried onwards into the stomach. It is possible however that if acute peptic ulceration of the œsophagus should occur in a person with extensive gastric heterotopia the spasm of the cardia which is liable to

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be set up may lead to the accumulation of considerable quantities of acid secretion above the closed orifice. This in turn may play a part in delaying the healing of the ulcer. In two other published cases (Fränkel 1899 and Tileston 1906) areas of gastric heterotopia were present.

Clinical aspects.

In order to analyse the symptomatology of this disease we have selected 8 undoubted examples of chronic ulcer from the cases collected by Tileston (numbers 5, 6, 10, 24, 27, 28, 33 and 41 of his series) and have added thereto our present case and 10 others from the literature (Barclay 1915, Christopherson 1917, de Lavergne 1921, Finlayson 1883, Fleischner 1925, Lindsay 1899, Miller 1912 and 1917, Robertson 1884 and Trotter 1850-52). Three of the latter belong to the category of latent ulcer, in that while the symptoms were of short duration (3 to 8 days), the lesion found post-mortem was regarded as definitely chronic. Of the remaining 16 pain, substernal or epigastric, was present in 15 (there is no mention of pain in Finlayson's case), dysphagia and hæmatemesis in 11 each. In three of the cases with hæmatemesis the bleeding was profuse, a terminal event from perforation of a large vessel. Including these, the three cardinal symptoms were present together in 7 of the 16 cases.

(1) The pain, sometimes slight but more often severe, comes on either in the later stages of the act of swallowing or immediately thereafter, contrasting with its rather later onset in gastric ulcer. Its severity may be such that the patient may be afraid to take food of any kind. Often definitely paroxysmal, its usual site is deep beneath the lower end of the sternum or high in the epigastrium, where there may be tenderness on pressure. Occasionally it radiates to the back, between the shoulder blades. As can be demonstrated radiologically (Barclay 1915) the pain is due to muscular spasm resulting from the passage of irritating food particles over the surface of the ulcer.

(2) Dysphagia in the earlier stages of the disease is also due to spasm, the obstruction to the passage of food passing off after a few minutes. Later the spasm may be more abiding and severe, leading to actual regurgitation, while in the stage of cicatricial stenosis the obstruction is continuous and the patient rapidly emaciates.

(3) Sudden fatal hæmorrhage, when it occurs, is due to the ulceration involving a large blood vessel, usually the aorta. More often there is moderate intermittent hæmatemesis of the "coffee grounds" variety. In the case reported by Zahn (1882) repeated hæmorrhages had so degraded the quality of the blood that the clinical diagnosis was one of pernicious anæmia.

Perforation of an œsophageal ulcer is usually rapidly fatal. In two cases in which the aorta was opened into, one died immediately, the other of a second hæmorrhage 24 hours after the first. In a case

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in which a small vessel was implicated death occurred 48 hours from the commencement of the hæmorrhage. Perforation into the pleura is usually fatal in from 3 to 6 days, and is associated either with pleural effusion or a pyopneumothorax. In a case reported by Lindsay (1899) perforation led first to the formation of a mediastinal abscess which in turn ruptured into the lung: death occurred 37 hours after the first onset of acute symptoms. In Finlayson's (1883) case, on the other hand, a perforation into the left bronchus had probably been in existence for some weeks before the patient succumbed to gangrene of the lung. In Trotter's (1850-52) case rupture into the pericardium was fatal in 24 hours.

Apart from "latent" cases, which give a history of a few days only, the duration of definite symptoms ranges from a few weeks to many months. Reher's (1885) case is exceptional in that symptoms had been present for four years.

In typical cases, the diagnosis may be made with considerable certainty from the clinical signs and symptoms. The X-rays may show nothing abnormal, but if the examination is made during an attack of pain the passage of food is seen to be obstructed at the lower end of the œsophagus. The diagnosis may be confirmed with the œsophagoscope, but the risk of confounding simple and malignant ulceration must be considerable.

Report of case.

The patient, a man of 53 at the time of his death, had enjoyed good health until his 44th year, when he began to have flatulence, with occasional fullness and discomfort about half an hour after meals. Five months before his death he developed a dull nagging pain in the epigastrium, with radiation to the right scapular region. The pain came on almost immediately after meals and was sometimes so severe that he made himself vomit in order to obtain relief. At first the attacks were irregular and intermittent, occurring about twice a week, but on 30th August 1928 the pain became continuous and presently he vomited about half a pint of blood. There were several further slight attacks of hæmatemesis and on 3rd September he was admitted to hospital as a case of chronic gastric ulcer. He was placed on appropriate dietetic and medicinal treatment but failed to obtain much relief. Continuous pain was present in the region of the xiphisternum, always worse a few minutes after food, and he also made some complaint of a feeling as if the food stuck in his gullet. There was persistent, almost daily vomiting of small quantities of altered blood. The vomiting was noticeably effortless; a small quantity of coffee grounds material would "rise in the throat" and be expectorated. Melæna in small amount was present daily.

There were many elements of uncertainty in the case and the possibility of a chest origin for the hæmorrhage was considered despite the absence of physical signs. X-ray examination of the chest showed no evidence of pathological change, either in the lungs or mediastina. On account of a strong alcoholic history the question of cirrhosis of the liver was considered, only to be rejected. At 9 A.M. on 13th October the patient was observed to have developed acute respiratory distress: he was orthopnœic and breathing rapidly, and complained of pain over the lower part of the chest on the right side. Examination revealed rales all over

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S. HARTFALL

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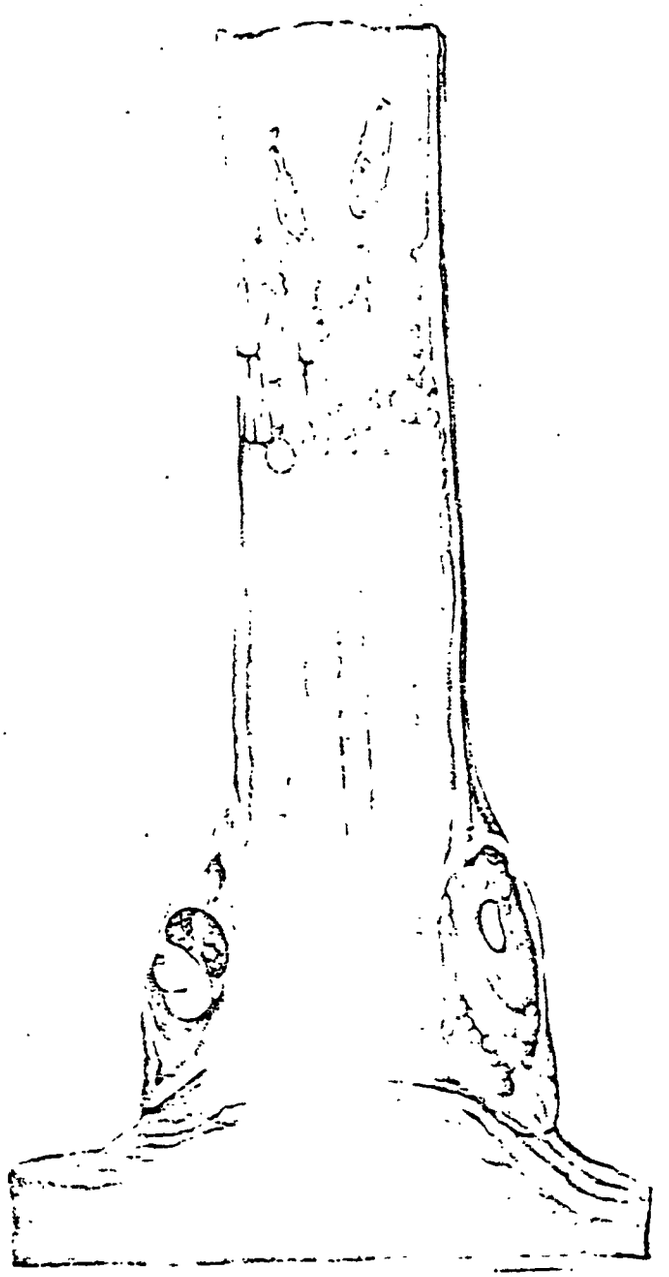
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CHRONIC PEPTIC ULCER OF ŒSOPHAGUS.



The œsophagus has been laid open along the middle of its posterior wall, with the result that the ulcer, situated immediately above the cardia, has been divided into two almost equal halves. In the left half, two lymph glands, the upper of which is anthracotic, protrude from the ulcer floor. In the upper part of the right half there is a perforation which communicated with the right pleural sac. On the anterior wall at approximately the same level as the ulcer there is a well-healed oval scar. The lower three-fifths of the œsophagus has been denuded of its epithelium by peptic (post-mortem) digestion. Near the upper end two elongated patches of heterotopic gastric mucous membrane are seen. (Slightly reduced.)

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the right lung with a slight degree of dullness at the base. This increased rapidly, and later in the day it became obvious that there was a large fluid effusion in the right pleural sac, but the patient's condition was such that interference was considered inadvisable. He died at 10 P.M.

At autopsy, there was a large simple chronic ulcer 30 mm. by 44 mm. in diameter immediately above the line of the gastro-œsophageal junction. It was situated mainly on the posterior wall but extended on to both lateral walls of the œsophagus, which was here much dilated, though slightly narrowed at the upper level of the lesion. On the anterior wall, exactly opposite the centre of the ulcer, there was a large, oval, well-healed scar. The open ulcer was callous and indolent, with rather flattened margins and a smooth densely fibrous floor, and there was much fibrosis of the surrounding mediastinal tissues. Two lymph glands, one of them anthracotic, protruded from the floor near the left margin of the ulcer. From a point a little above the centre of the right lateral portion of the ulcer floor a perforation about 1 cm. in length led through densely sclerotic tissue to the right pleural sac, which contained several pints of greenish yellow sero-purulent fluid. The rest of the œsophagus above the level of the ulcer was slightly hypertrophied and dilated and in its upper part, just below the level of the cricoid cartilage, there were two large symmetrically disposed areas of heterotopic gastric mucosa, each 25 mm. in length and 5 and 7 mm. in breadth respectively.

The other features of note in the case were a chronically inflamed gall-bladder with thickened walls firmly contracted down round a number of facetted gall-stones, early patchy fat necrosis in relation to the pancreas, and old degenerative thickening and partial fusion of the aortic cusps.

Histological examination.

Unfortunately the greater part of the specimen had been much damaged by post-mortem peptic digestion, which had completely denuded the lower three-fifths of the organ of both its mucosa and submucosa. The muscular coat was still intact, except where it had been destroyed by ulceration, and the upper two-fifths of the organ was well preserved.

(1) The ulcer had the usual characters of the chronic peptic lesion as seen elsewhere. The floor consisted of three zones: (*a*) a narrow, strongly eosinophilic, inner zone of fibrinoid necrosis (*b*) a granulation tissue zone which gradually merged into (*c*) an outer fibrous tissue zone. From the latter, broad fibrous bands radiated outwards into the surrounding mediastinal tissues. Many nerve trunks were implicated in the deeper zones of the fibrotic process and a few of them extended through the whole thickness of the ulcer floor to the zone of fibrinoid necrosis. The ulcer floor was highly vascular, and there were many small punctiform hæmorrhages within it. The muscular coat was completely breached, its severed ends inclining gently upwards to terminate in the floor close to the ulcer margins. The two lymph glands which projected into the left half of the ulcer floor were embedded in fibrous tissue, though not themselves fibrosed. One of them was heavily anthracotic, and much pigment was present also in the surrounding fibrous tissue and in the superficial fibrinoid zone covering the gland. In one portion of the floor a medium-sized artery had been laid open, but its lumen was now wholly occupied by an oldish fibrinous plug in process of organisation. Elsewhere other smaller arteries opened in the floor but were mostly plugged by recent thrombus or blood clot.

(2) The scar was composed of somewhat similar fibrous tissue to that seen in the deeper layers of the ulcer, but it was denser and there was definite obliterative endarteritis. The muscular coat thinned out at the margins, but owing to post-mortem digestion the whole of the mucous membrane and submucosa had

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disappeared. Again there was extensive involvement of nerve trunks in the outer part of the lesion.

(3) The two patches of gastric heterotopia in the upper œsophagus were composed of glands of fundal type with numerous oxyntic cells, and a well-formed muscularis mucosæ continuous with that beneath the surrounding squamous epithelium. The muscular coat was composed mainly of striped muscle but at one place there were considerable masses of unstriped muscle internal to and to some extent intermingled with it.

(4) The parietal pleura in continuity with the perforation was the seat of an acute inflammatory reaction. It was clothed by a broad band of fibrinous exudate, in the meshes of which were many cells in various stages of necrosis. Beneath this, and confined to a comparatively narrow zone, were many recently desquamated and well-stained pleural endothelial cells, together with numerous polymorphonuclear leucocytes, lymphocytes and plasma cells. The underlying adipose tissue showed a slight degree of similar infiltration.

Summary.

A case of chronic peptic ulcer of the lower end of the œsophagus is reported. The chief symptoms were high epigastric pain of five months' duration and recurrent hæmatemesis for a fortnight: dysphagia was not a prominent system. Death was due to perforation of the ulcer into the right pleural sac. An interesting feature of the case was the presence in the upper part of the œsophagus of two large patches of heterotopic gastric mucous membrane of fundal type: the possible relationship of this developmental abnormality to chronic peptic ulceration of the œsophagus is discussed.

We have pleasure in acknowledging our indebtedness to Dr G. W. Watson, who has allowed us to make full use of the notes of this case.

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"SMOOTH-ROUGH" VARIATION IN BACTERIA IN ITS RELATION TO BACTERIOPHAGE.

F. M. Burnet, Beit Memorial Research Fellow.

From the Bacteriological Department, Lister Institute.

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1. Introduction.

The recent publication by Hadley (1927, 1928) of two monographs which give an exhaustive review of the related fields of bacterial variation and bacteriophage phenomena makes it hardly necessary to attempt any review of the scattered literature here. The two papers mentioned contain extensive bibliographies and deal very fully with smooth-rough variation in general and in relation to resistance developed against bacteriophage. It will suffice, therefore, to state the main conclusions derived by Hadley from his review of the literature and to indicate their relation to the present work. He has made it clear that "resistant cultures" derived from normal strains are usually more or less outspokenly rough and are often completely identical with similar variants produced in the entire absence of bacteriophage. Hadley tends to stress the association of roughness with resistance more than seems justified from my experience. On the whole, phages lysing rough strains are more readily obtained than those which attack the normal smooth forms and it will be shown that amongst the resistant forms produced by lysis of a rough strain normal smooth types may occur.

Hadley endeavours to correlate the various phenomena of lysis and resistance by a theory which regards bacteriophage as a "cyclostage," forming part of a complex bacterial life-history, the normal smooth and rough forms being two other stages. The action of the phage, on

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this view, is to provoke bacteria to adopt a different form of existence. In most cases the alternative chosen is that of ultramicroscopic units—bacteriophage particles—but a certain proportion give rise to rough variants. He regards the production of these rough forms as due to the direct activity of the phage but is careful to stress that phage is only one of a number of agents that may provoke the change.

The work to be described is in several respects definitely opposed to these conceptions, but there can be no question that Hadley's work has been of the greatest value in directing attention to those aspects of bacteriophage phenomena which are practically neglected in d'Herelle's last monograph.

2. Scope of the present work and methods employed.

The present work is concerned with the part played by "smooth-rough" variation in strains of *B. enteritidis Gärtner* [*Salmonella enteritidis*] and some other intestinal bacilli in determining resistance or sensitivity to phage. The primary intention was to study the significance of bacteriophage phenomena in experimental infections, and *B. enteritidis* was chosen as a suitable infective agent for laboratory animals.

Preliminary work which has already been published (Burnet, 1927) showed that phages active against *B. enteritidis* were regularly active against corresponding phases of the other *Salmonella* species which possess the same or very similar heat-stable antigenic structure, viz. *B. pullorum* [*Salmonella pullorum*], *B. sanguinarium* [*Salmonella gallinarum*] and *B. typhosus* [*Eberthella typhi*] (Gruschka 1920, Bruce White 1925, Goyle 1926). The striking difference between the smooth and rough phases of a given strain in their behaviour toward phage and its apparent dependence on the nature of the heat-stable bacterial antigens suggested a study of "resistant cultures" from the point of view of their heat-stable antigenic structure.

Most of the experimental data given are concerned with (*A*) the qualitative sensitivity of bacterial strains (normal and variant) to a series of bacteriophages; and (*B*) the degree of "roughness" of the same strains as judged primarily by agglutination of suitably treated emulsions with pure "smooth" and pure "rough" antisera, but also by cultural appearances and salt stability.

A. The phages were obtained from any available source. Of those used 11 and 13 were derived from a phage received from Dr L. N. Asheshov, and 29 was obtained from Professor C. H. Browning. Numbers 8, 33, 31, 32, 34 and 35 were derived from the intestinal contents of infected or normal rats, 1, 12, 20 and 25 from lysogenic *B. enteritidis* cultures and 18 from normal fowl fæces. All phages were developed from single plaques, re-isolation being carried out if there were evident variation in the type of plaque developed and also in most cases whenever fresh stocks of phage were being prepared. As far as possible experiments with any particular strain were carried out with phages

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grown on the S or R forms of that strain, but for routine use the strain with which lysis appeared to be most complete was used as the substrate.

The phages when purified by successive isolation from single plaques were classified according to Bail's method by examining the qualitative sensitivity to phage of the resistant cultures produced with each phage. Details need not be given here as they will be found incidentally in various protocols below. No two of the phages used were completely identical but as regards their power of provoking resistance four groups could be distinguished comprising:—(A) phages 1, 12 and 33; (B) phages 8, 13, 28, 31, 34 and 38; (C) phages 20, 25, 35 and 32; (D) phages 11 and 13. Groups C and D were evidently closely related, but occasionally bacterial variants were encountered that had developed a resistance to phages of group D while remaining sensitive to those in group C. Group B was apparently heterogeneous but the six phages always reacted similarly in their resistance-provoking power. This group is analysed in detail in one of the concluding sections of the paper. As regards type of plaque, phages 11 and 13 gave very large plaques up to 8 mm. in diameter on 1·2 per cent. agar and phage 18 gave moderately large plaques with a characteristic halo. The phages of group A produced medium sized plaques usually showing a surrounding translucent halo. The others were "small hole" phages nearly always showing sharply cut edges to the plaques, though this character varied a little when different organisms were used as substrates.

The sensitivity of bacterial strains to phage was tested on agar. For most of the tests a diluted agar 1·2 per cent. was used containing a little gentian violet (approximately 1:10,000 of a saturated aqueous solution) to minimise air contamination during manipulations of the plate. Many "smooth" strains of *B. enteritidis* gave a moist growth on 1·2 per cent. agar obscuring the evidence of phage action and for these ordinary 2 per cent. nutrient agar was used. The plate was spread with a young broth culture and the surface dried in the incubator. A small loopful of each phage (undiluted) was then spread over small numbered areas of the plate and the result read after incubation overnight. The results in the tables are expressed according to the number of plaques appearing with each loopful, and the relative amount of secondary growth. In the vast majority of cases lysis, if it occurred at all, was uniform over the area (+ + +): in a few instances semiconfluent (+ +) or discrete (+) plaques were present. Uniform lysis without secondary colonies or with only a few isolated ones is reported as (+ + ±), with many discrete colonies (+ + ±) and with a continuous layer of secondary growth covering the lysed area (± ± ±).

It is recognised that lysis on agar may not signify that multiplication of the phage will occur with the same strain in broth culture. One or two instances of this will be noted in the present paper. It also happens occasionally that a phage with a weak action on some heterologous species may give irregular results when the test is repeated. In the great majority of tests clean cut results which could always be repeated were obtained and the method is sufficient to characterise clearly the groups of variants in their relation to bacteriophage.

B. The antisera used were obtained by immunising with a smooth (*Wallasey*) and a rough (127 R) strain of *B. enteritidis Gärtner*. Both sera contained H agglutinins to a titre of approximately 20,000. They also contained agglutinins corresponding to the respective heat-stable constituents of the strains used. When their titres were estimated against emulsions treated with alcohol according to Bruce White's method, the limit of macroscopically visible agglutination was at a dilution of about 1:2560 in both cases for the homologous emulsion. Emulsions prepared from typical cultures in the opposite (S or R) phase were not agglutinated between 1,80 and 1,5120. In some cases, when non-motile variants lacking H agglutinogen were being studied, untreated emulsions in dilute saline were used and the titres were then higher, up to 10,240. When rough

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strains were used in this way non-specific agglutination by normal rabbit serum frequently occurred. Alcohol treated emulsions do not show this agglutination (Bruce White) and for this reason they were preferred for general use.

The salt stability of the strains was tested by adding one drop of a thick suspension in distilled water prepared from a young agar culture to tubes containing increasing percentages of sodium chloride. The lowest concentration showing agglutination after incubation overnight at 37° was taken as the precipitating saline concentration (P.S.C.).

Throughout this paper the terms rough (R) and smooth (S) will be used with reference to the antigenic structure of the culture rather than to the actual appearance of colonies on agar. Hadley's suggestions as to nomenclature of the different bacillary antigens will be followed, H denoting the heat-labile flagellar antigen, O the heatstable antigen of normal smooth forms and R that of typical rough variants.

3. The sensitivity to phage of normally occurring S and R forms.

The sensitivity to phage of a series of normal strains of *B. enteritidis* and related organisms in both S and R phases has been reported previously and it is only necessary to present here a table showing the results obtained with a more extensive series of bacteriophages. The rough strains used were derived by the ordinary method of isolation from old broth cultures without the use of phage (table I).

The chief facts to be noted in this table are:—

(*a*) Phages, 1, 12, 33 lyse smooth forms only.

(*b*) Phages 18, 35, 11, 13 are active against rough types only.

(*c*) Phages 8 and 34 lyse all normally occurring variants.

The other phages may show individual variations but on the whole lyse rough forms actively and have only a weak action on smooth bacteria. Two phages, 32 and 38, act only on phases of *B. sanguinarium* and are omitted from most of the later tables.

The results make it clear that another criterion of "roughness" is available in the characteristically different range of sensitivity to phage of the two phases.

4. The antigenic changes in bacteriophage-resistant variants.

Although the subject is extensively discussed in Hadley's (1928) monograph, practically no experimental observations appear to have been published in which the antigenic nature of phage-resistant variants is studied in the light of recent conceptions of the agglutinogenic structure of bacteria. The work of Bruce White on the *Salmonella* group has resulted in a very clear picture of the antigenic nature of these organisms and supplies a basis for the serological study of the variants produced by phage.

In the course of the present work a large number of bacteriophage-resistant forms has been produced from *enteritidis* and *sanguinarium* strains and an attempt has been made to correlate their serological behaviour with their reactions toward phage. There is an embarrassing

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TABLE I.

Sensitivity to phage of normal S and R forms.

Strain.	Phages.														
	1	12	35	8	18	26	31	34	38	20	23	36	33	11	18
B. enteritidis, smooth, 617, 1010, etc.	± ± ±	+ + ±	+ + ±	+ + +	0	0	0	+ + ±	0	+ + +	0	0	0	0	0
B. enteritidis, smooth, 126	0	0	0	+ + +	0	± ± ±	± ± ±	+ + +	0	0	0	0	0	0	0
B. sanguinarium, smooth, 398	+ + +	+ + +	+ + +	+ + +	0	± ± ±	± ± ±	+ + +	0	+ + +	+ + +	0	+ + +	0	0
B. enteritidis, rough, BTMH, 127 R, etc.	0	0	0	+ + +	+ + +	0	+ + +	+ + +	0	+ + +	+ + +	+ + +	0	+ + +	+ + +
B. enteritidis, rough, 126 R	0	0	0	+ + +	+ + +	+ + +	+ + +	+ + +	0	+ + +	+ + +	+ + +	0	+ + +	+ + +
B. sanguinarium, rough, 396 R	0	0	0	+ + +	+ + +	± ± ±	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

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richness of material available but discussion will be limited to the variants obtained in three representative cases :—

(*a*) lysis of a smooth strain by a phage limited in its action to the smooth phase of the organism :
(*b*) lysis of a rough culture by a phage similarly limited to the rough phase :
(*c*) lysis of a smooth strain by a phage approximately equally active against both phases.

a. *Resistant derivatives of a smooth strain with phage* 12.

The smooth culture selected was a strain of *B. sanguinarium* (*N.C.T.C.* 398) which was found to be the most convenient organism to work with. This strain and its derivatives gave as a rule sharply defined positive or negative results when tested with the various phages on dilute agar. The absence of H agglutinogen made it unnecessary to treat emulsions with heat or alcohol before agglutination with the result that higher titres were obtainable. Suspensions of rough variants were stable in 0·25 per cent. saline and none of them showed the phenomenon of non-specific agglutination by normal rabbit serum (see Bruce White) which was fairly regularly present with *enteritidis* strains lacking H agglutinogen.

The strain 398 S was a typical smooth *sanguinarium* quite non-motile and showing no trace of floccular agglutination. Emulsions were quite stable in saline concentrations up to 5 per cent. and the colonies on agar were characteristically smooth and slightly fluorescent. The antigens of *B. sanguinarium* are practically identical with the heat-stable components of the corresponding phases of *B. enteritidis Gärtner* and this strain agglutinated with the stock "smooth" antiserum at a titre of 5120. It did not agglutinate at 1:80 with the "rough" *enteritidis* serum.

The strain was allowed to age in broth culture for over a month but despite numerous examinations no rough colonies were obtained from such cultures by simple plating methods. They were easily obtained however by the use of phage 12. The usual method adopted for obtaining resistant forms was to spread a suitable number of bacteria over an agar plate and after drying to cover an area with a drop of undiluted phage. Isolated colonies appeared on incubation and representative forms were purified from phage by successive plating and re-isolation and their characteristics examined.

A considerable proportion of the colonies appeared to be normal smooth types and all those tested by re-isolation showed bacteria still sensitive to phage 12. No persisting resistance had developed. The colonies which showed well-defined resistance on testing were of several types and, though only one was frankly rough, all had an opaque yellowish appearance which usually signifies essential "roughness."

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TABLE II.

Characteristics of the resistant strains derived from 398.S with phage 12.

Strain.	Precipitating saline concentration.	Agglutination. S.	Agglutination. R.	Phage sensitivity. 8	10	23	31	34	29	25	35	32	11	18	1	13	38
Normal S	>5 per cent.	5,120	< 80	+++	0	0	0	+++	+++	+	0	+++	0	+++	+++	+++	+++
R 1 (true rough)	0·8 ,,	< 80	10,240	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0	0	0
R 7	0·8 ,,	2,560	10,240	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	0	0	0
R 3	0·6 ,,	10,240	5,120	+++	+++	±	+++	+++	+++	+++	+++	+++	0	0	0	0	0
R 2	>5 ,,	5,120	5,120	+++	+++	+++	+++	+++	0	0	0	0	0	0	0	0	0

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The characteristics of four such types R 1, R 2, R 3, and R 7 are shown in table II. All the strains show the presence of R antigen and with the exception of the first they are also all agglutinated at fairly high titre by a "smooth" serum. The reactions to phage are essentially "rough" in type, but there is a general tendency for the phage sensitivity to be nearer that of the typical rough, R 1, when the "roughness" as judged by other characteristics increases. The multiplicity of types produced by a single phage should be noted.

b. *Derivatives of a rough strain with phage* 13.

The rough strain chosen was BTM-R, a non-motile derivative of an enteritidis strain BTM-HR, which was received already in the rough phase. This was lysed on agar by the "large plaque" phage 11, the activity of which is confined to the rough phase. It was found that the trace of gentian violet used as a routine in the agar helped considerably in the differentiation of the resistant colonies obtained. Except for an occasional tiny "nebenform" colony, the following list comprises all the types found:—

(1) typical smooth colonies semi-transparent, greenish and slightly fluorescent:
(2) irregular actively growing "rough" colonies brownish in colour and opaque:
(3) more regular and smaller "rough" colonies quite similar to those of the original strain:
(4) smooth round opaque colonies with deeply stained centre:
(5) small rough colonies appearing late:
(6) mucoid colonies of watery appearance taking up none of the stain from the agar.

Type 3 was found to be quite non-resistant: emulsified in broth and spread on agar *en masse* plaques appeared as soon as growth was visible and lysis was ultimately complete with the usual mixture of resistant colonies. Types 1, 2, 4 and 5 bred true to type on subculture but the mucoid form showed itself highly lysogenic and gave rise to several types. When a colony was spread heavily on agar the primary sheet of growth was rapidly destroyed within a few hours by the appearance of confluent plaques: a heavy secondary growth appeared on further incubation, apparently composed of pure smooth colonies. If one of the mucoid colonies was plated on MacConkey's medium to give discrete colonies a mixture of mucoid and smooth colonies was produced. Further direct subculture to the same medium of the mucoid colonies without intermediate growth in broth gave colonies of the same type; if transferred to broth culture growth was slight and a highly active phage of the 13 type appeared and on plating an almost pure smooth culture was obtained. One must assume that the mucoid

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colony is essentially rough and immediately gives rise to highly sensitive rough forms which are lysed by the phage present and give a proportion of smooth resistants. Such hypothetical sensitive rough forms were obtained in the primary plating of one mucoid colony. They appeared like a normal "rough" on incubation overnight, but when left a day at room temperature developed a mucoid "wall" around the colonies.

We have, therefore, in addition:—

(6) *a.* smooth derived from mucoid:
b. sensitive mucoid rough strain:
c. lysogenic mucoid.

The characters of these 6 persisting variants are given in table III.

These tables bring out clearly a point that is liable to be overlooked in considering the nature of resistance to phage, *viz.* the multiplicity of resistant types induced by the interaction of a pure phage and a single strain of bacteria. The example described is rather more striking than usual inasmuch as it shows practically all the commonly found types of resistance. These types are:—

(*a*) temporarily resistant colonies that immediately revert to the normal form on subculture (group 3):
(*b*) mucoid variants showing normal sensitivity on subculture (6*b*):
(*c*) specifically resistant colonies of the same phase (S or R) as the parent strain of which there may be more than one type (2, 4 and 5):
(*d*) colonies resistant in virtue of a change of phase to the normal S or R form (1 and indirectly 6*a*).

c. *Derivatives of a smooth strain BTM-O after lysis with phage* 8.

Amongst the resistant types so far described there is no example of a resistant derivative of a smooth strain that has retained its full smooth characteristics. Lysis by phages of the 1-12-33 group has so far only given smooth colonies which lost their resistance on subculture or rough and semi-rough forms. True smooth resistants however were obtained by the action of phage 8 which is equally active against the normal smooth and rough forms and thus has little tendency to induce a change-over of phase in the development of resistance.

An O strain of *B. enteritidis* (BTM-O) was used (strain 1 of table III.) and the resistant types were derived from lysis on agar. A few colonies only were present and three of these were taken for examination. After two replatings the colonies all appeared essentially smooth. Those of A were in all respects similar to the parent strain. B showed larger colonies with a tendency to umbilication on MacConkey's medium and frankly mucoid growth on ordinary agar, while the colonies of C

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TABLE III.

Derivatives of rough strain HTM-R with phage 13.

Strain.	Colony type.	Growth in broth.	Precipitating saline concent.	Agglutination. R.	Agglutination. H.	Agglutination. N.H.S.*	Remarks.
1	S	S	>5 per cent.	2560	160	< 80	True smooth.
2	R	R	0·6 ,,	480	2560	320	Rough resistants. Agglutination with smooth serum is non-specific.
4	S	R	0·4 ,,	1280	1280	960	
5	R	R	0·3 ,,	320	1280	320	
6a	S	S	>5 ,,	2560	160	< 80	True smooth.
6b	M	R	1·0 ,,	320	1280	< 80	Non-resistant rough mucoid.
Original HTM-R . .	R	R	0·8 ,,	320	6120	< 80	...

Strain.	1	15	33	8	16	25	81	84	20	25	85	11	18
1	0	±±±	±±±	+++	0	0	±±	+++	+±±	0	0	0	0
2	0	0	0	+++	+++	+++	+++	+++	0	0	0	0	0
4	0	0	0	+++	+++	0	+++	+++	0	0	0	0	0
5	0	0	0	+++	+++	+++	+++	+++	0	0	0	0	0
6a	0	+±±	±±±	+++	+++	±±±	±±±	+++	±±	±±	±±	+++	+++
6b	0	0	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Original . . .	0	0	0	+++	+++	±±±	+++	+++	+++	+++	+++	+++	+++

* N.H.S. = Normal rabbit serum. Untreated emulsions in 0·2 per cent. saline were used in this series of agglutinations.

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were small and rather dry and adhesive. All gave uniformly turbid growth in broth. Their sensitivity and other relevant characteristics are tabulated:—

TABLE IV.

Smooth derivatives of strain BTM-O following lysis by phage 8.

Strain.	P.S.C.	Agglutination of alcohol-treated emulsions.	
		S.	R.
Normal O	>5 per cent.	1280	<30
Resistant A . . .	>5 ,,	2560	<30
,, B . . .	>5 ,,	80	<30
,, C . . .	>5 ,,	240	<30

Strain.	Sensitivity to phage.												
	1	12	33	8	18	28	31	34	30	25	35	11	13
Normal O .	0	+++	++±	+++	0	0	±±	+++	++±	±	0	0	0
Resistant A .	++	++±	++±	0	0	0	0	0	+++	±	0	0	0
,, B .	0	±±	±±±	0	0	0	0	0	0	0	0	0	0
,, C .	+	++±	++±	0	0	0	0	0	0	0	0	0	0

Cultures B and C are rather aberrant, but A is a perfectly typical smooth form antigenically and is sensitive to the first phage group which is regularly limited in its action to smooth types. Specific resistance to the whole of the second group may be developed therefore without any change in the "smoothness" of the organism.

5. The derivation of smooth forms from rough cultures.

Opinion is divided as to the possibility of deriving smooth forms from rough cultures. The most generally accepted view is probably that of Bruce White that the so-called smooth cultures which can in some cases be obtained from rough cultures are still antigenically "rough" although their physical properties have been modified. Some authors have claimed that true smooths may be obtained by animal passage with rough strains, *e.g.* Jordan (1926) with *Salmonella* forms and Griffiths (1928) with pneumococci. It has already been mentioned that from one strain of *B. enteritidis Gärtner* antigenically typical smooth organisms were obtained from a rough culture by lysis with a phage active only against the rough form.

A further series of rough cultures has been examined for the production of smooth strains by this method. Six typically rough strains of *B. enteritidis Gärtner* were used comprising examples

* N.R.S. = Normal rabbit serum. Untreated emulsions in 0·2 per cent. saline were used in this series of agglutinations.

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showing most of the variations possible within this phase of the species. Four of them gave rise to smooth resistants on lysis with phages limited in their action to the rough phase. Two developed resistants that were always frankly rough in antigenic character although smooth-looking colonies were sometimes obtained.

The characteristics of the various cultures used in so far as they bear on the question of smoothness and roughness may be given in summarised form.

(1) *B. enteritidis*, 127 R. From N.C.T.C. Typically rough conical colonies on MacConkey's medium. Agglutinative growth in broth. Motile. Precipitating saline concentration 0·8 per cent. Agglutination of alcohol-treated emulsions with stock sera:—S 80, R 5120. Non-lysogenic.

(2) 410 R. From N.C.T.C. Typically rough conical colonies. Faintly granular growth in broth. Precipitating saline concentration 2·0 per cent. Non-motile and showing no H-antigen. Agglutination of alcohol-treated emulsion:—S 640, R 640. Non-lysogenic.

(3) WaR. By ageing of culture "Wallasey" received from Mr Bruce White. Typical rough colonies. Granular broth culture. Motile. Precipitating saline concentration 0·6 per cent. Agglutination of alcohol-treated emulsion:—S 160. R 1280. Non-lysogenic.

(4) BTM (HR). Received as *B. typhi murium* from Dr Ashcshov. Colonies slightly rough. Granular growth in broth. Precipitating saline concentration 1 per cent. Motile. Agglutination of alcohol-treated emulsion:—S 160, R 2560. Lysogenic toward many smooth *enteritidis* strains.

(5) BTM (R). Derivative of previous culture—a non-motile variant appearing spontaneously. Colonies similar to H form. Granular growth in broth. Precipitating saline concentration 0·6 per cent. Non-motile. Agglutination of alcohol-treated emulsion:—S 160, R 2560. Lysogenic (as 4).

(6) 126 R. From ageing of N.C.T.C. culture 126. Typically rough colonies. Granular growth in broth. Motile. Precipitating saline concentration 0·6 per cent. Alcohol-treated emulsions unstable:—S 640, R 320 (?), heated emulsion in 0·25 per cent. saline gave S 80, R 1280.

Sera were prepared against 5 of these strains and their content in H, O and R agglutinins examined by the use of appropriate emulsions (table V).

TABLE V.

To show content of H, O and R agglutinins in rough sera.

	Sera.				
	BTM-HR.	BTM-R.	410 R.	127 R.	126 R.
Normal BTM-HO emulsion (H)	40,000 F	< 80	< 80	20,000 F	10,000 F
,, BTM-O (O)	80	80	< 80	< 80	320
,, 393 S (O)	160	80	< 80	< 80	..
BTM-R in 0·25 per cent. saline* (R)	5,120	2,560	960	2,560	640
393 R in 0·25 per cent. saline (R)	10,240	2,560	960	10,240	1,280

* This emulsion showed non-specific agglutination by normal rabbit serum to a titre of 1 : 320.

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rough sera.

Sera.		
410 R.	127 R.	126 R.
< 50	20,000 F	10,000 F
< 50	< 50	320
< 50	< 50	..
960	2,560	640
960	10,240	1,280

serum to a titre of 1 : 320.

The sensitivity to phage of these cultures was as shown in table VI:—

TABLE VI.

Sensitivity to phage of "rough" enteritidis strains.

	1	12	33	8	18	28	31	34	20	23	25	11	36
127 R	0	0	0	+ + +	+ + +	0	+ + +	+ + +	+ + +	+ + +	+ + +	− − +	− + +
410 R	0	0	0	+ + +	+ + +	0	0	+ + +	0	0	0	0	− + +
Wa R	0	0	0	+ + +	+ + +	0	+ + +	+ + +	− + +	+ + +	+ + +	+ + +	− + +
BTM-HR	0	0	0	+ + +	+ + +	0	+ + +	+ + +	− + +	+ + +	− − −	− − +	− + +
BTM-R	0	0	0	+ + +	+ + +	0	+ + +	+ + +	− − +	+ + +	− + +	− − −	− + +
126 R	0	0	0	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	− − −	− + +

The cultures were all replated on MacConkey's medium and showed uniform colonies in every case. A single colony of each was transferred to broth and a loopful of phage 13 added. After two days' growth the broth cultures were plated out on MacConkey's medium and on agar. Any colonies that appeared to be smooth were then replated twice, or more frequently if any evidence of lysis was present, and the cultures so obtained were examined as to the various properties relevant to their "smoothness" or "roughness."

The strains WaR, BTM-HR, BTM-R and 126 R all gave typical smooth colonies with regularity. The other two strains 127 R and 410 R gave a proportion of smooth-surfaced colonies which, however, were always antigenically rough. The characteristics of these smooth cultures are shown in table VII:—

TABLE VII.

Smooth derivatives of "rough" enteritidis strains.

Strain.	Colony.	Growth in broth.	P. S. C.	Agglutination.		Lysogenesis.
				S.	R.	
WaR/13	S	S	> 5 per cent.	1280	< 80	0
BTM-HR/13	S	S	> 5 „	640	< 80	+
BTM-R/13	S	S	> 5 „	1280	< 80	+
126 R/13	S	S	> 5 „	1280	< 80	0

These four strains show all the properties of true smooth cultures and there can be no doubt therefore that at least from a considerable proportion of cultures "rough" by all the usual criteria true smooth types can be obtained. The two strains that gave no true smooths were treated with several other phages and under various conditions without giving rise to smooth variants in any instance.

The strains from which smooth resistant colonies were derived all

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showed a very slight power to induce O agglutinins in the rabbit. This trace of O agglutinin was absent in the case of the sera produced by the "irreversible" strains 127 R and 410 R and although the serological evidence is incomplete it is in accord both with the facts and with *a priori* considerations to believe that as long as any O antigen is present reversion to the smooth form may be accomplished.

It seems probable that by suitable manipulations it is possible to continue the alternation between the S and R form indefinitely. In the case of strain BTM-R the sequence R to S to R to S to R has been followed and, with strain 126, S to R to S to R. In both cases the alternate forms completely resembled each other and maintained their characteristic properties, *e.g.* each 126 R phase was sensitive to phage 28 while each BTM-R phase was resistant. It must be pointed out that to obtain such results careful selection of colonies at each stage is necessary, *e.g.* of the "roughs" obtained by allowing 126 S 2 to age in broth only 3 out of 12 colonies tested gave the typical 126 R 2 reactions.

6. Increase in virulence associated with the change from "R" to "S".

It is well known that the change from the smooth to the rough type is accompanied by loss of virulence. Within the *Salmonella* group this has been definitely established by Topley and Ayrton (1923) for *B. aertrycke*, by Bruce White (1926) for *B. suipestifer*, and by Goyle for *B. enteritidis Gärtner* (1926). Jordan (1926) obtained the same result with *B. paratyphosus B* and in addition succeeded in obtaining a virulent S strain from a non-virulent R culture by frequent transfer in broth.

The various types derived from strain BTM were examined for their pathogenicity toward mice and as would be expected the induced smooth forms were more virulent than the original rough cultures. It was found in fact that both HO and O smooth forms were highly virulent by feeding and the HO form was used for a considerable number of infection experiments. Of 25 mice each receiving about 1 c.c. of young broth culture of this form on bread, 2 died on the 6th day, 2 on the 7th, 7 on the 8th, 6 on the 9th, 4 on the 10th, 2 on the 11th and 1 on the 12th day after feeding. The following comparative experiments may be given:—

A. Mice were injected subcutaneously with broth cultures 20 hours old in the amounts shown in table VIII. The figures show the number of days before death occurred for each mouse while S signifies survival beyond 28 days. Three mice were used with each variant from strain BTM-HR, one receiving 0·2 c.c. of culture and two 0·02 c.c. Organisms of the type injected were recovered post-mortem from all the animals dying.

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d were recovered post-

TABLE VIII.

Comparative virulence of variants from BTM-HR.

Strain.	Days before death of mice occurred.	
	0·2 c.c.	0·02 c.c.
BTM-HR . .	9	S S
BTM-HO . .	5	4 15
BTM-R . . .	21	11 S
BTM-O . . .	4	6 7

B. About 2 c.c. of a day-old broth culture of the strain to be tested was poured over a small piece of bread and fed to each mouse. In this experiment six mice received the strain BTM-R and nine its smooth derivative BTM-O. The days on which each mouse died are shown as before. In this experiment also organisms of the type fed were recovered from the spleen of all the animals dying.

TABLE IX.

Virulence by mouth of rough strain BTM-R and its smooth derivative.

Strain.	Days before death occurred.
BTM-R . .	9 14 16 S S S
BTM-O . .	7 7 7 7 8 8 9 11 12

The development of smooth forms from rough by the use of bacteriophage is associated with greatly increased virulence. In agreement with Arkwright's (1927) results this virulence is independent of the presence or absence of H agglutinogen.

7. Reactions of pellicle-forming variants.

Amongst the colonies obtained from old broth cultures of *B. enteritidis* with or without phage a peculiar cohesive type is occasionally found. These have a granular wrinkled surface and are often adherent to the agar. A mass culture on agar shows a dry wrinkled sheet of growth very reminiscent of cultures of *B. subtilis*. In broth a surface pellicle is formed which readily falls to the bottom of the tube allowing fresh surface growth to occur. A 24 hours' broth culture therefore shows a surface pellicle, usually a light general turbidity and a sediment of small sheets of coherent growth.

This form of variation seems to be unassociated with any characteristic antigenic change. Three examples have been studied from the point of view of their reactions to phage. They have been called Q types since some of the forms described by von Lingelsheim (1913) under that name seem to have been of this variety. It is

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certain however that most of von Lingelsheim's variants were true R forms with which the pellicle-forming type has nothing fundamentally in common. Strain 126 Q was obtained from an old broth culture of the smooth strain 126 S along with rough and smooth colonies. BTM Q 1 was obtained by plating a culture of BTM (HO form) with phage 18: no apparent lysis occurred but a number of these Q colonies was obtained. The second variant BTM Q 2 was obtained from an old broth culture of the same HO form without any added phage.

Their sensitivity to phage and serological behaviour were as follows:—

TABLE X.

Pellicle-forming variants.

Strain.	Agglutination of alcohol-treated emulsion.	
	S.	R.
126 Q	1600	< 30
BTM Q 1	5120	320
BTM Q 2	2560	2560

Strain.	Phage sensitivity.												
	1	12	33	5	13	23	31	34	20	25	35	11	3
126 Q	0	0	0	+ + +	0	± ± ±	0	+ − −	0	0	0	0	0
BTM Q 1	0	± ± ±	+ + ±	+ + +	0	0	0	+ − −	+ +	0	0	0	0
BTM Q 2	0	0	0	+ + +	+ + +	0	+ + +	+ − −	0	0	0	+ + +	− − −

These forms may therefore be either smooth or rough antigenically and their behaviour toward phage is determined solely by their antigenic nature. 126 Q shows the usual reactions of smooth strains of 126, despite the extreme superficial difference in their cultural appearances. The variants from BTM are very similar in their mode of growth but are antigenically distinct and each possesses a corresponding phage sensitivity.

The results give definite support to the view that change in bacteriophage sensitivity is directly associated only with variation in the heat-stable antigenic structure. Adoption of the pellicle type of growth in broth or complete loss of motility and H antigen is equally without effect on the reactions towards phage.

8. The origin of bacteriophage-resistant cultures.

D'Herelle takes the view that the secondary cultures of resistant bacteria are the descendants of organisms which have successively destroyed an invading phage particle: in so doing they have acquired

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an active immunity that may be transmitted to their descendants: this immunity is held to be fundamentally similar to that developed by vertebrates against pathogenic micro-organisms. The analogies are not very impressive and a more popular view has been developed by Bordet (1925) Gratia (1921) and others who hold that any ordinary bacterial population is not homogeneous in its sensitivity to phage. The general variability of a species around its mean character will provide a proportion of organisms more sensitive than the average and others more resistant. A minute proportion, one in a million or less, is sufficiently resistant to give rise to secondary cultures.

It will probably be admitted by all that the capacity to develop resistance must depend primarily on some degree of variation pre-existent in the bacterial population. The alternative views differ as to the extent and type of this pre-existent change. D'Herelle would consider it as some slight modification that allows the invaded organism to cope better with the parasite and so develop an active immunity. The modifications observed in secondary cultures are manifestations of this immunity. Bordet and Gratia apparently consider that the organisms which escape lysis and give rise to resistant descendants do so only in virtue of modifications present before the addition of phage, *i.e.*, if the original culture were suitably diluted to give a million discrete colonies one of these would be found exactly similar to the one resistant colony produced from this population by bacteriophage. The action of the phage is purely selective.

The only real ground for an experimental distinction between the two theories lies in the behaviour of the resistant cultures. On d'Herelle's theory we should expect all the resistant colonies derived from lysis by a single pure phage to be approximately identical and resistant only to phages of the same group as the one causing lysis. If the selection hypothesis is correct the only common factor to be expected amongst the secondary colonies is resistance to the phage being used.

A given culture will often give rise to resistant colonies even when lysed by a combination of phages from two or more groups. On the theory of selection these resistant organisms insensitive to, say, two distinct phage groups are present in the original culture and should give rise to secondary colonies on lysis by phages of either group as well as by the combination. But on d'Herelle's hypothesis, resistance to two groups of phages should only develop under their actual influence.

The experimental results given earlier in this paper are in complete accord with the demands of the selection theory and the great variety amongst the resistant colonies resulting from lysis of a pure culture by a single pure phage immediately invalidates any simple form of d'Herelle's hypothesis. When strain 398 S is lysed by phage 12 (p. 20)

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two of the four rough derivatives show loss of sensitivity to one and two groups respectively in the absence of any phage of either of these groups.

Variation in the absence of any phage may also give rise to "specifically resistant" forms. In connection with the experiment described on p. 28 broth cultures of the reverted smooth strain 126 S 2 were allowed to age for some weeks. Plating then showed a mixture of smooth and rough colonies. Twelve apparently rough colonies were tested for their sensitivity to phage. Three of these were pellicle forming cohesive variants antigenically smooth. Of the others three were true normal rough types whose characters are described above as 126 R 2, the remaining six colonies were sensitive only to phages of the second group (8-34), *i.e.*, they behaved precisely like true resistants derived from 126 R 2 by the action of phage 13.

Further evidence in favour of the view that selection of "spontaneous" variants is the most important factor in the development of resistant cultures can be found from a consideration of the numerical relationships between the numbers of resistant colonies produced on agar and the amounts of phage and bacteria used. The following experiment shows the effect of varying the concentrations of phage and of bacteria on the numbers of resistant colonies of each phase obtained in the interaction between strain BTM-R and phages 11 and 13.

A four-day old agar slope culture was washed off with 5 c.c. of saline. By plating suitable dilutions this emulsion was shown to contain approximately eleven thousand million living organisms per c.c. Another suspension one-tenth the concentration of the first was prepared in saline. One drop (0·02 c.c.) of each suspension was then spread over agar plates that had previously been uniformly spread with phage, either undiluted filtrate or a 1 : 10 dilution of this, five drops per plate in each case. The numbers of smooth and rough colonies obtained after incubation are shown in the table. Only those colonies were counted as smooth whose appearance was absolutely typical. A number were tested serologically and none showed deviations from the typical behaviour (table XI.).

TABLE XI.

To show relations between the numbers of resistant colonies produced and changes in the concentration of phage and bacteria.

	Phage 11.				Phage 13.			
	Undiluted.		Diluted 1/10.		Undiluted.		Diluted 1/10.	
	S.	R.	S.	R.	S.	R.	S.	R.
Stock emulsion of BTM-R.	16	78	12	79	14	24	12	42
Stock emulsion of BTM-R diluted 1/10 . . .	1	10	1	7	2	0	0	4

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l bacteria.

Phage 13.			
... spelt.		Diluted 1/10.	
S.	R.	S.	R.
24	12	42	
0	0	4	

This experiment exemplifies the following general rules that were found to apply to all the experiments carried out along these lines.

1. The number of resistants of opposite phase (here the smooth colonies) is proportional to the number of bacteria submitted to lysis and independent of the concentration of phage.
2. The same numbers of resistants of opposite phase are produced by phages of different origin provided these are strictly confined in their action to the one phase. The numbers of resistant colonies of the same phase usually vary with the particular phage used.

In an experiment with exactly analogous technique, in which the smooth strain 398 S was lysed by phage 12, the figures in table XII were obtained: the experiment was done in duplicate.

TABLE XII.

Numbers of S and R colonies appearing after lysis of a smooth strain on agar.

Bacterial concentration.	Colonies with phage 12.			
	Smooth.		Rough.	
Stock emulsion . .	265	204	72	64
.. .. 1:10 .	3	1	6	5
.. .. 1:100 .	0	0	0	0

This shows a characteristic difference between the numbers of smooth (same phase) and rough (opposite phase) resistant colonies in that the number of smooth colonies is not proportional to the size of the population lysed. In general it was found very difficult in experiments with this and other smooth strains to obtain reproducible results as to the number of smooth colonies appearing after lysis while the numbers of rough colonies were extremely consistent. Most of the "resistant" smooth colonies gave rise immediately to sensitive cultures on subculture and it seems that some other mechanism is at work than is responsible for the appearance of the stable rough variants.

The two experiments described are typical of a considerable number of others carried out along the same lines. As long as the phages used were strictly confined in their action toward the one phase the number of resistant colonies of the opposite phase depended only on the size of the population available for lysis. Even when lysis by a large plaque phage such as 13 was barely confluent from the development of only a few hundred plaques the number of colonies of opposite phase was approximately the same as when a thousand times this concentration of phage was used. In such a case three or four hours unhindered growth of bacteria must have occurred in the

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regions corresponding to the peripheral zones of the eventually formed plaques, but this growth apparently has not increased the number of points from which a resistant colony can develop. It seems that in the originally spread population (from a culture at least one day old) there are a certain number of resistant or potentially resistant organisms and that, in the first few hours' growth on agar, resistant bacteria are produced only by direct multiplication of these organisms. By far the simplest and probably the only valid explanation of this constancy of numbers of colonies in the opposite phase is that they are derived from organisms actually in this phase in the original population.

Other factors are evidently involved in the production of temporarily resistant cultures, but the two lines of evidence discussed indicate that the typical persistently resistant variants whether of the same or opposite phase result from the selection of variants occurring "spontaneously" in the substrate culture.

9. Bail's theory of the nature of bacterial resistance.

Before proceeding to a discussion of the general significance of these results it is necessary to consider in some detail the most important current interpretation of the nature of acquired bacterial resistance—that of Bail (1923-1925). Of the theories opposed to the independent virus conception of d'Herelle, Bail's is the only one that attempts an exhaustive examination of the essential objection to any "ferment" theory, namely the fact that several different phages may maintain easily recognised individuality although developed at the expense of one strain of bacteria. The different phages may often be distinguished by the type of plaque produced on agar—its size and the nature of its edge—but more precisely by a study of their power of provoking the appearance of specifically resistant forms. If a colony derived from the secondary culture appearing after lysis of (say) a *Shiga* strain by a particular phage, is purified from phage by replating and tested against a series of phages all active against the original bacterial strain it will be found that the new strain is resistant to the homologous phage and to a certain number of others but still sensitive to the rest. By examining in this way resistants to all the *Shiga* phages available, a grouping of the bacteriophages into types is possible.

Another method of classification depends on the use of anti-bacteriophage sera and Watanabe has shown that the results obtained parallel closely those from Bail's method. Antilytic activity within the group of *Shiga* phages is practically confined to phages of the same type as that from which the serum was produced. Similar results are obtainable with other members of the intestinal group of bacilli, but irregularities are more frequent than when *Shiga* bacilli are used.

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Bail's well known "splitter" theory of bacteriophage is fundamentally an attempt to co-ordinate these findings, and his interest in other manifestations of phage activity is quite secondary. He considers that each phage type represents a particular unit (gene) of the hereditary make-up of the sensitive bacterium. The gene is considered to have undergone some "pathological" alteration by which its normal balance of constructive and disintegrating power is upset, with the result that it now exercises a predominantly destructive action on the bacterial cell. Only bacteria containing the particular gene involved are sensitive to bacteriophage of a given type and resistance is interpreted as loss of the unit from the hereditary make-up. The essential point in which this theory becomes completely incompatible with that of d'Herelle is its explanation of the nature of multiplication of bacteriophage. For d'Herelle, there is a direct genetic relationship between the attacking phage particle and the particles resulting from the subsequent lysis of the bacterium. For Bail the action of the attacking phage unit is to cause the bacterium to liberate in abnormally active form one of a certain limited number of pre-existent bacterial constituents.

The distinction is clear enough to allow of a crucial experiment to distinguish between the two views. Suppose we select from a large series of "pure" phages derived from various sources and active against a single bacterial strain, a certain number all belonging to one of Bail's types, *i.e.* all producing resistant strains that are equally resistant to all the others. According to Bail these phages should be identical, at least in their qualitative behaviour, while on d'Herelle's view each may be capable of maintaining individual differences through numerous passages with the one strain. Further, if the series of phages is now developed at the expense of another bacterial species in which the specific resistance relation holds, any change found in the behaviour of one member, should on Bail's hypothesis similarly affect every other. On d'Herelle's theory any individuality possessed by the phages when developed at the expense of one substrate organism will show itself, perhaps slightly modified, on their transfer to another sensitive species.

It was found possible to examine these points experimentally with a series of phages primarily developed against a rough strain of *B. enteritidis Gärtner* but also active against *B. sanguinarium* and *B. dysenteriæ Shiga* and the results are unequivocally in favour of d'Herelle's conception.

Phages 3, 18, 28, 31 and 34 were diluted suitably and isolated plaques obtained on *B. enteritidis* (strain 126 R).

From single plaques of each phage, broth cultures of 126 R were inoculated and after lysis sterilised as regards bacteria by heating to 56° for 30 minutes. The same procedure was gone through with *B. sanguinarium* (398 R) and with *B. dysenteriæ Shiga* (752 R), a further phage, 38, being used with *B. sanguinarium*.

Resistant strains of each organism toward all the phages developed at its expense were obtained, in most cases by plating broth cultures showing secondary

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growth after lysis but when, as in some cases, no true resistant forms were obtained in this way they were secured by replating resistant colonies appearing in the course of lysis on agar. Phage 28 seemed to lyse *B. sanguinarium* on agar, but the secondary colonies were quite non-resistant and no multiplication of the phage occurred in broth cultures of this organism. Similar findings apply to the action of phage 38 on the *Shiga* strain 752 R. The last phage was quite without action on the rough *enteritidis* strain 126 R. All the resistants obtained from strains 398 R and 752 R were typically "rough" bacilli with characteristic colonies and precipitating growth in broth. With 126 R (*B. enteritidis*) several of the phages gave as well as rough resistant forms a certain number of true smooth colonies. The appearance of S or R types from a lysing culture of the opposite phase is a phenomenon apparently of a different nature from that responsible for the development of specific resistance within the same form and for that reason only the rough resistants have been considered.

The resistant colonies were replated and from a single colony stock cultures were obtained. These were then tested in the usual fashion with each of the phages being studied and with phage 13 as a member of an unrelated group. The phage filtrates used for testing the resistant strains of each organism were developed at the expense of the original strain concerned, *i.e.* resistant cultures from strain 398 R were tested with phages grown on that strain and similarly with the two other substrate organisms. Except for the culture obtained after apparent lysis of 398 R by phage 28 all the resistant cultures were completely insensitive to the action of phages 8, 18, 28, 31, 34 and 38 and all were lysed by phage 13. The resistant cultures from strain 398 R were tested with a sample of phage 28 developed on *B. enteritidis* 126 R. As has been mentioned above this phage appeared to lyse 398 R on agar, but no resistant colonies developed nor could any multiplication in broth culture be detected. The resistant strains produced by phages 8, 18, 31, 34 and 38 showed no lysis of any sort by this phage.

These results make it clear that the six phages all fall into one of Bail's groups. A series of smooth and rough strains of various intestinal bacilli chosen with the aim of bringing out differences amongst the phages was similarly tested against all of the stock phages, each phage being developed where possible on each of the three substrate organisms. The usual method of testing on agar was employed and the results are combined in table XIII.

The table shows clearly that despite the fact that the 6 phages all belong to a single type in Bail's sense each one can be readily differentiated from any other by the use of a number of heterologous bacterial strains. For instance, the two phages which most closely resemble one another are 8 and 34 but, while 8 actively lyses every strain tested, 34 is only very weakly active against the smooth *enteritidis* strain 126 S and one or two other strains. Further this individuality of phage behaviour persists with occasional slight modification no matter which of the three species is used as substrate. A good example is given by phage 31 which regularly lyses three out of four rough *enteritidis* strains and fails to lyse the fourth on all three substrates.

If Bail's theory is taken strictly at its face value the above experiments constitute a formal disproof of its validity. All the phages produce resistants of the same type—resistant to each phage

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of the group but still sensitive to other phages such as 13—yet specific differences between all of them can be demonstrated. It follows that if each phage represents a single "gene" at least 5 other "genes" must be lost whenever resistance is induced toward a particular phage. Bail describes lesser degrees of "linkage" between "genes" (Koppelung) but it is obvious that any unlimited extension of such an idea must make the theory quite useless as a working hypothesis.

TABLE XIII.

To show the existence of individuality amongst phages all of which belong to a single type in Bail's sense.

Strain tested.	Reaction with phages developed on the sensitive rough strains shown.								
	Phage 8 on			Phage 18 on			Phage 25 on		
	398 R	126 R	732 R	398 R	126 R	732 R	398 R	126 R	732 R
sanguinarium 398 R	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	0	(+ + +)	0
enteritidis 126 R	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	0	+ + +	+ + +
Shiga 732 R	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	0	+ + +	+ + +
enteritidis BTMR	+ + +	+ + +	+ + +	+	+	+	0	0	0
,, 127 R	+ + +	+ + +	+ + +	+	+	+	0	0	0
,, 410 R	+ + +	+ + +	+ + +	+	+	+	0	0	0
typhosus 901 R	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	0	+ + +	+ + +
Shiga 753 S	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	0	± ± ±	± ±
sanguinarium 398 S	+ + +	+ + +	+ + +	0	0	± ±	0	0	0
enteritidis 1010 S	+ + +	+ + +	+ + +	0	0	0	0	0	0
,, 126 S	+ + +	+ + +	+ + +	0	0	0	0	±	± ±
typhosus 2135 S.	+ + +	+ + +	+ + +	0	± ±	± ±	0	+ + +	+ + +
,, 2137 S.	+ + +	+ + +	+ + +	0	0	± ±	0	+ + +	+ + +

Part II.

Strain tested.	Reaction with phages developed on the sensitive rough strains shown.								
	Phage 31 on			Phage 34 on			Phage 38 on		
	398 R	126 R	732 R	398 R	126 R	732 R	398 R	126 R	732 R
sanguinarium 398 R	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	...	...
enteritidis 126 R	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	0	...	...
Shiga 732 R	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	(+ + +)	...	...
enteritidis BTMR	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	0	...	...
,, 127 R	+ + +	+ + +	+ + +	±	± ±	± ±	0	...	...
,, 410 R	0	0	1	+ + +	+ + +	+ + +	0	...	...
typhosus 901 R	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	...	...
Shiga 753 S	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	0	...	...
sanguinarium 398 S	+	0	± ± ±	+ + +	+ + +	+ + +	±	..	...
enteritidis 1010 S	±	± ±	±	± ± ±	±	± ± ±	0	...	...
,, 126 S	0	0	± ±	0	0	± ± ±	0	...	...
typhosus 2135 S.	+ + +	+ + +	+ + +	± ±	0	±	0	...	...
,, 2137 S.	+ + +	+ + +	+ + +	+ + +	±	+ + +	± ±	..	...

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It is much simpler to assume that each phage gives rise to actual descendants in the ordinary sense of the word, which maintain the general characters of the parent phage although they may be modified somewhat when passed from one species of bacteria to another.

Sufficient evidence has been given earlier in the paper to make it clear that the source of the apparent discontinuity of the changes that occur when resistant variants appear must be sought wholly within the bacteria themselves. Although more than one factor may be operative it is simplest to consider that there is some functional entity in the bacterial make-up which is responsible for determining the sensitivity to phage of any given organism and that this entity can change its character only in a certain limited number of discontinuous steps. With each such change one group of phages loses its power to lyse or another group shows an activity previously absent. The phages within each group may possess widely different activities, but all seem to be related in some way to a single unit in the bacterium in the absence or latency of which they are all equally unable to lyse the organism.

It remains to determine whether this hypothetical entity, a simple "mosaic" composed of a certain small number of units, can be co-ordinated with any of the known functions of bacteria.

In a previous communication (1927) it was shown that amongst the *Salmonellas* there was a definite though not quite complete correspondence between the heat-stable antigenic structure and the sensitivity to a series of bacteriophages. Further evidence of the importance of changes in the heat-stable agglutinogens in determining or accompanying changes in phage-sensitivity has been accumulated throughout the whole of the present work. This has led to the hypothesis that what primarily determines whether or not lysis by a given phage will take place is the capacity of the phage to be specifically adsorbed by the same entity that functions in serological reactions as the heat-stable (O or R) agglutinogen. The evidence for this view may be briefly summarised:—

1. the presence or absence of H (heat-labile) antigen is without influence on the behaviour of the strain towards phage:
2. changes in cultural behaviour unassociated with antigenic change, such as to the pellicle-forming Q variant, have no influence on phage sensitivity:
3. change in the nature of heat-stable antigen from O to R is associated with a sharp change in phage reactions:
4. organisms having common heat-stable antigens show a very close correspondence in the phages to which they are sensitive:

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5. organisms that have become resistant to a phage no longer absorb it from solution to the same degree as the sensitive form, *i.e.* a specific adsorption seems to be at least one determinant of lysis (d'Herelle, Lepper, 1923, Prausnitz and Firle 1924):

6. sensitive organisms killed by heat absorb a normally active phage even after being heated above 100° (Prausnitz and Firle).

It is evident that there is some co-ordination between changes in the heat-stable antigens and in sensitivity to phage but it may be held that this co-ordination is more or less accidental. Important changes in the functional elements of the organism are involved in the S-R interchanges and these may be reflected in more or less parallel fashion in the two properties under consideration although no actual functional relationship between the two exists. But the evidence is strong enough to suggest that the same surface constituents of the bacterium which provoke and react with the stabilotropic (O or R) agglutinins also absorb specifically the active particles of those phages that are capable of lysing the organism. In particular the power of sensitive bacteria to absorb an active phage from a solution containing it, the inability of resistant strains to do so and the retention of this power to a considerable degree by bacteria heated to 100°, are very difficult to interpret on any other hypothesis.

The experimental work at present available is not sufficient to allow a detailed elaboration of this hypothesis, but some of its implications and the difficulties they provoke may be discussed. In the first place it implies that bacteria are subject to certain discontinuous variations in their heat-stable antigenic structure, variations that occur in culture without known cause and are most readily detected by the specifically selective action of bacteriophage. The smooth to rough change is the best known and most striking of these variations, but other discontinuous modifications within the limits of each of these main phases must also occur.

According to White the relation between the smooth and the rough heat-stable antigens in *Salmonella* is quite similar to that in the more extensively studied pneumococci. The smooth O antigen possesses a double structure: part is identical with the rough R antigen; the other portion, which is responsible for the whole of its specific behaviour, is a complex carbohydrate. The rough antigen lacks the carbohydrate and provokes a totally different agglutinin response much less specific than the smooth O agglutinins. As far as the serological analysis of *Salmonella* variants has gone at present there seems to be no evidence that any changes occur within the heat-stable antigen beyond quantitative variations in the amounts of O and R antigen available for reaction, *i.e.* in the proportion of nucleoprotein units that carry the specific carbohydrate. The serological work reported in the present

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paper has been confined to an estimation of such quantitative differences and offers no direct evidence of qualitative changes in the antigen associated with phage resistance apart from change of phase.

If antigenic differences do occur in association with the development of resistance within the smooth phase (as in the example described on page 23) they must involve a change in the nature of the specific carbohydrate. This seems *a priori* very unlikely although Griffith's experiments (1928) with pneumococci suggest the possibility of such an occurrence. However, the problem is susceptible of direct experimental attack and it is hoped to follow up this work with a more detailed serological study of resistant variants which do not undergo change of phase.

10. Conclusions.

Bacteriophage lysis is a complex process and interference with its course at any point will usually result in the appearance of apparently resistant cultures. Investigation of each resistant culture is therefore necessary to determine at which of several points the course of lysis has been checked. What is probably the commonest form of resistance seems to be due to a simple accumulation of products of lysis. A secondary culture appears after lysis but on subculture to fresh media (either broth or agar) it is rapidly lysed by the phage carried over with it (Gohs 1926). The resistance of the common mucoid variants found after lysis is probably rightly attributed by Kimura (1925) to the simple physico-chemical protection of the mucus as an emulsoid colloid similar to gelatine.

The third group contains variants showing what Bail terms "true resistance," *i.e.* they are stable non-lysogenic variants which maintain their characteristic properties on subculture through several generations at least. This type of resistance is associated with and is probably determined by a lack of power to absorb the phage specifically. Finally, even when the lytic particle has become incorporated in the bacterial cell it may still encounter conditions which result in resistance usually in a lysogenic form rather than lysis. Two of the cases analysed by Flu (1923) are undoubtedly of this nature.

In the present paper only the third of these groups is discussed, the stable non-lysogenic resistant cultures. Two chief conclusions have emerged. (1) They are probably produced in virtue of the general variability of a bacterial species and not in any sense by the active participation of the phage. Phage acts merely by selectively sparing those variants to which it is not specifically adsorbed. (2) The appearance of this type of resistance is frequently associated with a change in the phase (smooth or rough) of the organism. This denotes a gross change in the heat-stable antigenic structure and evidence is adduced that even when there is no such change-over the development of resistance is associated with change in that bacterial constituent

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which determines its "heat-stable" serological behaviour. In this connection the discontinuous nature of the changes in the bacterium is emphasised. There are only a certain number of variant types that can appear of which the classical S and R types are two.

It is probably legitimate to consider the typical S form as the normal genetically complete form and all other variants as mutations by loss of certain units comparable to the genes of Mendelian theory. As is found in studies of higher organisms such mutations by loss may result in the appearance of superficially positive characters that are normally held inactive.

Summary.

1. There are characteristic differences in the behaviour of the smooth and rough forms of *Salmonella* strains toward bacteriophage.

2. Certain phage types are limited in their action to smooth forms, others only attack rough strains, while a third group may lyse either form.

3. The type of resistant colony produced is related to these characteristics of the lysing phage. "Smooth" phages tend to give rough or partially rough resistants, "rough" phages with certain rough cultures provoke the appearance of true smooth forms, while those phages which lyse both forms impartially are more liable to show specifically resistant forms in the same phase as the parent strain.

4. From cultures that are rough by all the usual criteria, it is possible by the use of suitable phages to derive true smooth forms showing all the characters of the type including active virulence.

5. Some rough cultures could not be so converted to the smooth type and the evidence, which is incomplete, suggests that the conversion can only occur so long as a trace of smooth antigen is retained in the antigenic make-up of the bacillus.

6. The quantitative relations between the numbers of bacilli lysed and the numbers of the various types of resistant colonies strongly suggest that the appearance of a new phase (R or S) under the influence of phage is purely a selection phenomenon.

7. Experiments are presented to disprove Bail's contention that phages which produce the same type of resistance with a single bacterial strain are necessarily qualitatively identical.

8. A general discussion of the nature of bacterial resistance to phage is attempted from the standpoint that its most important factor is change in the bacterial constituent which also functions as the "heat-stable antigen."

This paper comprises work accepted by the University of London as a thesis for the degree of Ph.D. (1927). I am greatly indebted to Prof. J. C. G. Ledingham and Dr J. A. Arkwright for their advice and criticism during the course of this work.

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RETROPERITONEAL GANGLIONIC NEUROMA.

D. F. CAPPELL.

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(PLATES IV.-VI.)

WHILE tumours of the central nervous system are comparatively common, new growths of the peripheral nervous system composed of true nerve elements are of much less frequent occurrence. Within recent years the true nature of certain tumours previously confused with the sarcomata has been recognised, and the constituent elements have been identified as undifferentiated nerve cells, the group being designated *neuroblastoma*. Tumours composed of adult nerve elements are still regarded as uncommon, and are of special interest in that they are made up of cells which have in the adult practically no powers of regeneration, and whose origin therefore must be sought in a displacement of an embryonic rudiment or at least in some abnormality originating at an early stage of development. Such growths are most commonly met with in connection with the abdominal sympathetic chain, and may attain a large size, but they generally present an admixture of adult and embryonic tissue. The specimen which forms the subject of this communication appears to be one of the largest if not actually the largest on record which consists solely of adult cells and fibres.

Clinical History.

The patient, a woman aged 27, was admitted to hospital complaining of slight pain in the right side of one year's duration. She stated that the pain was never severe and did not cause her to lie up; it came on two or three times a day but lasted for only a few minutes, and it occasionally radiated to the vulva. The bowels were costive, and she took medicine every other day. Menstruation was normal. She had one child, aged 3 years, healthy in every respect.

On examination the woman looked healthy and her weight was normal. A tumour, which felt about the size of a small melon, smooth in outline and freely movable, could be palpated in the right lumbar region; it could be pushed up into the right kidney pouch but would not remain there. Chemical examination of the urine revealed no abnormality. Cystoscopic examination by Mr W. W. Galbraith showed that the bladder walls and trigone were healthy. Both ureters were seen to be functioning and were healthy in appearance. Five c.c. of 0·4 per cent. indigo carmine were injected intravenously and the dye was seen to be excreted from the left ureter in 3½ minutes, but appeared from the right ureter only after 20 minutes. A right ureteral catheter was inserted easily for a distance of 25 cm. and a pyelogram was taken, 12 c.c. of a 12½ per cent.

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solution of sodium iodide being injected. The pyelogram (fig. 2) showed that the right ureter was very abnormal in situation, passing across the middle line over the mid part of its course, that it was considerably stretched and that the catheter introduced a distance of 25 cm. reached only about two-thirds of the way along it. The kidney was seen to be in its normal position but the pelvis was distinctly horse-shoe shaped with the concavity downwards. A further cystoscopic examination was made a week later, the left ureter being catheterised and a pyelogram taken. This showed the ureter and pelvis of the left kidney to be normal; two calcareous glands were seen lying adjacent to it. Bacteriological examination of the urine from both kidneys showed that there were no organisms in film or culture.

As a result of these investigations a diagnosis of retroperitoneal tumour on the right side was made, and the opinion was expressed that whatever abnormality was present in the kidney on that side was due to mechanical pressure from the tumour. Operation was performed by Mr M. Logan Taylor, assisted by Mr W. W. Galbraith. The abdomen was opened by a right rectus incision, and a large retroperitoneal tumour was found. The growth was adherent to the inferior vena cava and common iliac veins, which were both spread over its inner side and firmly adherent to it. The ureter was lying over the front and inner aspect of the tumour, and was reflected along with the peritoneum. The inferior vena cava and common iliac veins were dissected free, and the tumour was then shelled out. The right kidney was felt lying in its normal bed but rotated, the lower pole being pushed upwards and outwards, the kidney thus lying transversely. The abdomen was closed in layers, and the patient made an uninterrupted recovery from the operation. Two months later a pyelogram was taken on the right side, and it was then seen (fig. 3) that the kidney had come down a little and had resumed its normal position, that the ureter had not yet contracted and was lying in folds, and that there was a considerable degree of hydronephrosis. The indigo carmine test was repeated with a result similar to that on the first occasion. Bacteriological examination of the urine from the right kidney showed that it was sterile. The patient was examined three years later, and was then in perfect health.

The tumour was received within a very short time after removal from the body. Its external characters were noted and the growth was weighed. It was then sectioned and about half the tumour was preserved as a museum specimen, while the remainder was cut into small pieces and portions from many different parts were fixed in each of the reagents. The fixatives employed were Zenker's fluid with acetic acid, Bouin's aqueous solution, saturated corrosive sublimate solution, formol alcohol, and 10 per cent. formalin in saline. Paraffin sections were prepared and were stained for general histological structure by hæmatoxylin and eosin, eosin and methylene blue, iron hæmatoxylin and van Gieson's stain. As special stains for connective tissue Murray's nile blue—van Gieson method, Mallory's original anilin blue and Masson's trichromic stains have been found to yield instructive pictures. A variety of neuroglial stains including the original Weigert method and nearly all its modifications, Mallory's phosphotungstic acid hæmatoxylin, and Bailey's ethyl-violet orange G mixture have all been employed; in some cases the special fixative for these staining methods has been used after previous formalin or other fixation. Numerous silver impregnation methods have been tried, the

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GANGLIONIC NEUROMA.



FIG. 1.—Coronal section of retroperitoneal ganglionic neuroma showing typical whorled fasciculated structure. × ⅔.

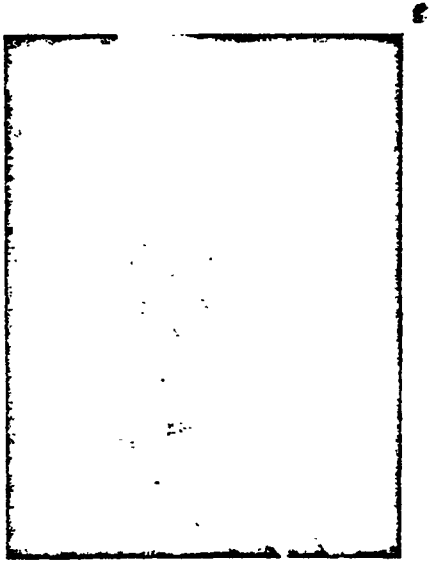


FIG. 2.—Pyelogram before operation: extreme displacement of ureter with kidney in normal situation but rotated to lie transversely. Pelvis dilated, horse-shoe shaped: upper part of ureter not shown owing to density of underlying tumour.

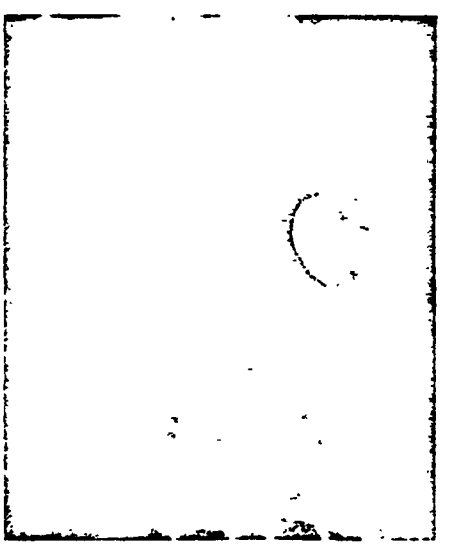


FIG. 3.—Pyelogram two months after operation: kidney rotated back to its normal position. Ureter still tortuous, considerable hydronephrosis.

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most consistently successful being Ransom's pyridin silver technique, but the Bielschowsky method for blocks also gave good results. It was not found possible to cut frozen sections even after gelatin embedding owing to the soft and varying consistence of the tissue, so that the Bielschowsky technique for sections was not applicable. Portions of the tumour were also embedded in celloidin after mordanting by the Weigert process, both slow and rapid methods being tried, and sections were subsequently stained by Weigert's original method and by the Pal, Kulchitsky and Wolter's modifications.

Naked-eye appearance.

The tumour is roughly ovoid and somewhat flattened antero-posteriorly. It weighs about 1020 grms., and measures 18 × 13 × 8 cm. The surface is smooth and the growth is obviously well encapsulated, but a large bundle of nerve fibres is seen entering the upper and medial aspect of the growth. A large number of blood vessels with well-formed walls enter the tumour at various points. The growth is of fairly soft consistence, and the capsule is tense and elastic. On section the substance of the mass bulges over the cut surface, and the tumour has a characteristic appearance of interlacing irregularly-arranged bundles of nerve fibres embedded in a soft translucent myxomatous tissue (fig. 1). These bundles are of varying size and run in all directions giving the cut surface a curiously whorled appearance not unlike that of an œdematous fibromyoma. The tumour appears to be of uniform character throughout, and no areas of more cellular primitive tissue are seen. Blood vessels are not very numerous on the cut surface; they have thick well-formed walls and appear to be surrounded by a denser stroma. No areas of necrosis or degeneration are present but a few small calcified nodules are scattered throughout the growth.

Microscopic examination.

Microscopically the tumour is seen to be a typical ganglionic neuroma in which the constituent elements are large unipolar and bipolar ganglion cells, while the bundles of nerve fibres are of *non-medullated* type. The interstitial tissue is of an unusually myxomatous nature with characteristic stellate cells and abundant mucoid intercellular matrix. The ganglionic cells tend to occur in foci, the size of individual groups varying from a few cells up to many hundreds (fig. 4). They are generally large, resembling those of sympathetic ganglia (figs. 5, 7), and possess a large nucleus with well-defined nuclear membrane and a large single karyosome; binucleate cells are not uncommon. The cytoplasm of the majority of the ganglion cells is like ground glass, with the deeply staining Nissl bodies tending to be disposed in a circle towards the periphery of the cell while the

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...ormis after operation: ... to its normal posi... ...considerable hydro-

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centre is relatively free (fig. 7). The nucleus in such cells is generally situated at one side, and it is probable that the condition is one of mild degeneration, such as occurs in spinal ganglion cells soon after severance of the axon. A considerable number of apparently healthy ganglion cells with central nucleus and evenly distributed Nissl bodies are present, but there are also many cells in a state of more advanced degeneration with complete loss of Nissl substance and accumulation of pigmentary granules in their cytoplasm. These granules appear to be of *lipochrome* nature, as they stain black with the Weigert myelin sheath stains and with osmic acid, orange with sudan III and scarlet red, and reddish purple with Mallory's hæmatoxylin. They are not wholly soluble in alcohol or ether, and appear in paraffin sections as yellowish-brown granules. In silver impregnation preparations many of the ganglion cells exhibit an extremely foamy structure which is probably due to degenerative changes. The healthy cells here present a uniform finely granular appearance (fig. 10). In the more degenerate cells the nuclei are frequently shrunken and atrophic, and karyorrhexis and karyolysis are found. Finally the cell body has become shrunken and appears to disintegrate. No evidence of phagocytosis on the part of the capsule cells has been seen.

Each ganglionic cell is surrounded by a sheath of fine connective tissue between which and the cytoplasm of the ganglion cell lie one or two nuclei belonging to the capsule cells or *amphicytes* (fig. 5). This membrane is prolonged over the axon in the form of a sheath which appears to be composed of fine collagen fibres, as it stains pink with van Gieson, blue with the anilin blue and trichromic stains, and reddish brown with phosphotungstic acid hæmatoxylin. The groups of ganglionic cells sometimes consist of a large number of isolated cells (fig. 6) each with its capsule and axon covered by a sheath, but frequently a considerable number of ganglion cells occur closely packed together (fig. 4), the cell bodies being polygonal from mutual pressure. In such groups each individual cell does not appear to possess a capsule, but the whole group is invested by a common connective tissue sheath on the inner aspect of which there is a nucleated layer. From such groups a large number of axons may emerge, but each appears to receive a covering as it pierces the investing membrane. Many of the ganglionic cells in these closely packed groups are degenerate, and in some cases the investing sheath appears to be incomplete, as if it had been ruptured by the growth of the enclosed cells. In such groups it is not uncommon for two ganglion cells to be joined by a thick protoplasmic process, such as those described by Adams (1914).

The numerous nuclei seen in the bundles of nerve fibres correspond to the nuclei of the cells of the cytoplasmic sheath which surrounds each fibre like the sheath of Schwann (fig. 8). With careful focussing in suitably stained preparations (*e.g.* by Mallory's or Masson's anilin

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GANGLIONIC NEUROMA.



FIG. 4.—Large group of ganglionic cells showing isolated cells and small closely packed groups. Majority of axons of fine type, a number of coarse variety also present. Bielschowsky's silver technique. ×80.

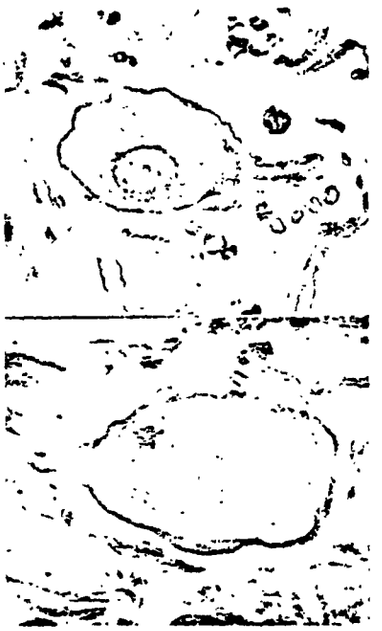


FIG. 5.—Two large ganglion cells with amphicytes and connective tissue capsules continued over emerging axons. Nerve fibres cut obliquely and transversely. connective tissue sheaths well seen. Mallory's anilin blue method. ×600.

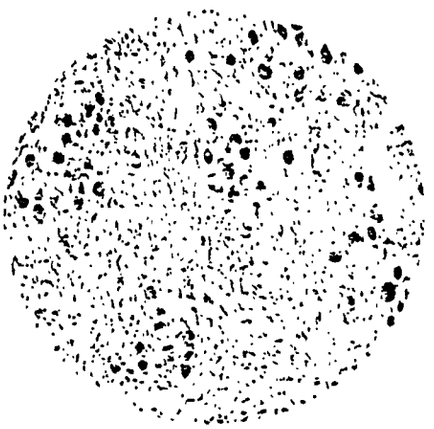


FIG. 6.—Ganglion cells scattered among bundles of nerve fibres with neurilemma sheaths. Capsule visible around almost every ganglion cell. Iron haematoxylin and van Gieson. ×70.



FIG. 7.—Relatively healthy ganglion cells, the majority with excentric nuclei. Binucleate cell with peripheral Nissl bodies; several contain lipochrome droplets. Eosin-methylene blue. ×300.

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blue method), it can be seen that the nuclei of the neurilemma cells are situated internal to the fine collagen fibrils of the sheath and that their cytoplasm forms a delicate reticulated structure around the central axon separating it from the outer connective tissue sheath. This appearance is strongly suggestive of the structure of a medullated fibre, as the reticulation above described closely resembles the neurokeratin network while the outer collagen fibrils correspond to the sheath of Henle. The application of myelin sheath stains has however failed to demonstrate any trace of medullation except in a very occasional fibre which is usually solitary in a non-medullated bundle. These myelinated fibres are more numerous in the neighbourhood of the groups of ganglionic cells, but the myelin sheath is always in a fragmentary condition as if degenerate. In some cases a nerve fibre, myelinated when near a group of ganglion cells, has been observed to lose its myelination and to join a bundle of non-medullated fibres after its myelination has disappeared, but it is of course impossible to know the condition of the axon in such fibres.

In sections of tissue impregnated by Ransom's and Bielschowsky's methods it is seen that each nucleated sheath contains an axis cylinder process, but there appear to be at least two types present. The most numerous axons are rather fine and exhibit the characteristic varicosities of non-medullated fibres (figs. 9, 10, 11). The other type of axon is much stouter and is of uniform calibre throughout as much of its length as can be traced in a single section or even a series of sections (fig. 10). In some of the ganglionic cells silver impregnation reveals intracellular neuro-fibrils which can be seen running together to leave the cell in the emerging axon (fig. 10). By counterstaining Bielschowsky sections by the fuchsin-ponceau-lightgreen method of Masson the structure of the nerve bundles is well brought out, and the central axon can be clearly recognised while the nucleated and connective tissue sheaths are also clearly demonstrated. In spite of the enormous number of ganglionic cells present in the tumour the presence of so many apparently healthy axons is difficult to account for, especially in view of the widespread degenerative changes in the ganglionic cells. Undoubtedly the greater part of the large mass of tumour tissue is made up of bundles of apparently healthy axons with their nucleated and connective tissue sheaths, and it is difficult to conceive that each is connected with a ganglionic cell in the tumour. The highly convoluted course of the nerve bundles and their branching anastomotic character render it probable that each is cut in more than one plane, but the number of nerve bundles is nevertheless remarkable.

In successful silver impregnation preparations, a few structures suggestive of terminal arborisations of axons around certain ganglionic cells have been observed (fig. 10). Occasionally an axon or axons have been observed to form an ill-defined network within the capsule

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of certain ganglionic cells, but it is difficult to be sure of the appearances since in such cases thick sections are of course required and the cytoplasm of the ganglionic cells is generally obscured by a very heavy deposit of silver.

The *interstitial tissue* is of very loose texture, myxomatous in appearance. The essential interstitial cells are of fairly large size and of characteristic stellate shape with long processes which can sometimes be traced along the collagen fibres for a considerable distance. These fibres are of varying thickness and can readily be distinguished owing to their wide separation by the intercellular mucoid matrix. The stellate cells possess a rather vesicular nucleus poor in chromatin and the cell body and processes are well brought out in silver preparations by a fine granular precipitation of the silver. In thick celloidin sections stained with iron hæmatoxylin and van Gieson these stellate connective tissue cells are clearly shown in relation to the fine collagen fibres. The interstitial tissue also contains a considerable number of lymphocytes and mast cells scattered irregularly throughout the mucoid matrix, and in a few places numerous polymorphs are also present. These leucocytic cells are generally more numerous near small blood vessels which course through the interstitial tissue carrying with them a rather firmer and more definite sheath of collagen fibres. Even the smallest vessels have thick well-formed walls, and in the walls of the small arterioles there is an abundance of elastic tissue. Elsewhere in the tumour elastic fibres appear to be completely absent. In the loose intercellular matrix the mucin has been precipitated by some of the fixatives in the form of fine filaments which branch and anastomose with one another, and often appear to form definite networks around the bundles of nerve fibres. With the phosphotungstic acid hæmatoxylin stain these filaments are deep blue, and their finer forms were at first confused with neuroglial fibrils. Further investigation showed that they gave characteristic staining reactions with mucicarmine and mucihæmatin and they were then recognised as precipitation artefacts.

Commentary.

The specimen here described is a typical ganglionic neuroma, and has probably arisen in connection with the abdominal sympathetic chain. Owing to its large size (1020 grms.) the exact point of origin could no longer be recognised with certainty, but it is clear that the growth had not originated in connection with the suprarenal gland or cœliac plexus, as it lay below and behind the lower pole of the right kidney. The literature of the subject has not been investigated fully, but this specimen appears to be the largest on record in which the tissue is of uniformly adult type throughout. In view of the remarkably complete structure and relatively healthy condition of many of

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GANGLIONIC NEUROMA.



FIG. 8.—Bundle of fine nerve fibres showing numerous elongated nuclei of the sheath of Schwann. Nile blue—van Gieson. × 300.



FIG. 9.—Similar fine fibres showing thin axons with varicosities of sympathetic type: surrounding sheaths faintly indicated. Bielschowsky's silver technique. × 300.



FIG. 10.—Ganglionic cells: one shows intracellular neurofibrils converging into emerging axon. Numerous course and fine axons; indications of some pericellular networks. Ransom's pyridin silver method. × 300.



FIG. 11.—Ganglion cell with two emerging axons, another with one axon: in both indications of intracellular neurofibrils. Ransom's pyridin silver method. × 300.

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the ganglion cells, it is strange that the growth did not give rise to more pronounced visceral disturbances, since large bundles of nerve fibres were observed to pass between the growth and the main abdominal sympathetic chain.

Tumours of similar structure were recorded by Ribbert (1904) in connection with the suprarenal glands, and many other examples have been found since that time. Dunn (1915) has collected the literature up to that time, and in reporting two cases discusses the relation of ganglionic neuroma to neuroblastoma. In the present case no trace of neuroblastoma cells has been found; the small lymphocyte-like cells present in the stroma appear to be true lymphocytes and are usually associated with other leucocytic cells; their morphology and arrangement are in no way suggestive of neuroblasts. A very interesting feature of the case is the extraordinary number of nerve fibres, for these elements, consisting of axon and sheath, form the chief bulk of the tumour, and it is difficult to conceive how they have been formed. Though the number of ganglionic cells is great, the number of very fine fibres seems quite out of proportion. It may be that the ganglionic cells, probably functionless, are engaged in constantly adding to the length of the fibres which curl and form the bundles observed, after the manner of what is seen in amputation neuroma. Many of the ganglionic cells may have disappeared but, in this case, one would expect that the fibres would disappear also; degenerated ganglionic cells from which the emerging axon is absent from its sheath have indeed been observed. Some such view of indefinite formation of axons by the ganglionic cells seems necessary, though of course the now generally discarded view of axon formation by neurilemma cells would afford an explanation of the findings in this and similar cases (*see* Bethe, 1903). Falk (1907) and Friedrich (1912), for example, consider that some of the axons in their specimens may have been formed from the neurilemma cells. Pick and Bielschowsky (1911) have shown however that many of the fibres branch, and they indicate that this may be an important factor in producing an apparent excess of fibres. They also observe, as in the present case, that many of the ganglionic cells may have degenerated and disappeared. Adams (1914) described a ganglionic neuroma of fairly adult structure, and commented on the apparent new formation of axis cylinders by neurilemma cells, but in his case one area of primitive cells of neuroblast type was present; it may be noted also that the ganglionic cells exhibited neither capsule cells nor sheaths. Beneke (1901) and Wegelin (1909) have observed sheaths around the ganglionic cells in their specimens and Wegelin noted that the sheath passed out over the nerve fibres and was continuous with the sheath of Schwann. This has been confirmed in the present case. The general absence of myelin sheaths is apparently the usual finding in such growths, but it is interesting to note that the structure of the nerve fibres with a

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"neurokeratin-like" reticulum is more suggestive of myelinated than non-myelinated fibres. The varicosities of the axis cylinder processes on the other hand are characteristic of non-medullated sympathetic fibres. It is probable however that the fibres, being of neoplastic formation, are not perfectly formed, and do not correspond exactly to any normal adult structures.

Summary.

A case is recorded of an unusually large ganglionic neuroma which presents throughout a uniform structure of adult ganglionic cells and nerve fibres. The majority of the fibres are of non-medullated type and the remarkable feature is their enormous number. They possess a well-formed sheath of Schwann and outer connective tissue sheath of Henle. Two varieties of axis cylinder processes have been recognised; coarse relatively uniform fibres, and fine fibres with the varicosities of sympathetic type. The interstitial tissue is unusually myxomatous, but no neuroglial elements other than the neurilemma cells have been recognised. No trace of rosettes or other neuroblastic elements has been found, and the subsequent history of the case confirms the view that the tumour is of simple type.

I am indebted to Mr Logan Taylor for the clinical history of the case. Mr W. W. Galbraith kindly supplied the pyelograms from which figures 2 and 3 are reproduced. The photo-micrographs are the work of Mr John Kirkpatrick of the Pathological Department, Glasgow University, and I desire to thank him for the trouble he has taken to secure satisfactory preparations.

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THREE NOTES ON TUBERCULIN.

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It is necessary to preface the following three notes with the remark that the standard of comparison in all the experiments was a laboratory standard equal in potency to the Frankfurt standard tuberculin. It is also necessary to refer to the paper by the authors on the standardisation of tuberculin (1927) which indicates the limits of accuracy of the methods of measurement employed, and therefore the degree of significance of the results which follow.

I. The stability of Old Tuberculin.

Old Tuberculin, human (T) or bovine (PT), is a remarkably stable preparation in the undiluted state. For example, on intradermic test in the tuberculous guinea-pig no difference was detectable between a sample of standard Old Tuberculin presented to these Laboratories in 1911 by Ehrlich and kept for some years in the cold room and later at room temperature, and the present Frankfurt Standard. This is but one of a series of observations on samples of Old Tuberculin, all of which point to indefinite stability. We have never found any evidence of instability in any sample of tuberculin, T or PT.

Tuberculin bouillon filtrate, human (TOA) or bovine (PTO), which has a glycerine content of approximately 5 per cent. instead of approximately 50 per cent. as in the concentrated product (Old Tuberculin), may be regarded as equivalent to a 1 in 10 dilution of Old Tuberculin in glycerine veal broth. Samples of TOA and PTO have frequently been re-tested after being kept for 2 years at room temperature and have shown no loss of potency, although a few samples of PTO or TOA have lost 40 per cent of their potency after 3 years.

Jowett (1914) states that some investigators have found that diluted tuberculin is unstable and "undergoes more or less profound deterioration as a result of storage." In our hands it has been reasonably stable. A 1 in 10 dilution of Old Tuberculin in saline kept at room temperature for 4 years lost about 50 per cent. of its efficiency. One in 500, 1 in 1000, 1 in 2000 and 1 in 4000 dilutions, kept at 4° C., at room temperature and at 37° C. were slightly weaker after 3 months than dilutions which had been freshly prepared; after 1 year, a difference was readily detectable on a large number of guinea-pigs. It is probable therefore that the loss in potency was about

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60 per cent. There was no significant variation in potency between samples kept in the refrigerator, at room temperature and in the incubator. As a working rule, therefore, it would appear that ordinary dilute tuberculin may be used for at least 1 month after dilution, but preferably not for longer than this.

When undiluted tuberculin, bovine (PT), was autoclaved for half an hour at a pressure of 15 lbs. it was still apparently of full strength. After autoclaving for 2 hours at 10 lbs. we found a loss of potency just detectable by the intradermic test on six guinea-pigs, representing a drop of about 40 per cent. Dilutions of Old Tuberculin, 1 in 500, 1 in 1000, 1 in 2000 and 1 in 4000 were less heat-stable than the undiluted product, after 2 hours at 10 lbs. they had lost at least 60 per cent. of their efficiency.

Similar results were obtained with a sample of Old Tuberculin of quadruple strength which had been prepared by the method of Douglas on potato broth medium.

Conclusions.

(1) Undiluted Old Tuberculin is stable for an indefinite period.

(2) Tuberculin bouillon filtrates (TOA) and (PTO) are stable for at least 2 to 3 years.

(3) Dilutions of 1 in 500 to 1 in 4000 of Old Tuberculin may show a loss in potency of 40 per cent. after 3 months.

(4) Undiluted Old Tuberculin may be autoclaved for half an hour without detectable loss, and after 2 hours may retain 60 per cent. of its original value. Dilutions of Old Tuberculin are less heat-stable but may retain 40 per cent. of their original value after autoclaving for 2 hours.

II. The "Old Tuberculin" content of New Tuberculins.

Koch's New Tuberculin (BE) is essentially a vaccine consisting of pulverised tubercle bacilli suspended in glycerine, and Tuberculin R is an extract of ground-up bacillary bodies. A comparison has been made of BE and TR with Old Tuberculins by injecting suitable doses intracutaneously, subcutaneously and intravenously into tuberculous guinea-pigs.

Altogether 7 samples of BE or (PBE) and 5 of TR from british and foreign sources have been used for the experiments. The strength of the BE was approximately 5 mgrms. of ground bacilli per c.c. By the intracutaneous method of testing, the potency of one of the samples of BE was approximately 1/5th that of a sample of Old Tuberculin of Frankfurt standard potency. Dilutions of 1 in 100, 1 in 200, 1 in 400 and 1 in 800 gave rise to reactions in tuberculous guinea-pigs at 20 hours which approximated to those given by 1 in 500, 1 in 1000, 1 in 2000 and 1 in 4000 respectively of standard tuberculin (= Frankfurt). In normal guinea-pigs which were used as controls

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no reactions were produced. One sample of BE was about 1/10th of the potency of standard Old Tuberculin, three samples were about 1/15th, and two were about 1/20th. Of the five samples of TR, one was approximately 1/15th and two 1/20th; two contained very little Old Tuberculin and were probably 1/50th to 1/100th of standard value.

A suspension of dead tubercle bacilli of strength 5 mgrms. per c.c. induced reactions when suitably diluted which suggested that it was approximately 1 20th of Old Tuberculin standard value.

Heating of dilutions of BE to 100° C. for 20 minutes on 3 consecutive days caused no diminution in the size of the skin reactions obtained by intracutaneous injections. This observation was made in connection with some routine sterility tests on BE. Although no experiments have been made on the stability of BE after autoclaving, this result suggests that the Old Tuberculin present in BE is probably as heat-stable as Old Tuberculin in its usual form.

The same relative potency between the seven samples of BE and five of TR was obtained by the subcutaneous method of testing as by the intradermic. Doses of 0·1 c.c. of Old Tuberculin and 1 to 5 c.c. of the BE or TR were injected into comparable groups of tuberculous guinea-pigs, *i.e.* guinea-pigs which had been infected at the same time and with the same inoculum of virulent culture. The samples of TR which were of low potency by the intracutaneous test were the only ones to fail to kill any of the guinea-pigs which were injected subcutaneously with 5 c.c. doses. The other tuberculins, including the standard Old Tuberculin, killed approximately 50 per cent. of guinea-pigs at the doses selected. At autopsy the dead animals exhibited the well-known signs of death from tuberculin.

In a few experiments in which tuberculin has been injected intravenously, the intravenous dose for Old Tuberculin has been about half the subcutaneous dose, that is, 0·05 c.c. suitably diluted and given intravenously has killed the same percentage of tuberculous animals as 0·1 c.c. injected subcutaneously. The intravenous dose for BE has been less than half the subcutaneous dose. BE or TR which was approximately 1, 20th the potency of standard Old Tuberculin by the intradermic test killed tuberculous guinea-pigs in doses of 0·5 to 1·0 c.c. given intravenously, producing the typical post-mortem appearances of death from tuberculin.

Conclusion.

When samples of BE and TR are injected intracutaneously, subcutaneously or intravenously into tuberculous guinea-pigs, they produce effects which are qualitatively indistinguishable from those caused by Old Tuberculin. BE varies in potency between 5 per cent. and 20 per cent. and TR between 1 per cent. and 5 per cent. of Old Tuberculin.

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III. The development of skin-sensitiveness to Old Tuberculin in the tuberculous guinea-pig.

Fifteen guinea-pigs were infected with 0·5 mgrm. of a virulent human strain (H 12) of *B. tuberculosis*. The animals were injected intradermally with 0·2 c.c. of two or more dilutions of standard tuberculin bi-weekly from 1 to 3½ weeks and then weekly to 9½ weeks after infection. The figures given in the table represent the reciprocals of the highest dilutions of standard tuberculin which gave well-developed positive reactions, approximately 8 mm. in diameter, 16 to 20 hours after injection.

Ten of the 15 guinea-pigs gave definite positive reactions one week after infection at dilutions of 1 in 50 to 1 in 200; 7 of the 15 gave similar reactions 1 week later at 1 in 1000 to 1 in 4000. The development of skin sensitivity to high dilutions of tuberculin varied considerably from animal to animal. In many instances it was exceedingly rapid, the minimum amount of tuberculin necessary to produce a standard reaction falling 20 to 40 fold in 1 week. In one guinea-pig, however, 1 in 50 was the highest dilution which at any time caused a standard reaction, in another 1 in 500, and in two 1 in 1000.

It will be seen from the table that more guinea-pigs were skin-sensitive to high dilutions of tuberculin at about the third week than at any other period of the infection. Although some animals are extremely sensitive for much longer periods to tuberculin given intradermally, the third week is probably the most suitable for tuberculin standardisation if the guinea-pigs are infected in the manner described. Guinea-pigs which were apparently in good condition, had well-nourished skins and showed enlargement of the right inguinal glands (the nearest to the site of infection) tended to be the best reactors. The mode of development of skin sensitivity in this experiment is confirmatory of many casual observations made by the authors in the course of work on the standardisation of tuberculin extending over several years. It should be noted however that many of the reactions recorded after the second or third week are probably less marked than they would have been on other tuberculous guinea-pigs which had not been subjected to bi-weekly depilation and injections. We think that this loss of sensitivity is to be ascribed partly to desensitisation to tuberculin as a result of repeated intradermic injections, and partly to impaired general health of the animals from frequent handling; the mortality in the early weeks from tuberculosis and infections due to organisms of the *Salmonella* group was comparatively high, only 6 of the 15 guinea-pigs living for a longer period than 7 weeks.

There was considerable variation among the individual guinea-pigs in the course as well as in the mode of onset of tuberculin sensitivity.

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Development of skin sensitiveness to tuberculin.

Guinea-pig	Weeks after infection. 1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
1	< 50	D	...	...	...	...	...	...	...	...
2	50	D	...	...	...	...	...	...	...	...
3	50	50	D	...	...	...	...	...	...	...
4	50	2000	2000	...	D	...	...	...	...	...
5	200	2000	2000	...	D	...	...	...	...	...
6	< 50	50	500	D	50	...	...	...	...	...
7	50	1000	1000	500	1000	D	...	...	...	...
8	50	100	4000	4000	4000	D	...	...	...	...
9	< 50	50	4000	1000	2000	500	...	...	...	...
10	< 50	50	100	1000	1000	1000	D	...	...	...
11	50	1000	1000	8000	2000	1000	D	...	...	...
12	50	500	1000	500	500	1000	...	...	...	...
13	50	1000	2000	2000	2000	500	100	...	...	...
14	< 50	100	2000	1000	1000	1000	100	...	...	D
15	100	4000	8000	8000	8000	4000	2000	...	...	1000

Figures represent reciprocals of the highest dilutions of standard Old Tuberculin which gave definite positive reactions. The tests shown under week 1 were done at 7 and 10 days after infection, and so on. D = death of guinea-pig.

Thus, of the only two guinea-pigs which were skin-sensitive to 1 in 4000 2 weeks after infection, one became skin-sensitive to 1 in 8000 at the third week and remained so for 2½ weeks, but the other was sensitive at 1 in 4000 for a few days only, and became markedly less sensitive to the end of the 5th week when it died with post-mortem appearances of advanced tuberculosis. The majority of guinea-pigs have shown a fall in skin sensitivity for some time before death. Reactions which occur in wasting animals shortly before death tend to have an atypical pallor due to the failure of the rather atrophic, poorly nourished skin to give a hyperæmic response; the pallor gives way to necrosis rather than to hyperæmia when lower dilutions are injected.

In conclusion, although the number of guinea-pigs under experiment is small, the results obtained confirm views based on casual observation that sensitiveness develops progressively although often quite quickly once it has started. Frequently guinea-pigs are relatively insensitive or only slightly sensitive one week, and the next week or the week after are perfectly suitable for ordinary testing. The subsequent progress of sensitiveness varies considerably and frequently shows a fall for some time before death.

Boquet and Nègre (1926) state that sensitiveness to tuberculin sometimes appears suddenly in all its intensity and that more frequently it is preceded by a short period of transition (24–48 hours). We have not been able to confirm this opinion by intradermic methods, and it has been our general experience that sensitiveness becomes detectable at the end of a week after infection and advances to a maximum in 2 to 3 weeks.

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THE RELATIONSHIP BETWEEN THE HÆMOLYTIC COMPLEMENT AND THE OPSONIC POWER OF NORMAL GUINEA-PIG SERUM.

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THE relationship between the opsonic power of normal serum and the power to hæmolyse sensitised red corpuscles has been studied by numerous authors and in many instances evidence has been produced to show that the opsonic action of a normal serum involves the action of complement or perhaps a more complex system consisting of a thermostable substance and a thermolabile component of the complement type. If the latter view is correct, the opsonic system may be regarded as being similar to the hæmolytic system in consisting of a relatively heat-stable and probably specific amboceptor and a heat-labile non-specific complement.

Muir and Martin (1906) produced evidence to show that there is a close similarity between normal thermolabile opsonins and hæmolytic complement, for they found that substances which remove hæmolytic complement from normal guinea-pig serum also remove the opsonic power. The absorbing substances used for this purpose were (*a*) sensitised red cells (*b*) serum precipitates formed by mixing antigen and antibody and (*c*) sensitised bacteria, and in all cases the absorption of hæmolytic complement and opsonins were closely parallel. Bächer (1907) and Levaditi and Inmann (1907) produced further evidence pointing to a similarity between normal opsonin and complement, whilst Neufeld and Hüne (1907) showed that treatment of normal serum with yeast cells removes both hæmolytic complement and opsonin.

Other authors however have expressed the opinion that a thermostable opsonic constituent (amboceptor) is concerned, together with complement, in opsonic action. Dean (1907) showed that the opsonic power of heated immune serum was greatly increased by the addition of a small amount of unheated normal serum. Cowie and Chapin (1907, 1) obtained similar results with normal human serum and found that the addition of a small amount of normal human serum could restore the opsonic power of human serum which had been heated at 55° C. for ten minutes. From these experiments the conclusion was reached that the opsonin of normal human serum has a double amboceptor-complement structure like haemolysins and bacteriolysins. Further evidence in favour of this view was presented in a later paper (Cowie and Chapin 1907, 2), when it was shown that *Staphylococcus* treated with heated serum and then washed is more readily opsonised by a normal serum than is the untreated organism. In addition, treatment of normal human serum with staphylococci results, under certain conditions, in the loss of practically all the opsonic power but leaves behind

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a substance which can reactivate a heated serum for opsonic activity. The same authors (1907, 3) submitted further evidence in support of the amboceptor-complement view of opsonic action. They found that *Staphylococcus*, treated with normal human serum at 0° C. and then washed with cold salt solution, is not much more susceptible to phagocytosis in the absence of serum than the untreated organism, but is more readily opsonised by a dilute normal serum or by a serum which had been inactivated by contact with staphylococci in the cold. Thus at 0° C. the bacteria absorb the amboceptor component only of the opsonic system. Browning (1925) states that the taking up of opsonins at low temperatures without the removal of complement may be attributed to the existence of a natural immune-body which plays a part in the opsonic effect, leading to the union of complement, or it may be due to the existence of multiple complements.

It is possible to study what rôle, if any, is played by complement in opsonic action by observing the effect on the opsonic power when complement is "split" into fractions, or when one or more components are inactivated. Simon, Lamar and Bispham (1906) found that the opsonin of serum is associated with the globulin fraction and Heinemann and Gatewood (1912) similarly found that the opsonins of antistreptococcic and antigonococcic sera are precipitated with the globulins. Ledingham and Dean (1912), however, showed that mid-piece as prepared by the CO_2 method was unable by itself to sensitise *B. typhosus* for phagocytosis.

In an investigation of the properties of hæmolytic complement the present authors have shown (1926, 1) that small amounts of ammonia inactivate hæmolytic complement by the almost specific destruction of a component which is relatively heat-stable and which is associated with the albumin fraction of the serum. Thus ammonia-treated serum has no hæmolytic action on sensitised red cells but is reactivated by the addition of serum heated at 56° C. or heated "end-piece" (albumin fraction). It has been shown later (Gordon and Wormall 1928) that ammonia has a similar action on the bactericidal power of normal guinea-pig serum against *B. dysenteriæ* (Flexner), and it was concluded that this bactericidal system consists of hæmolytic complement and an immune-body present in the normal serum. Preliminary investigations of a similar nature (Gordon, Whitehead and Wormall, 1926, 2) showed that the fourth component of hæmolytic complement is not essential for opsonic action, and the investigations described below form an extension of this observation.

Treatment of normal guinea-pig serum with small amounts of ammonia results in the complete loss of hæmolytic action towards sensitised red cells, due to the inactivation of the fourth component of complement, but no corresponding loss of opsonic power occurs. Thus the conclusion is reached that although the other components of hæmolytic complement may function in the opsonic system, the fourth component is not essential.

The treatment of serum with bacteria usually results in the removal of both hæmolytic complement and the opsonic power, and this is quoted in support of the view that the opsonic system of normal serum is a complement or amboceptor-complement system. If suitable

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amounts of the organism (*Staphylococcus aureus* in these experiments) are added to normal guinea-pig serum and the organisms removed by centrifuging after incubation of the emulsion for about 2 hours, it is found that the serum has no opsonic action and no hæmolytic action on sensitised red cells. The centrifuged bacteria however, after being washed with 0·9 per cent. NaCl solution, are readily phagocyted, and the loss of opsonic power by the serum is attributed to the absorption or adsorption of the opsonins by the bacteria. If the view expressed above is correct—that the fourth component of complement is not essential for opsonic action—then the fourth component of complement need not necessarily be absorbed by the bacteria, and indeed the non-removal of this component by the bacteria would furnish evidence in support of the hypothesis. Experiments carried out to test this point have shown that treatment of the serum with bacteria under conditions which lead to the complete loss of hæmolytic and opsonic functions does not cause the removal of the fourth component of complement from the serum. The bacteria, which are removed from the serum by centrifuging, are however fully sensitised for subsequent phagocytosis, and the conclusion is drawn that these bacteria have removed the whole of the opsonic system; this system therefore does not include the fourth component of complement.

Experimental.

To determine the opsonic power of the normal or treated guinea-pig serum 0·1 c.c. of an emulsion of *Staphylococcus aureus* was added to 0·5 c.c. of the serum in a centrifuge tube; the mixture was then incubated at 37° C. for 1 hour and washed guinea-pig leucocytes added. After a further incubation for 1 hour, films were made, stained and examined for phagocytosis. For our purpose it sufficed to determine only the presence of a good phagocytosis or the absence of phagocytosis and it was not found necessary to make opsonic counts.

The hæmolytic complement activities were determined by diluting the sera with 0·9 per cent. NaCl solution and adding various amounts of the diluted sera to 1 c.c. of 4 per cent. sensitised ox cells. The total volume in each tube was adjusted to 2·0 c.c. where necessary by the addition of 0·9 per cent. NaCl solution, and the tubes were then incubated at 37° C. The amount of hæmolysis after incubation for ½ hour, ½ hour and 1 hour was recorded thus:

++++ complete hæmolysis. − no hæmolysis.
+++, ++ or + intermediate grades of hæmolysis.

The action of ammonia on the opsonin and complement of normal guinea-pig serum.

In the earlier experiments normal guinea-pig serum was treated with one-quarter of its volume of N/0·5 NH_3 solution, but it was found later that treatment with a smaller volume of stronger ammonia solution was preferable for the opsonic experiments. To inactivate the hæmolytic complement, 3 c.c. of guinea-pig serum were treated with 0·05-0·10 c.c. of 0·87 N. NH_3 solution and the mixture incubated at 37° C. for 1½ hours. The serum was then neutralised by the addition

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of the necessary amount of N. HCl (determined by the titration of an aliquot portion) and then tested for opsonic and hæmolytic action. The following is a typical result from a large number of experiments in which the amount of NH_3 used and the time of incubation were varied.

Opsonic power.—No differences could be detected between the opsonic powers of normal and ammonia-treated sera. Controls with 0·9 per cent. NaCl solution showed no phagocytosis.

Hæmolytic activity—

Hæmolysis of sensitised red cells after incubation for 1 hour :—

1·0 c.c. normal guinea-pig serum (1 : 10)	++++
0·5 c.c. „ „ „ (1 : 10)	++++
1·0 c.c. NH_3-treated guinea-pig serum (1 : 10)	−
0·5 c.c. „ „ „ (1 : 10)	−
0·5 c.c. „ serum (1 : 10) plus 0·5 c.c. heated serum	++++

The heated serum was guinea-pig serum heated at 56° C. for 30 minutes and then diluted with 9 volumes of 0·9 NaCl solution.

These experiments show that destruction of the fourth component of complement by ammonia results in a complete loss of hæmolytic action for sensitised cells, whereas no detectable loss of opsonic power occurs.

Removal of hæmolytic complement and the opsonins of normal guinea-pig serum by bacteria.

A heavy emulsion of *Staphylococcus aureus* was added to normal guinea-pig serum and the mixture incubated at 37° C. for 2 hours with frequent shaking. The bacteria were then removed by centrifuging for 1 hour at high speed, and the opsonic and hæmolytic powers of the supernatant serum determined. The NH_3-treated serum was prepared as described for the preceding experiment.

Opsonic activity	Normal guinea-pig serum	Good.
	NH_3-treated guinea-pig serum	Good.
	Serum treated with bacteria	None.

The bacteria removed from the last serum were washed with 0·9 per cent. NaCl solution and were found to be readily phagocyted in NaCl solution.

Hæmolytic activity.—All the sera were diluted with 9 volumes of 0·9 per cent. NaCl solution.

Hæmolysis of sensitised red cells after incubation for 1 hour :—

1·0 c.c. normal guinea-pig serum	++++
0·5 c.c. „ „ „	++++
1·0 c.c. NH_3-treated guinea-pig serum	−
0·5 c.c. „ „ „	−
0·5 c.c. „ serum + 0·5 c.c. heated serum	++++
0·5 c.c. „ „ + 0·5 c.c. bacteria-treated serum	++++
0·5 c.c. „ „ + 0·5 c.c. heated bacteria-treated serum	+++ or ++++
1·0 c.c. bacteria-treated serum	−
0·5 c.c. „ „	−

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From these results it will be seen that neither NH_3-treated serum nor serum treated with bacteria had any hæmolytic complement activity. When combined, however, they show strong hæmolytic action towards sensitised red cells. Thus the treatment of normal guinea-pig serum with *Staphylococcus aureus* in amounts sufficient to remove the opsonic power does not result in the removal of the fourth component of complement.

Summary.

Destruction of the hæmolytic complement activity of normal guinea-pig serum by small amounts of ammonia does not reduce to any significant extent the opsonic power of the serum for *Staphylococcus aureus*. Incubation of the normal serum at 37° C. for 2 hours with this organism in amounts sufficient to remove the opsonic and hæmolytic complement activities does not remove the fourth component of complement. The conclusion is drawn from this evidence that one component of complement at least, the fourth component, is not essential for the opsonic action of the normal serum.

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THE NERVOUS SYSTEM IN RATS FED ON DIETS DEFICIENT IN VITAMINS B_1 AND B_2.

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(PLATE VII.)

SINCE Eijkman (1897) first described polyneuritis in hens as the result of an exclusive diet of polished rice a very large amount of work has been carried out on the relations between dietary deficiencies and lesions of the nervous system.

The pathological changes originally described by Eijkman consisted of non-inflammatory atrophic degeneration of the medullary sheaths of the nerves with chromatolysis and atrophy of the ganglion cells in the anterior horn of the cord. Vedder and Clark (1912) confirmed these results in fowls, chromatolysis being found both in the nerve cells of the cord and dorsal root ganglia together with degeneration in the medullary sheaths of the peripheral nerves and in the nerve roots and tracts of the cord. Kimura (1919), M'Carrison (1919) and Findlay (1921) obtained similar results in rice-fed birds. M'Carrison and Findlay however both found that complete starvation in pigeons and fowls led to chromatolysis in the nerve cells and degeneration of the medullary sheaths of the nerves, confirming the earlier work of Chamberlain, Bloombergh and Kilbourne (1911) and Eijkman and van Hoogenhuyze (1916). Culley (1927) believed that the degeneration of the medullary sheaths of the nerves in fowls fed on polished rice is not identical with Wallerian degeneration.

Polyneuritis with similar lesions has also been produced in mammals by deficient diets. Thus Shiga and Kusama (1911) fed monkeys on boiled rice and caused degeneration of the peripheral nerves as did M'Carrison (1919). Andrews (1912) produced degenerative lesions in the nervous system of puppies by feeding them on the milk of women whose children had died from beriberi, while Voegtlin and Lake (1919) produced polyneuritis in cats and dogs in from four to six weeks by feeding them on a diet of lean beef heated for three hours at 120° C. in the presence of alkali. The sciatic nerves of these animals stained by the Marchi method showed all stages of myelin degeneration.

When rats, on the other hand, are fed on diets which are complete except for the antineuritic vitamin, polyneuritis is only produced in a small number of animals, the others dying with symptoms of inanition only. Examination of the nervous system in rats fed on diets lacking the antineuritic vitamin has been carried out by various observers. Schaumann (1910) produced paralysis of the hind legs by feeding rats on an exclusive diet of horseflesh which had been sterilised at 120° C. Examination of the nerves showed no typical degeneration of the myelin sheaths, but a foamy appearance which was considered to be evidence of the breaking up of myelin. Hofmeister (1922) fed rats on a diet which was deficient only in vitamin B. In a few animals a spastic condition of the limbs occurred, accompanied in some cases by tremors and convulsions, but in most there was at first a definite ataxia of the hind limbs followed within

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a day or two by paralysis. In the nervous system there were no degenerative changes in the peripheral nerves with the exception of slight swelling of the myelin sheaths in three rats and an increase in the nuclei of the sheath of Schwann in two others. In the brain, brain stem and cerebellum small capillary hæmorrhages were however observed, the changes corresponding roughly to the severity of the symptoms observed during life, since hæmorrhage was absent in those rats which had not shown any nervous symptoms. Degenerative chromatolytic changes were present in the nerve cells of the brain. Hofmeister regarded the hæmorrhages as evidence of a hæmorrhagic encephalitis in spite of the absence of inflammatory change. Woollard (1926) confirmed Hofmeister in so far as the absence of Marchi degeneration in the cord and nerves of rats on a diet deficient in vitamin B is concerned. There was an absence of chromatolysis in the nerve cells of the anterior horn of the cord but changes were present in the fine intermuscular medullated motor and sensory nerves: their terminal ramifications were swollen, particularly in the most peripheral parts which showed loss of fine differentiation, the myelin being irregular in outline. Similar changes though of a less pronounced character were however noted in rats which had been completely starved except for water for from four to seven days and in rats also which had been given marmite as their sole source of nourishment. Woollard suggests that the more pronounced manifestations of experimental beriberi may be explained by the different duration of the conditions and that beriberi occurring in rats may be equal to starvation from inability to assimilate food in the absence of the appropriate vitamin. In this connection it is interesting to note that Sundwall (1917) has confirmed in the rat the findings of earlier workers on pigeons and hens in regard to the production of nervous lesions by simple inanition. Examination of the spinal cord disclosed congestion of the vessels, vacuolation of the cellular elements of the cord suggesting extreme œdema and certain abnormalities of the anterior horn cells, some of which were shrunken and stained darkly whilst in others the vesicular appearance of the cytoplasm was presumptive evidence of chromatolysis. In a recent communication Tsunoda and Kuro (1923) have noted swelling in the nerve-endings of pigeons fed on a diet of polished rice. After the administration of the antineuritic factor the swelling is said to have disappeared.

The exact interpretation of the lesions produced by lack of vitamin B has been rendered still more difficult by the work of Goldberger, Wheeler, Lillie and Rogers (1928) who have shown conclusively that vitamin B obtained from yeast consists of at least two factors, one thermolabile which prevents and cures polyneuritis in birds and rats, the other thermostable, lack of which leads to the appearance in rats of a condition resembling, according to Goldberger and Lillie (1926), pellagra in man and black tongue in dogs. Since both vitamin B_1 (the antineuritic factor) and vitamin B_2 (the antipellagra factor) are present in autolysed yeast, it is not possible by feeding rats on a diet containing no vitamin B but consisting of caseinogen, starch, cod liver oil and inorganic salts to determine the nervous lesions due solely to deficiency of either vitamin B_1 or vitamin B_2. The following investigation was therefore undertaken to determine the changes produced in the nervous system by diets deficient in either vitamin B_1 or vitamin B_2.

Technique.

The pathological material was obtained from a series of experiments which have previously been described (Findlay 1928). Young rats were placed on the following basal diet:—

Purified caseinogen	25 parts
Rice starch	70 „
Salt mixture (M'Collum and Davis 1915)	5 „
Cod liver oil	0·2 grm. per rat per day.

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VITAMIN B DEFICIENCY.



FIG. 1.—Anterior horn of spinal cord: group C: toluidin blue. Several swollen vacuolated nerve cells, distorted in shape. Distribution of Nissl granules irregular except in two cells below. (Rat 49. × 350.)

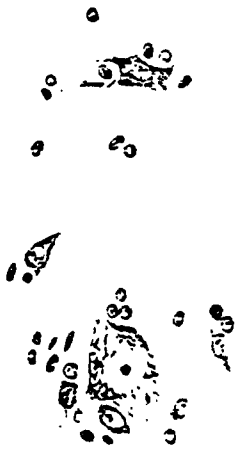


FIG. 2.—Anterior horn of spinal cord: group C: toluidin blue. Two large nerve cells: in lower, Nissl bodies fairly distinct, but cell swollen and surrounded by many satellite cells: in upper, much distortion of cell body, Nissl granules have disappeared, many satellite cells. (Rat 50. × 350.)



FIG. 3.—Anterior horn of spinal cord: group C: rat had chronic skin lesions. Scharlach R and hæmatoxylin. Cells distorted in shape, contain many vacuoles: in cell in middle of field an accumulation of lipochrome pigment, surrounding almost unrecognisable nucleus. (Rat 52. × 350.)



FIG. 4.—Anterior horn of spinal cord: group C rat had acute skin lesions: toluidin blue. Slight swelling of nucleus and cell body in large nerve cells, with an increase in satellite cells. Probably represents earliest stage of degeneration. (Rat 51. × 350.)

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The animals were divided into four groups.

Group A: basal diet alone: *lacking* B_1 *and* B_2. Two out of ten rats showed symptoms of paralysis which appeared 24 hours before death. Average duration of life 34 days, the animals being killed when moribund.

Group B: 0·5 grm. per day per rat of yeast autoclaved at 120° C. for 5 hours: *lacking* B_1. Paralysis appeared in six out of ten rats, the first symptoms occurring 2 to 3 days before death. Average duration of life 37 days.

Group C: 0·2 c.c. per day per rat of Peters' (1924) yeast extract: *lacking* B_2. This group was further subdivided; some rats were killed when they showed definite skin lesions, others were given a little dried yeast and then allowed to relapse on the diet without B_2: by this means it was hoped to produce a condition resembling more closely the chronic form of pellagra in man. In group C no nervous symptoms were noted except in one rat which had peculiar side-to-side movements of its head; it died three hours later despite the administration of yeast extract and at the post-mortem a severe hæmorrhage into the stomach was found but no macroscopic changes in the nervous system.

Group D: yeast extract and dried yeast: *receiving* B_1 *and* B_2. The animals developed no symptoms and served as controls.

The weight changes are given by Findlay (1928, p. 357); the controls nearly doubled their weight but the others grew little or not at all or declined.

Peripheral nerves were fixed in Müller's fluid and thereafter used for the study of π granules by the toluidin blue method and for myelin degeneration by the Marchi method. Portions of the cervical dorsal and lumbar regions of the spinal cord were also stained by the Marchi method. Similar portions of the spinal cords and brains were sectioned by the freezing method and stained with toluidin blue for Nissl granules and with scharlach R and alum hæmatoxylin for fatty changes and lipochrome pigment. Celloidin and paraffin sections of the brains and cords of many of the animals were made and stained with toluidin blue for cellular detail. Portions of the cord and brain were also fixed by the Schridde method for studying the mitochondria. In addition to the control animals in group D, other rats fed on a laboratory diet rich in vitamins were killed by coal gas or by bleeding to exclude the possibility of any nervous changes being due to the method of killing.

Pathological changes.

In group A, consisting of rats which had been deprived of both vitamins B_1 and B_2, the changes found in the nervous system were slight. There was no pathological evidence of polyneuritis corresponding to the clinical symptoms. The only abnormality seen in the sciatic nerve was an increase in the π granules of Reich. These are small granules which lie scattered along the sheath of Schwann and which stain metachromatically with toluidin blue. Attention was called to their presence in human pellagra by Mario Zalla and Kinnier Wilson (1914), who pointed out the great increase in their number in that disease. They were considered to be an index of myelin metabolism and their increase an evidence of an early degenerative change. The increase of these granules in the nerves of this group was less than in either of the groups deprived of only one factor of vitamin B. The shorter duration of life may account for this. There was no degeneration seen in the nerves by the Marchi method. The spinal cords of

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animals in this group showed early chromatolysis in some of the anterior horn cells, whilst others were shrunken, had lost their processes and stained darkly and diffusely. There was no increase in glial tissue nor any cellular infiltration. Marchi preparations were negative and there was no accumulation of lipochrome pigment in the nerve cells. The spinal cords of this group in common with those of the other groups, including the normal group, were greatly congested, but this was due probably to the effect of the coal gas asphyxia. The brains appeared normal.

In group B, which received only the B_2 factor, the sciatic nerve contained large numbers of π granules. In one rat which had shown symptoms of paralysis for as long as twelve days a foamy appearance was seen in Marchi preparations of the sciatic nerve. According to Schaumann (1910) and Voegtlin and Lake (1919), this is the earliest sign of myelin degeneration given by the Marchi method. It is therefore reasonable to suppose that if the other animals of this group had survived longer similar degeneration might have been seen. In the spinal cord the changes in the nerve cells were similar to those in group A but more advanced. Most large cells were shrunken and stained darkly, the Nissl granules being generally indistinct and "powdery" in appearance. In some there was a clumping of Nissl substance round the nucleus. Eccentric nuclei were rare, but many cells had lost their processes and had become rounded. There was a moderate increase in the number of satellite cells around the anterior horn cells but no general cellular infiltration of the cord. Marchi preparations were negative and again in this group there was no accumulation of lipochrome pigment in the nerve cells. The brains appeared quite normal.

In group C, which received B_1 but not B_2, the changes in the sciatic nerve consisted solely in a large increase in the number of π granules. As there had been no nervous symptoms of any kind it was not surprising to find no demyelination by the Marchi method and the increase in π granules was regarded as evidence, not of a degeneration, but of a functional exhaustion, such as occurs in senility, in which there is a great increase in π granules. The spinal cord presented a striking appearance. The large nerve cells were swollen, much vacuolated and distorted in shape. The Nissl substance did not form recognisable granules in most cells. In some it occurred in threads, in others it had completely disappeared, leaving a pale "honeycomb" cell. In certain of the rats some groups of cells were definitely more affected than others. The number of satellite cells around the nerve cells was enormously increased and many nerve cells were completely surrounded by them. Marchi preparations were normal. In the nerve cells of the rats which had survived for some months after the appearance of the skin lesions, there was a considerable accumulation of lipochrome pigment and a smaller but definite amount was found

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in the cells of the cerebral cortex of the same animals. The brains otherwise appeared normal, there being no hyaline changes in the blood vessels.

Mitochondrial changes.

Vedder and Clark (1912) were unable to determine any changes in the mitochondria of the nerve cells of fowls suffering from polyneuritis, results which were similar to those of Clark (1914). Ma (1925) however showed that in certain of the spinal ganglion cells of fowls with beriberi the mitochondria had changed from short rods to tiny granules and had also decreased in number.

Examination by the Schridde method of rats deprived of vitamins B_1 and B_2 showed no qualitative or quantitative changes in the mitochondria of the nerve cells of brain or cord. There was a similar absence of change in the brains and cords from rats deprived of vitamin B_1 alone. The brains of rats deprived of vitamin B_2 also showed no abnormality in the mitochondria, but in the cords the mitochondria of many nerve cells were reduced in number and were small and granular in shape, in contrast to the more rod-shaped mitochondria found in the ganglion cells of control rats.

Discussion.

The result of the examination of the nervous system of rats which have been fed on diets lacking one or both of the factors making up the vitamin B complex shows that such diets produce results of a totally non-specific character. The absence of degenerative changes recognisable by the Marchi method in the peripheral nerves agrees with the observations of Hofmeister and Woollard. This absence of myelin degeneration in the peripheral nerves of the rat is in striking contrast to the myelin changes found in the peripheral nerves of other mammals and of birds as the result of deprivation of vitamin B.

Absence of the B_1 antineuritic factor alone produced for the most part chromatolysis in the nerve cells only, though in the sciatic nerve of the rat which had been paralysed for twelve days there was evidence by the Marchi method of very early degeneration.

The main point of interest in regard to the nervous lesions found in rats fed on the diet lacking vitamin B_2 lies in the resemblance of these lesions to those found in human pellagra. According to Winkelman (1926) the essential nervous lesions of human pellagra consist in a primary degeneration of nerve cells throughout the brain and spinal cord with the deposition in them of large quantities of a fatty substance and a hyaline degeneration of the blood vessels with the deposition around them of the same fatty material. This fatty substance is presumably similar to the yellow pigment described by Kozowsky (1912) as occurring in large quantities in the nerve cells in human pellagra, the accumulation of which he considered to be the

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most constant and general alteration in the nervous system in this disease. In our material also, pigment of a lipochrome nature was present in the nerve cells of certain of the rats lacking vitamin B_2. The amount of pigment present was directly proportional to the length of time that the animal had been on the diet and it was absent in those rats which had been killed during the first acute attack of dermatitis. The largest amount was found in the nerve cells of a rat which had survived for a year on the diet, though lacking hair and appearing prematurely old. In this rat there was fatty degeneration of the heart muscle and early atheroma in the aorta. Normal rats of the same age did not show any lipochrome pigment in the nerve cells.

The presence of π granules in nerves is not a lesion peculiar to pellagra, since they are present in many other conditions, including spinal syphilis (Doinikow). The finding of these granules in the nerves of this series of rats is of interest only in that it shows that there is some alteration in myelin metabolism.

The great vacuolation and swelling of the anterior horn cells of the spinal cord which was so constantly found in our animals whose diet was deficient in B_2 was similar to one of the types of degeneration of nerve cells described by Kozowsky in human pellagra. The satellitosis also was comparable with that found in human pellagra. Sundwall (1917) also obtained identical changes in the anterior horn cells of rats deprived of all food, though he makes no mention of satellitosis. In a recent communication Denton (1928) has compared the nervous changes in acute human pellagra with those of black tongue in dogs and has found that chromatolytic change was the only constant lesion in both conditions. This agrees with our findings in rats deprived of vitamin B_2.

The three findings, accumulation of lipochrome pigment, the presence of π granules in the peripheral nerves and changes in the ganglion cells of the cord, if considered together, bear some resemblance to the lesions of the nervous system in human pellagra, while the skin lesions described in the same series of rats (Findlay (1928)) resemble very closely those of human pellagra.

Summary.

1. Rats fed on a diet deficient in both vitamin B_1 and B_2 exhibit only slight chromatolytic changes in the ganglion cells of the cord.

2. Rats fed on a diet deficient in vitamin B_1 exhibit the same changes but to a greater extent for the same duration of life. In addition, when symptoms of paralysis are of long standing, early degeneration may be found in the myelin of the peripheral nerves.

3. The histological changes found in the nervous system of rats fed on a diet lacking vitamin B_2 consist in swelling and vacuolation of the anterior horn cells of the spinal cord with the deposition in them of

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lipochrome pigment, a noticeable increase in the surrounding satellite cells and an increase in the number of π granules in the peripheral nerves.

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A CASE OF DIABETES MELLITUS WITH ACROMEGALY AND LIPÆMIA.

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Department of Pathology, Aberdeen University.

(PLATE VIII.)

On first admission (to Aberdeen Royal Infirmary) the patient, a married woman, was 39 years of age. Four and a half years before the cervix uteri had been amputated for prolapse and two years before a "tumour," histologically a chronic mastitis, had been removed from the left breast. Since the latter date, she had complained of general weakness tiredness and breathlessness and, for the four months prior to admission, of thirst and polyuria as well. In the last six months failing eyesight had been complained of, and she had had pain at the back of the eyes. Menstruation had occurred only once in the previous two years, and had been irregular, once in six to eight weeks, "for years" prior to that. She was often rather constipated.

Examination on admission (26 9/25):—Height 5 ft. 2 in. Weight 7 st. 13 lbs. The skin and mucous membranes were very pale. The hands were slightly spade-like, but patient stated they had always been like that. The lower jaw was somewhat protruding and the nose coarse. The hair of the head was thick. There was some mastitis in both breasts.

Urine S.G. 1040: 82 oz. in 24 hours: sugar 19·8 gns. per oz.: trace of acetone: no albumen (faint trace on two days only during rest of stay in hospital).

Blood sugar curve: (28/9/25) 0·312 per cent., 0·387 per cent., 0·353 per cent., 0·309 per cent.

Ophthalmologist's report: fields of vision and fundi normal: spectacles required.

X-ray examination: large sella turcica, irregular in outline.

Fractional test-meal: no abnormal findings.

Wassermann reaction negative.

Other systems: nil important noted.

There was no diabetic family history.

Patient improved on a basal diet (blood sugar, 20/10/25, 0·173 per cent.), the urine became sugar free and eventually acetone free, and she was discharged (28/10/25) on a basal diet plus 1 oz. of cheese daily.

She was re-admitted to hospital almost three years later (26/8/28). She had been unconscious for 24 hours. No detailed history of the period since her last admission was available, but she had not been very well for several months. Her temperature was normal.

Urine: acid: albumen 0·75 per cent.: practically no chlorides: sugar 48·7 gns. per oz.: acetone + + + : fairly numerous hyaline and granular casts.

Patient was given 60 units of insulin but died the same day.

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Post-mortem examination.

There was slight bony enlargement of the face, especially of the nose and lower jaw. The hands and feet were slightly enlarged. There was no marked stubbiness of the fingers. The body was well developed and well nourished. The *blood* was mostly fluid and very pale pink: on standing, it soon separated into a normally coloured portion and a white supernatant layer of fat. Portions collected in test-tubes from various levels of the (recumbent) body showed varying depths of this fat layer, but on the average the layer of fat was rather deeper than the underlying blood (fig. 1). Very little of the blood had clotted save in the heart, where large clots almost filled the dilated right auricle and the right ventricle. These heart clots were almost dead white and on lying exuded a milky fluid. The smaller vessels of the meninges showed as opaque white lines from the contained fat.

There was a small amount of *pericardial exudate* which had an appearance almost exactly like milk (fig. 1). *Heart* muscle anæmic. *Aorta* showed marked superficial fatty change. *Lungs* congested, with a little mucus in the larger bronchi.

Kidneys enlarged: capsules stripped readily: outer and cut surfaces pale: the cortex broadened, and the appearance that of a "subacute nephritis." *Liver* enlarged, anæmic and fatty. Wall of *gall-bladder* not thickened, but it contained five facetted mixed stones of the ordinary type. Bile ducts patent.

Stomach greatly dilated; contained a large amount of altered blood. Its mucosa was catarrhal throughout. On each side of the lesser curvature, 2 in. below the œsophageal opening, there was a recent active ulcer. Both ulcers were oval and over ½ in. in long diameter. Two other ulcers, almost healed over, were seen lower down, also near the lesser curvature. No chemical irritant could be found in the gastro-intestinal contents.

Spleen was moderately enlarged, pale pink, soft though not diffluent. Much of the cut surface was of a pinkish gelatinous appearance, and slightly sticky, resembling blood clot of poor clotting quality.

Thyroid: distinctly enlarged, symmetrical, of uniform normal consistence.

Pituitary: greatly and asymmetrically enlarged (fig. 3), of uniform soft consistence and pale yellow colour. Its left half showed a smooth upward projection about the size of a hazel nut. The dura overlying this projection was complete, but greatly thinned and adherent. The overlying brain substance showed a localised hollow corresponding to the projection. The optic nerves were slightly elongated. The right optic nerve passed back clear of the projection, but the left was stretched over its left margin. The optic chiasma was displaced slightly backwards and lay behind the tumour. Further dissection showed

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DIABETIC LIPÆMIA WITH ACROMEGALY.

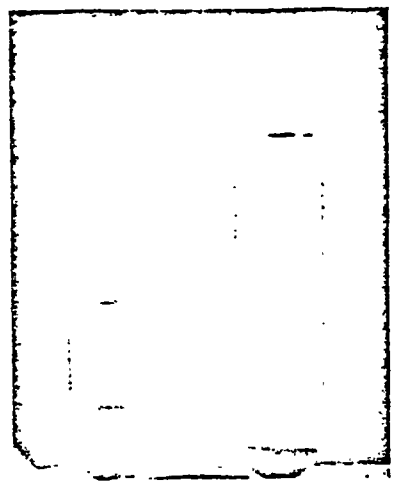


FIG. 1.—Left: blood from femoral vein. Right: pericardial fluid mixed with a little blood.

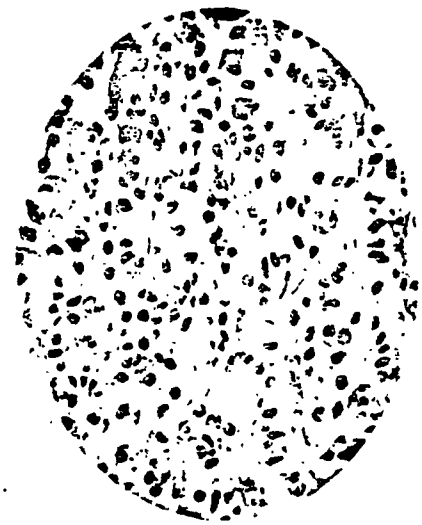


FIG. 2.—Pituitary adenoma: tall columnar cells: thin-walled vessels.



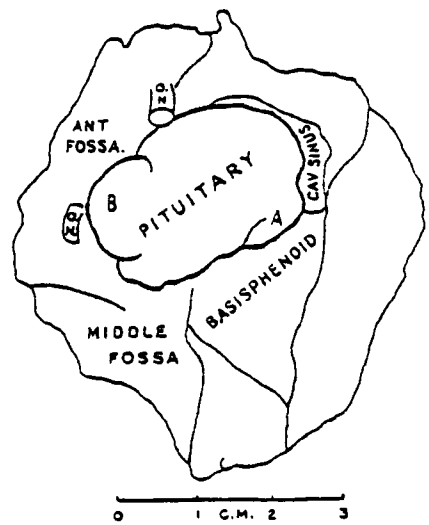


FIG. 3.—Pituitary after removal of part of the egg-shell bone and dura of the dorsum sellæ.

especially of the slightly enlarged. The body was well fluid and very pale ly coloured portion ected in test-tubes varying depths of was rather deeper e blood had clotted the dilated right were almost dead ller vessels of the tained fat.

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fig. 3), of uniform showed a smooth ne dura overlying d adherent. The corresponding to ated. The right out the left was displaced slightly dissection showed

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that the gland projected on both sides into the cavernous sinuses, displacing the carotid arteries downwards and laterally. The posterior clinoid processes were eroded, that on the right side having almost completely disappeared. Posteriorly, the gland had thinned out the dorsum sellæ till only a thin transparent egg-shell of bone survived on the left side, whilst on the right side, where the backward projection was greater, even this had disappeared, and the bony layer became continuous with a thin but complete dural covering. The sella was greatly increased in depth also. The surface of the gland was smooth and lobulated by the upward projection on the left side (B) and the backward projection on the right side (A). There was no perforation of the dura.

Greatest transverse measurement . . .	2·9 cms.
Greatest antero-posterior measurement . .	2·7 cms.
Greatest depth	2·4 cms.

Suprarenals: normal size; cortex appeared to the naked eye to contain a normal amount of lipoid. *Ovaries*: atrophic. *Pancreas*: appeared normal to the naked eye.

Microscopic examination.

Blood. The fat present was in the form of droplets taking sudan III and scarlet R readily, and staining a bluish pink with nile-blue sulphate. A smaller amount of fat was shown in osmic acid preparations. Anisotropic material was present, but was very scanty. All the droplets were very small.

Liver. The liver showed a fine fibrosis of slight degree and rather patchy distribution. There was only a slight degree of increase of connective tissue in the portal tracts, most of the new connective tissue being intralobular, lining the sinusoids. Circumscribed patches of liver tissue here and there near the centres of some lobules showed marked fatty infiltration without nuclear degeneration. These patches showed fat in sudan, scarlet, nile-blue and osmic acid sections, as also did the lumen of the vessels, both the sinusoids and the larger vessels: the amount of fat in the vessels was very striking. Similar appearances were seen in the vessels of the kidney, heart and other organs.

Heart. Nothing abnormal save the fat in the vessels.

Spleen. The sinusoids contained a fair amount of fatty material, stainable with sudan, scarlet and nile-blue, and with osmic acid to a less extensive degree. The nuclei of many of the reticulo-endothelial cells stained poorly. The Malpighian bodies were rather small and were interpenetrated by an increased number of reticular cells. In frozen sections, there was a large amount of granular pigment in the splenic pulp, taking the sudan stain faintly (much scantier in paraffin sections). The striking feature was the presence of numerous large round and very faintly staining globules in the sinusoids. These were

from two to three times the diameter of red blood corpuscles and were mostly non-nucleated. In paraffin sections stained with hæmalum and eosin they showed merely as clear areas bounded by a thin rim taking on the hæmalum stain faintly. With Weigert's iron hæmatoxylin, they were rather better defined, and though still only the rim was stained in most cases, in others there was a faint granular grey staining throughout. Save for a faint greyish staining of the periphery, they did not stain with osmic acid. In frozen sections stained for fat, they did not stain at all with sudan, scarlet or nile-blue, taking up rather the nuclear (hæmalum) stain throughout, much more deeply than in paraffin sections stained with hæmalum. The material was therefore apparently of a somewhat similar nature to myelin. No globules of anything like similar size or staining reactions were found in the other organs or in the blood. The small globules of fat in the blood of the spleen, as in the blood elsewhere, showed readily with all the fat stains. There were also present in the splenic sinusoids, in smaller numbers, definite large mononuclear cells, of the same size and the same spherical shape as these globules. The cytoplasm of these also was clear except at the very periphery (in hæmalum stained paraffin sections), and with other methods it gave identical reactions to those detailed above for the globules, save for at times a rather deeper staining with iron hæmatoxylin. These mononuclear cells had very small eccentric nuclei, often compressed against the margin of the cell to produce a signet ring appearance, and, as transitional forms with vague nuclei were found, it was concluded that the globoid bodies represented similar cells whose nuclei had disappeared or were in some cases not in the plane of section. Occasional transitional forms were observed also between these mononuclears and the splenic cells—the reticular rather than the endothelial. Apparently the condition was a splenic reaction to the presence of lipæmia such as has been recorded in a number of diabetic lipæmias (see Dyke, 1928). The staining reactions of the cells in the recorded cases vary considerably, and those in the present case approximate most closely to those in Oppenheimer and Fishberg's case, where the lipoidal cells of the spleen did not stain with sudan or Smith-Dietrich although their cytoplasm dissolved out in the preparation of paraffin sections. The material was also apparently not unlike that in Dyke's case of hypercholesterolæmic splenomegaly. The appearances in my case are however modified by the widespread degeneration of the cells concerned. It seems surprising that the lipoid should remain "masked" in spite of such degeneration.

Kidneys. A "subacute nephritis" was present, with tubular catarrh and fibrous thickening of the capillary walls throughout practically all the glomeruli, and a similar finely distributed increase of connective tissue between almost all the tubules of the cortex. The uniformity of the picture did not correspond exactly with the ordinary

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types of subacute nephritis and inclined one rather to describe the condition found as a fine cirrhosis of the kidney, of a type analogous to that found in the pancreas and liver. The interstitial tissue did not show anywhere appreciable accumulations of lymphocytes or plasma cells.

No *anisotropic material* was present in sections of any of the above organs, save in the blood, where it was very scanty.

Pituitary. Sections showed an adenomatous growth reproducing closely the normal appearance of anterior lobe tissue (fig. 2). The cells were arranged in typical groups and columns, there was a supporting network of fine connective tissue and there were numerous thin-walled blood vessels like those of the normal gland. A large number of cells were of columnar shape, often tall columnar, and in places the demarcation between individual cells was difficult to make out. The nuclei varied somewhat in size. In all these features, the tumour corresponded completely with the descriptions of Bailey of typical chromophobe-celled adenomata. Though here and there, there were a few multinucleated cell forms more suggestive of the chromophil adenoma (α granule type, "eosinophile") these were very scanty, and the predominant appearance in every field was that of the chromophobe adenoma. Specific granule stains confirmed this impression. The great majority of the cells were chromophobe, but a minority showed a fine dusting with small α granules (acid violet-acid fuchsin stain, Mallory's phosphotungstic acid hæmatoxylin). Eosin-stained sections showed a uniformly pink cytoplasm to all the cells, but this was not regarded as of assistance in differentiating the type of cell. The outer surface of the tumour was covered over by a very thinned-out layer of anterior lobe substance, compressed so as to appear laminated.

Pancreas. The striking feature was a moderately marked but universal fine cirrhosis, similar to that in the liver but more widely distributed. Individual acini were all more or less separated up by a network of delicate but not very cellular connective tissue. Around the larger ducts also there was an increase of connective tissue, but the change was not specially marked there. Islets were identified (tail of pancreas) in very small numbers only. They were small, with somewhat shrunken cell nuclei, and the capillaries within them had thickened walls.

Suprarenals. The cortical cells contained a fair amount of fatty material, the cells of the deeper parts containing much more fat than the superficial layers. Only a small proportion of this fatty material was anisotropic. The medulla showed a slight diffuse increase of connective tissue, but was otherwise normal.

Thyroid. Practically all the epithelial cells, which were cuboidal in shape, were arranged in single-layered rings round colloid containing spaces. There was no fibrosis or cellular infiltration.

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Ovaries. There was marked thickening of the smaller arteries with considerable hyaline change in both media and intima. The appearance throughout was that of the normal involuting ovary.

The organs were stained for the presence of waxy change, but none was found anywhere. As the autopsy was performed fourteen hours after death, the tissues were not examined for glycogen.

Summary.

In a case dying of diabetic coma and presenting also certain features of acromegaly, the following lesions were noted post-mortem:—lipæmia, consequent mononuclear hyperplasia in spleen, pancreatic, hepatic and renal cirrhosis, cholelithiasis, chromophobe adenoma of pituitary gland, gastric catarrh with multiple gastric ulcers and hæmorrhage into stomach.

Discussion.

In a case such as the present, with all the classical symptoms of diabetes mellitus, with lipæmia and death in coma, a pancreatic origin can hardly be doubted, and the changes found in the pancreas may be taken as accounting for the diabetes. It is more difficult to decide whether this pancreatic lesion was related, either as cause or effect, to the pituitary lesion. In spite of the intimate inter-relation of the endocrine organs, it seems unlikely that a pituitary lesion of tumour nature could be brought about by a lesion of the islets of Langerhans. Such evidence as exists regarding pituitary changes in pancreatic diabetes is rather conflicting. Some observers have described a diminution in the "eosinophile" cells, but Sakakibara, recently, has failed to find any constant changes in ten cases examined. No description of pituitary adenoma in pancreatic diabetes has been found in an admittedly incomplete study of the literature. Regarding the possibility of the pituitary lesion causing the pancreatic one, this also seems unlikely in the present case, inasmuch as the latter lesion seems to be but part of a fine fibrosis affecting the whole of the pancreas, the liver and probably the kidneys also. An infective ætiology which might explain the changes in at least liver and pancreas is suggested by the cholelithiasis present. At any rate, the diabetes seems to have originated in a more or less accidental involvement of the islets of Langerhans in a widespread fibrosis, not confined to the pancreas. A "replacement" fibrosis, following on a primary lesion of the islets, would not explain even the pancreatic picture.

It seems, therefore, most reasonable to postulate two independent diseases, a pituitary adenoma and a pancreatic diabetes, probably of infective or toxic origin. The history provides no clue as, when first seen almost three years before death, both lesions were well developed.*

* The X-ray of the sella turcica taken in 1925 shows an enlargement apparently about sufficient to accommodate the gland as found post-mortem.

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Granting this independence of origin, it is unlikely that two such lesions could run their course without mutually affecting one another. It may well be that the paucity of "eosinophile" cells found in the adenoma and the stationary nature and slight degree of the acromegaly present were related to the absence or diminution of the pancreatic hormone, as those observers who have found alterations in the pituitaries of diabetics have described deviations in the direction of diminution of the "eosinophile" cells. There are no symptoms of hypopituitarism in the case record, yet the histological appearances (chromophobe adenoma) are such as to lead one to expect this rather than acromegaly. It appears that such evidences of acromegaly as were present had existed for over three years, and had not increased since. The contradiction between the clinical acromegaly and the chromophobe tumour found post-mortem may be due to the latter having had a more "eosinophilic" character at its commencement, and the later alteration in cell type may have been stimulated by the pancreatic change; at the same time it must be remembered that spontaneous transition from acromegaly to hypopituitarism is not infrequent.

I can suggest no explanation of the gastric lesions found, beyond the surmise that they are merely an example of the proneness of diabetics to all sorts of secondary infections. Severe gastro-intestinal symptoms, with melæna, were prominent terminally in Smith's case of diabetic lipæmia: the lesion found post-mortem was a diphtheritic and hæmorrhagic ileocolitis.

I wish to thank Professor A. W. Mackintosh for permission to use the clinical history of the case, and to Mr W. Nelson likewise I owe a debt of gratitude for taking the photographs and microphotographs.

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THE ISOLATION OF *BACTERIUM MORGANI* FROM THE MAMMALS, BIRDS AND REPTILES AT THE ZOOLOGICAL GARDENS.

REGINALD LOVELL.

London School of Hygiene and Tropical Medicine.

THE frequency with which *Bact. morgani* * has been isolated from some of the cases of enteritis occurring amongst the animals in the gardens of the Zoological Society of London appears worth reporting.

Bact. morgani was first described by Morgan (1906), when working upon the bacteriology of summer diarrhœa in infants. He examined the stools of 58 affected infants and isolated what he termed his No. 1 bacillus from 28 of them: he failed to find it in normal human stools, sewage or drinking water. Experimentally he showed that it may cause death preceded by diarrhœa in young animals, and could be recovered in pure culture from the spleen and sometimes from the heart blood. The following year he caused death in young rabbits, rats and monkeys by feeding experiments. Four monkeys were used and the onset of diarrhœa varied from 2 to 12 days after feeding: the bacillus was recovered from all four animals after the onset of the diarrhœa and from three of them after death: it was not isolated from the spleen, nor was it found there in infants dead from diarrhœa. An intravenous injection caused the death of a goat in 18 hours: the organism was recovered from the heart's blood, spleen and liver. Morgan and Ledingham (1909) brought forward further evidence of the association of *Bact. morgani* with summer diarrhœa in children. They also suggested that the bacillus may be found in bovines, for, in 18 samples of cows' fæces examined, *Bact. morgani* was isolated once.

There are numerous references in the literature dealing with the isolation of this organism from cases of human intestinal disturbance but very few connected in any way, except experimentally, with the lower animals. Scott (1926) reports the isolation of an organism giving the morphological, cultural and biological characters of Morgan's No. 1 bacillus from two patas cercopitheques. Both animals had died from an enteritis and the organism was isolated in pure culture from the bile, liver tissue, spleen pulp and blood of one animal and from the first three sites of the other.

* *Salmonella morgani* according to Bergey. Thjötta (*J. Infect. Dis.* 1928, xliii. 351) gives reasons for putting it in *Eberthella* rather than *Salmonella* and would prefer to call it *Bacillus morganii*. See also below p. 91.

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Technique.—The autopsy was carried out as soon as possible after the death of an animal. Cultures were prepared from the liver, bile, spleen, blood and intestine, and sometimes from the mesenteric glands. Preliminary searing of the surfaces of all organs with a hot iron was carried out. The intestine was cut open with sterile scissors and a tube of broth liberally seeded with loopsful of contents. A similar technique was employed with the blood and bile cultures. Small portions of liver, spleen and mesenteric gland were excised in a sterile manner and dropped into tubes of broth. All cultures were incubated at 37°C. and no tube was discarded as sterile until it had been incubated for at least 5 days. All tubes showing growth were plated on to MacConkey's agar plates and these were incubated for 24 hours at 37° C. Non-lactose fermenting colonies were studied morphologically and biochemically, and if considered to be typical Morgan bacteria were tested for pathogenicity in mice. The following characteristics were regarded as identifying an organism as *Bact. morgani*: a gram-negative, non-sporing bacterium, giving acid and gas in dextrose, but producing no change in maltose mannite lactose sucrose or salicin; failing to liquefy gelatine; producing indol in peptone water after 5 days' incubation, and rendering litmus milk markedly alkaline within 10 to 15 days. Motility varied, a few strains being non-motile on isolation, but all strains except one appeared motile after sub-culturing.

Bact. morgani was isolated from the following.

AVES.

(1) A green-billed toucan (*Rhamphastos dicolorus*) of Guiana had been in captivity for about three years. It died from an acute enteritis and showed post-mortem an acutely inflamed intestine, the mucosa being of a uniform deep red, swollen and œdematous. *Bact. morgani* was isolated from the intestinal contents and the blood.

MAMMALIA.

(1) An orang-utan (*Simia satyra*) from Sumatra had been in this country for two and a half years. It was ill for about a month before death, refusing food, vomiting and suffering from diarrhœa. Post-mortem revealed a severe colitis of a dysenteric nature. The intestinal tract appeared to be normal until the large intestine was reached; from the hepatic flexure the mucosa was inflamed with scattered petechiæ which increased in number and size as one descended the bowel: from the descending colon to the rectum the whole mucosa was inflamed, with numerous raised areas terminating in petechiæ or crateriform ulcers. The mesenteric glands were swollen and congested. No amœbæ or cysts were seen in smears prepared from beneath the ulcers. *Bact. morgani* was isolated from the intestine, and no other pathogenic organism could be found.

(2) An orang-utan (*Simia satyra*) had been in contact with the previous case and died about six weeks later. Previous to her death she had been ill with diarrhœa for about three weeks. The autopsy revealed a similar condition, the small intestine being normal, and the large intestine acutely inflamed: from the cæcum to the anus the mucous membrane was almost entirely replaced by shallow greenish-grey ulcers with sloughs. The mesenteric glands were swollen and congested. No amœbæ were found in smears from the intestinal mucosa. *Bact. morgani* was isolated from the intestine, cultures from the spleen, kidney, liver, mesenteric glands, bile and blood proving sterile.

(3) A squirrel monkey (*Saimiris sciurea*) of South America died after six weeks in captivity. The intestine was deeply congested, the vessels engorged and the intestinal contents mucoid and blood tinged. The mesenteric lymph glands were swollen and congested. *Bact. morgani* was isolated from the intestinal contents; cultures from the liver, bile, spleen and blood were sterile.

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(4) An Abyssinian monkey (*Cercopithecus aethiops*) died after only a few weeks in the gardens. The latter few inches of the small intestine and the whole of the large intestine were inflamed, the mucosa was thickened and showed numerous small shaggy sloughs in minute ulcers. The liver and spleen were studded with greyish deposits varying from 2 to 3 mm. in diameter. Several of the mesenteric glands were swollen and one was very large measuring 2·4 by 1·3 cm. and contained pus. *Bact. morgani* was isolated in pure culture from the blood, liver, spleen and pus of the mesenteric lymph gland.

Reptilia.

(1) A pine snake (*Pituophis melanoleucus*) had been in captivity for nearly two years before its death. Post-mortem showed various abscesses in the lung, and a membranous enteritis. Just above the cloaca there appeared a small patch, three to four inches in length, where the mucosa was replaced by an ashy grey false membrane: the base of this was inflamed and the wall considerably thickened. *Bact. morgani* was isolated from the blood; from the blood, bile, liver and lung abscesses was also isolated an organism biochemically resembling *Bact. aertrycke* but showing no serological relationship to that group.

(2) A pine snake (*Pituophis melanoleucus*) of South America died showing a similar but less acute condition. There appeared to be no abnormality in the intestine till just above the cloaca, where there was a small area of inflammation two inches in length showing early signs of ulceration. *Bact. morgani* was isolated from the bile and was the only pathogenic organism recovered.

(3) A large-scaled mamba (*Dendraspis viridis*) of West Africa died shortly after admission to the gardens. The lung was congested and the intestine acutely inflamed, the terminal portion and the cloaca showing marked congestion. The spleen was enlarged and pale. *Bact. morgani* was isolated from the bile, spleen and cloaca. Lactose fermenting and unidentified non-lactose fermenting bacteria were also cultivated.

(4) A false mocassin (*Natrix fasciata*) of North America had been in the gardens about a month before death. There was at post-mortem an enteritis, the intestinal mucosa was much inflamed, raised and granular and could be easily scraped off. The lung was slightly congested but no further abnormality was found. *Bact. morgani* was isolated from the liver.

Bact. morgani has therefore been isolated from the following:—

Animal.	Site.
Green-billed toucan	Intestinal contents.
	Blood.
Orang-utan	Intestine.
Orang-utan	Intestine.
Squirrel monkey	Intestine.
Abyssinian monkey	Blood.
	Liver.
	Spleen.
	Pus of mesenteric gland.
Pine snake	Blood.
Pine snake	Bile.
Large-scaled mamba	Spleen.
	Bile.
	Cloaca.
False mocassin	Liver.

Pathogenicity of Bact. morgani.—There is a diversity of opinion regarding the rôle which *Bact. morgani* plays in the causation of

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bacillary dysentery and summer diarrhœa in humans. Thjötta (1920) describes nine cases of diarrhœa from which he isolated *Bact. morgani*. He could not find any serological connection between the strains studied, neither could he find any evidence of antibodies in the sera of the patients from which the bacterium was isolated. He considers that it is not enough that the organism is merely present in the intestine, for the intestinal flora may alter considerably under the pathological conditions that take place in the intestines during a severe attack of colitis. He has recently (1928) described a case of cholecystitis in which *B. morgani* was isolated from the pus and from the blood and agglutinated by the patient's serum. Tribondeau and Fichet (1916), referring to their researches on dysentery in the Dardanelles, maintain that the lack of agglutinins and complement-fixing bodies in the sera of patients is no criterion. They refer to other dysentery cases where they are lacking and show that blood culture for Morgan's bacillus was never successful. Morgan had previously produced evidence that the bacillus did not frequently penetrate beyond the intestinal mucosa, even in his feeding experiment in monkeys. Wilson (1927) has shown that Morgan's bacillus, under certain conditions which he is unable to define but which are probably associated with the dietary, may assume the power of spreading epidemically and of giving rise to fatal infections in mice.

Of the nine cases dealt with in this report, *Bact. morgani* was isolated three times from the intestine only and all three of these cases were monkeys, and in each case it was the only organism of a pathogenic nature isolated. From the fourth mammalian case, also a monkey, it was isolated in pure culture from the blood, spleen, liver and pus of a mesenteric gland. When one takes this into consideration there can be little doubt that *Bact. morgani* can assume a pathogenic rôle in such animals. The part played in the causation of the death of the reptiles is not so certain since other organisms were present: the organism resembling *Bact. aertryeke* and the unidentified coliform bacteria may have played some part.

All strains were tested on mice and proved to be pathogenic, 0·5 c.cm. of a 24-hour broth culture being injected intraperitoneally. Although the time of the death of the mice varied from 5 hours to 3 days, the organism could always be isolated from the heart blood. In view of the rapidity with which death occurred in many cases, an attempt was made to determine whether a soluble toxin was present in the broth cultures. A strain which in a dose of 0·5 c.cm. had killed a mouse in 5 hours was selected and grown in broth at 37° C. for varying periods and filtered through a Seitz filter. Inoculated into mice intraperitoneally or intravenously in doses up to 1 c.c., the filtrate proved harmless and there is no evidence that *B. morgani* produces a soluble exotoxin.

Summary.

(1) *Bact. morgani* was isolated from one bird, four monkeys and four reptiles, in circumstances which suggest that this organism was ætiologically related to the infection from which they died.

(2) No evidence was obtained that *Bact. morgani* is capable of producing a soluble exotoxin.

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NOTES ON INTESTINAL BACILLI WITH SPECIAL REFERENCE TO SMOOTH AND ROUGH RACES.

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The object of the present notes is to outline very briefly certain diverse observations bearing for the most part on the relative behaviour of smooth and rough organisms.

I. A simple method of studying the differential distribution of the soluble specific carbohydrate factor in smooth and rough bacilli.

In earlier communications (White, 1927 and 1928), extending the original observation of Reimann (1926) on the pneumococcus, I have pointed out that the essential serological event of rough change in *Salmonella* and related groups is the loss, complete or partial, of that non-protein carbohydrate-containing soluble specific component which as a result of the investigations of Avery and Heidelberger, of Landsteiner and Furth, of Zinsser and others has come so much to the fore in recent serological studies. The essence of my own observations was that while I could without difficulty obtain soluble specific substances of this class from smooth cultures of all of several *Salmonella* types I failed at repeated trials to extract any corresponding serologically active material from agglutinatively pure rough strains. The same relation I have since found to hold good for the corresponding variants of Shiga's dysentery bacillus.

The soluble specific substances in their stability and in the quality and range of their precipitation reactions declare themselves unequivocally as the O-*receptors* (as opposed to the entire "O-*antigen*") of Weil and Felix. In my hands, as in those of others, they have reacted negatively in the biuret and Millon tests, while giving a strong Molisch reaction for carbohydrate. Precipitatively potent solutions of these substances obtained from *B. aertrycke*, *B. enteritidis*, *B. typhosus* and *B. suipestifer*, thrice injected intravenously into rabbits at weekly intervals, have not stimulated recognisable antibodies for themselves or for the corresponding whole organisms—a finding which tallies with that of Zinsser and Parker (1923) with *B. typhosus*, but which seems at variance with that of Day (1928) writing of the same

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organism, and of Branham and Humphreys (1927) dealing with *B. enteritidis*.

The following appears to be a satisfactory general method for preparing solutions of the soluble specific substances in question for serological purposes and of demonstrating the presence and absence of these components in smooth and rough bacilli respectively.

Agar-grown bacilli are suspended in N/5 acetic acid made up with physiological saline, 3 well-grown plate cultures to 100 c.c. of the acid saline being a convenient proportion. The suspensions are heated by immersion of the container—preferably fitted with a reflux condenser—in a bath of boiling water. The same end may be attained by boiling the bacilli in acid of similar or lesser concentration but the frothing which occurs renders the proceeding difficult. So treated rough suspensions suffer prompt coagulation, leaving the menstruum clear save for circulating flakes. Under the same circumstances smooth bacilli may show some initial aggregation, doubtless due to coagulation of the flagella; such clumping, should it occur, is transient and for a considerable period (which may endure for 2-3 hours) the suspension remains uniform. Eventually however progressive clumping and sedimentation set in until finally an appearance is reached identical with that rapidly developed in the case of rough cultures. This may be taken as indication that the reaction is at an end—that the great bulk of the serologically active carbohydrate factor has been separated from the bacilli. Only traces of this substance are obtained by further acid washings of the bacilli, but should it be desired to obtain a quantitative yield, a second extraction with a smaller volume of acid saline should be carried out. Smooth and rough "suspensions" which have passed through this process side by side are centrifuged till clear, while yet strongly acid. With neutralisation of the supernatant a further precipitate forms and must be centrifuged or filtered off. Serological tests may be made with the solutions so obtained or the active principle may be concentrated either by boiling off excess of water, by precipitation with lead acetate or best by precipitation with alcohol. The proportion of added alcohol required to effect precipitation of the serologically active substance appears to vary with the bacterial type, but as a rule the precipitate thrown down from a definitely acid solution such as has usually been employed by 2 volumes of alcohol is serologically inert and may be discarded, 3-4 volumes being required to eject the specific substances entirely. The corresponding precipitates derived from smooth and rough bacilli are made up each in a small volume of saline (10-15 c.c.), acidulated with acetic acid and boiled, after which the protein-containing precipitate is centrifuged down; the supernatants may or may not give a *just discernible* biuret reaction.

The smooth-derived solutions so obtained cause vigorous precipitation with type homologous smooth antisera (prepared against whole bacilli) and the reaction may still be seen at a solution dilution of 10,000 or more; with typical rough antisera no precipitate results. The solutions derived from rough bacilli are to all intents serologically inert: occasionally they cause trivial clouding in type homologous *smooth* antisera; with the truly homologous rough antisera no reaction occurs.

Experiment shows that the process of heating for 2-3 hours in N 5 acetic acid causes no significant reduction in the serological value of the soluble specific substance in question; even considerably higher

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concentrations of acetic acid and HCl are tolerated. I have been unable to detect the presence of reducing sugar in any of the mixtures.

Since the rough-derived solutions contain a considerable amount of serologically inactive matter, it is probable that such substances contaminate the solutions of smooth origin; but as the aim of the work has been essentially serological no zealous attempt has yet been made at further purification; complete extraction of the active principle without damage, concentration of the extract and removal of protein have been the only serious objects. It appears that by the method given the soluble specific substance may be extracted quantitatively in a given volume of saline; it should, therefore, be possible to determine serologically the relative richness, as regards this component, of two strains of like type—a point which must be of value in any attempt to trace a relationship direct or indirect between "degree of smoothness" and infective virulence.

Serological examination of the curd of bacilli left by the extraction process is as yet incomplete. It has been found that rough bacilli may emerge from the treatment still possessing considerable, if much reduced, power of agglutinative reaction to ordinary rough antisera. (Tests must of course be carried out at a low saline concentration—0·1 to 0·2 per cent. of NaCl—in view of the denatured state of the bacterial proteins.) With smooth cultures it was found that from the time of their coagulation the bacilli became insensitive to the somatic agglutinins of smooth antisera. There was some expectation that they would now be found to react to rough antisera; this expectation has only been partially fulfilled for in no case did the observed titre of agglutination attain to more than $\frac{1}{4}$ of that achieved in the case of similarly treated rough variants; with various rough sera clumping was obtained up to dilutions 200 or 400. The matter is being further examined with the use of sera prepared against bacilli, smooth and rough, which have passed through the extraction process described. Up to the present the clearest indication obtained of the presence of the rough agglutinative complex within the smooth organism has been the selective action of sera, prepared against solutions of smooth bacilli in N/10—N/8 NaOH solution, on rough as opposed to smooth organisms. On this point the parallel with Reimann's observations on the pneumococcus, if it exists, has not been so clearly traced as it has in the matter of the specific carbohydrate component.

II. The reaction of smooth and rough bacilli to Millon's and to Molisch's reagents.

A striking difference in the behaviour of smooth and rough *Salmonella* bacilli towards Millon's reagent has been noted. When a large loopful of rough growth is emulsified in approximately 3 c.c. of water and about 1 c.c. of Millon's solution is added thereto, the bacilli

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are at once clumped and, on boiling of the mixture, collected in dense masses and quickly assume the deep red-pink coloration of the positive test. Under the same conditions typically smooth bacilli of the *Salmonella*, colon and dysentery groups are relatively little affected; the bacilli remain dispersed and the colour of their suspension does not deepen during a few minutes of boiling beyond a yellowish or ochre tint. The deposit which settles on standing commonly varies in colour from yellow to ochre; occasionally it is pale pink—a variation probably related to the degree of smoothness—; in all cases it is fine and readily dispersible.

Smooth bacilli from which the soluble specific non-protein factor has been extracted (*e.g.* by prolonged boiling with dilute acid) or the proteins thrown down by acid from a solution of smooth bacilli in NaOH solution react precisely like fresh rough bacilli, and it would seem to be an inevitable conclusion that in the smooth organism the soluble specific carbohydrate intervenes—probably mechanically—between the tyrosin-containing complex and the reagent. The negative reaction of the smooth form is seemingly not due to any injury to the reagent by components peculiar to smoothness, for rough bacilli react in a perfectly typical manner when tested in mixture with smooth bacilli or the freed soluble specific component.

It has further been observed that smooth bacilli usually react far more vigorously in Molisch's test for carbohydrate than do rough bacilli, always provided the test be applied to both under exactly parallel conditions.

III. Observations on the toxicity of smooth and rough *Salmonella* bacilli.

Goyle (1926) concluded that autolysates of smooth cultures of *B. enteritidis* are more toxic to mice by intraperitoneal injection than are those of the rough derivative races.

In my own comparisons of the relative toxicity of smooth and rough races, made intermittently during the past 5 years, rabbits mice and rats, all injected intravenously, have been employed for test purposes. So used the rat is particularly useful in testing for toxic activity in *Salmonella* strains in that all the commonly occurring species, after an incubation period of 35–45 minutes, set up an acute intoxication with prostration and diarrhœa and tending to death in a few hours. The intoxication set up by *B. typhosus* is probably to be regarded as most acute, that due to *B. enteritidis* least so; intermediate in effect are *B. aertrycke* and *B. paratyphosus B* [*Salmonella schottmülleri*]. In the rabbit, as is well-known, *B. typhosus* and *B. paratyphosus A* [*Salmonella paratyphi*], dose for dose, greatly surpass *B. enteritidis* *B. aertrycke* and *B. paratyphosus B* in the acuteness of the intoxication which they occasion under ordinary circumstances,—that is to say

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when saline suspensions killed at 60° C. are injected intravenously. In the mouse, according to my own experiments, *B. enteritidis* has exhibited the most intense "immediate" toxicity, that of *B. aertrycke* and *B. paratyphosus B* being definitely less; *B. typhosus*, like *B. dysenteriæ Shiga*, tends to occasion late death after 2-3 days of apparent well-being. When broth culture filtrates or saline autolysates are injected in place of whole bacilli intertypal differences of toxicity for any particular host are inevident. Furthermore, I have found that when before injection the bacilli are extracted with alcohol (½ hour at 50° C.), a process which never reduces toxicity, a considerable raising and levelling of the poisonous properties of the types results. These facts render it probable that such apparent differences in toxic value as are seen among the types of the *Salmonella* series depend not on any specific differences in the activity of the toxins or in their amount, but on the varied capacity of the different animal species to break down bacilli of the several types and liberate the toxic substances which they contain.

That saline suspensions of smooth heat-killed bacilli excel those of rough bacilli in toxicity for laboratory rodents is not difficult of demonstration. The action of *B. typhosus* on the rabbit furnishes an excellent example: in this case ¼ to 1 agar slant culture of the smooth form constitutes a probably fatal dose for an animal of medium size, whereas 2 to 3 agar slant cultures of the rough organism are usually tolerated. Should the living organism be employed, as is in this case feasible, 2 agar slant cultures of the rough race commonly cause an almost symptomless septicæmia or a chronic emaciating disease of several weeks' duration.

In a series of experiments with Shiga's bacillus and mice the same relative impotence of the rough form was patent. Furthermore filtrates from broth cultures of rough *Salmonella* bacilli—and apparently also of Shiga dysentery bacilli—do not, at least as a rule, approximate in toxicity to those obtainable in the instance of the smooth organism. From these facts it might be argued that coincidently with loss of soluble specific carbohydrate and of infective virulence the organism in roughening suffers loss or severe reduction of the poisonous principles which it normally contains. This does not however appear to be the case. Rough *Salmonella* bacilli after treatment with alcohol assume toxic properties at least as great as those of similarly treated smooth bacilli and sometimes seemingly greater; even rough coliform bacilli, so treated, exhibit for rabbits and rats a toxicity which hardly falls short of that of smooth *B. typhosus*. In an experiment in which equivalent dry weights of smooth and rough alcohol-treated *aertrycke* bacilli were injected in graded amounts intraperitoneally into mice no difference in the toxic value of the variants was obvious. It seems probable that the potential toxicity of the rough derivative approximates to that of the smooth parent organism and that contrasts in

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actual toxicity are due to differences in the rate of liberation of toxic substances within the animal body or into a fluid culture medium.

So far as I have been able to ascertain the solutions of soluble specific carbohydrate substance are to all intents non-toxic. The entire yield of this substance from 2 agar plate cultures of *B. typhosus* produced no symptoms in a rabbit, nor that from 1 agar plate culture of *B. enteritidis* in a rat; 1 agar slope culture of the former organism and 1 platinum loopful of the latter, both killed at 60° C., proved fatal by intravenous injection to a rabbit and a rat respectively. Without destruction or dislodgment of the soluble specific substance the toxicity of *B. aertrycke* for the rat was found to be practically abolished by autoclaving a suspension for 30–40 minutes at 15 pounds pressure.

IV. Observations on mucoid growth.

In the pneumococcus smoothness and the presence of the soluble specific carbohydrate seem to be associated with ability to form a capsule; the rough cocci are non-capsulate. The same relation between smoothness and capsulation has been described by Julianelle (1926) for the pneumo-bacillus, while Tomcsik (1927) apparently looks upon the capsule as the source of the soluble specific carbohydrate-like substance which he prepared from *B. lactis aerogenes*.

Though in mucoid growth of *Salmonella* the bacilli are not strictly capsulate but merely suspended in a communal mass of secreted slime, it has seemed desirable to consider the possibility of a relation between such mucoid growth and smoothness, more particularly in connection with those irregularities which are encountered in old laboratory strains in applying the "Schleimwall" test for the differentiation of *B. paratyphosus B* and *B. aertrycke*. There is a *prima facie* argument against any connection between mucoid growth and hypertrophy of the specific carbohydrate component, in that mucoid forms react particularly poorly to the homologous somatic agglutinins, only recovering normal behaviour after the suspensions have been boiled (White 1926). To support this argument smooth and rough variants of several strains of *B. paratyphosus B* and *B. abortus equi* were cultured as well-separated "macrocolonies" on agar; after 24 hours' incubation the cultures were allowed to continue their development at room temperature. All, smooth and rough, developed in typical fashion the marginal wall of turbid and slimy secondary growth. In a further experiment 2 plate cultures of a rough paratyphoid B strain, showing well-formed mucoid rings about the evenly dispersed colonies, were taken up into 10 c.c. of N/5 acetic acid and heated in a water bath for 2 hours. The clear supernatant finally obtained by centrifuging gave no precipitate with smooth or rough paratyphoid B antisera. These results indicate that no relation exists between mucoid growth

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of *Salmonella* and the smooth state, and that this growth habit is not in any sense homologous with encapsulation as seen in the pneumococcus.

V. The possible taxonomic value of the rough variant.

Within each of the great "biochemical" groups of "intestinal" bacilli serological methods have revealed the existence of more or less numerous types or species—elementary species differing from one another now as regards one group of antigenic properties, now as regards another; some are defined by the serology of their flagella alone, some only by that of the bacillary bodies, some by that of both. In the *Salmonella* group the measure and nature of these differences have been more or less effectively elucidated; the exact relationships of monophasic types, *B. typhosus B. paratyphosus A* and *B. enteritidis*, to the diphasic organisms of the old "paratyphoid B" group have been determined; the generic unity of the whole has been established beyond reasonable doubt; nigh on 30 types, common infrequent or rare, have been rallied by unequivocal serological argument to the clan *Salmonella*. But there remain in doubt a variety of organisms, undemonstrative of any salmonellar affinity in their normal serology, which various systematists, seeking to juggle the intestinal bacilli into pigeon holes on the score of their biochemical reactions, have ascribed to *Salmonella*, apparently regarding that ill-used group as a very fitting dumping-ground for their difficulties; *e.g. B. columbensis* and Morgan's No. 1 bacillus, which Bergey (1926) dignifies with the title *Salmonella*. Moreover the experience of most of those who have attempted serological analysis of the great coliform groups is that the diversity of behaviour encountered renders any comprehensive classification on these lines unthinkable. But it is clear that the continued use of "functional" criteria—the use of biochemical reactions—in bacteriological taxonomy cannot lead to a real classification; it is a proceeding which the biologist has condemned but in which the bacteriologist ingenuously persists. Somehow and by some means the substance of the organisms themselves must be utilised in tracing their generic affinities. To this end I have examined the possible value of the rough variant in effecting serological grouping of strains, seeking, more particularly, answer to the question: what is a *Salmonella*?

Schütze (1921) pointed out the remarkable latitude of cross agglutination among rough variants of the *Salmonella* types, and White (1926) has shown that this depends on similarity of the rough somatic antigens throughout the *Salmonella* series and carries with it unity in complement-fixing behaviour: even by means of the absorption test it is difficult to effect a differentiation of the rough bacillary bodies of the various species. Exactly analogous is the case of the pneumo-

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coccus types. The question for present consideration is as to how far such community in roughness is limited; how far the rough variant of the *Salmonella* group differs from the corresponding variants of other intestinal bacilli.

Rough variants of 9 different *Salmonella* types [*B. typhosus* (3), *B. paratyphosus A* (1), *B. enteritidis* (3), *B. aertrycke* (4), *B. paratyphosus B* (2), American *B. suipestifer* (2), European *B. suipestifer* (2), Newport type (1) and Stanley type (2)] were isolated and antisera, at least one per type, were prepared. Sera were also prepared against rough races of Shiga's bacillus (2), Sonne's bacillus (3), Flexner's bacillus (4), *B. columbensis* (1), Morgan's bacillus (2), hæmolytic *B. coli* strains (3), saccharose non-fermenting *B. coli* (3), and saccharose fermenting *B. coli* (3).

For agglutination purposes the rough cultures, grown on agar, were treated with absolute alcohol or a mixture of alcohol and chloroform for an hour at 50-53° C. to destroy flagellar antigens if present, and to render the bacillary bodies insensitive to the precipitating action of electrolytes and normal serum (White 1927 and 1928). In many cases the bacilli were stored dry after the extraction process, to be made up in saline as required.

Agglutination tests were performed in the usual manner (4 hours in the water bath at 50° C. and standing for a few hours at room temperature). In all cases agglutination, where it occurred, was of the characteristic rough type, either in fine dispersible granules or in coarser but equally dispersible flakes, unlike either the "sandy" agglutination of smooth bodies or the loosely flocculer agglutination of flagella. The titres of the sera for the homologous organisms, treated as described, ranged between 2500 and 5000, except in one anti-dysentery (Shiga) serum of which the value was only 1000 and an anti-*aertrycke* serum of titre 10,000.

With the many *Salmonella* rough sera tested the numerous rough *Salmonella* races agglutinated in indistinguishable manner to the same titre; with no other rough serum tested did any of these races react to above 5 per cent. of the homologous titre; the genus *Salmonella* stood defined in a striking manner.

In tests made in the reverse sense with the rough variants of coliform strains, Sonne, Shiga and Flexner dysentery bacilli, *B. columbensis* and Morgan's bacillus, results were rather more complex. With rough typhoid antisera none of these organisms showed appreciable reaction; *B. columbensis*, all the hæmolytic coliform races, Morgan's bacillus, Shiga's bacillus, Sonne's bacillus and most of the other strains tested refused to react to any typical *Salmonella* rough serum; the rough races of certain Flexner strains and of 3 of 12 saccharose non-fermenting coliforms agglutinated up to 25 per cent. of the titre of several of the rough *aertrycke paratyphosus B* and hog cholera antisera employed.

It is true that Schütze (1921) indicates considerable cross agglutination of a rough variant of Shiga's bacillus with rough *Salmonella* antisera; but since, as I have shown, the rough races of this organism normally agglutinate to titre between 2000 and 5000 with normal rabbit serum, the significance of the observation is doubtful.

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The results obtained support in no uncertain way the inclusion of *B. typhosus* in the genus *Salmonella*, and impress the injustice of any attempt to sunder that organism from its gas-forming congeners or to associate it, as does Bergey in his genus *Eberthella*, with a congeries of organisms with which it has no serological link whatever, and with which its special biochemical similarities are of a purely negative nature. *B. columbensis* and *B. morgani* are sharply excluded from the genus *Salmonella*.

Study of the groupings of the non-*Salmonella* strains has been cursory. The rough variants of the several Flexner strains examined showed the same community of agglutinative behaviour which marks the *Salmonella* types, and with them reacted the rough variants of Sonne's organism; all these organisms showed marked affinity with the same cluster of saccharose non-fermenting, non-hæmolytic strains of *B. coli* which evinced some relation to the Salmonella series; from the rough variants of Shiga's bacillus they stood sharply distinct. The four hæmolytic coliform races formed a definite cluster in close agglutinative harmony with the rough variants of two strains of *B. columbensis*, an observation which supports Dudgeon's (1927) view as to the relationships of that organism. Four rough saccharose-fermenting races of *B. coli*, by the special vigour of their reaction to any one of the corresponding sera, stood out from the saccharose non-fermenting forms.

These observations, desultory as they be, suggest that study of the rough variant along these lines may be of some real value in checking the present formal groupings of bacteria and may give some indication of a more rational arrangement of species. In the wide range of rough agglutination we have perhaps an expression of the biological truth that those elements of the organism which are least exposed to contact and conflict with the environment are least subject to evolutionary change and longest maintain their primitive characters; between the basal proteins of the bacterium and the medium of its life—in the case of the parasitic species between these proteins and the reaction of the host—stand the soluble specific substances as a shield. It is hardly to be expected that any such order of naturally related organisms as that formed by the "intestinal" bacilli can be, by any means, resolved into sharply defined genera; but much can doubtless be done to map out the broad lines of divergent evolution and to discount groupings based only on the superficial similarities of convergence.

Summary.

1. The soluble specific substance may be extracted quantitatively from *S. Sonnei* and similar bacilli by hot dilute acid.

2. Rough variants give an immediate reaction with Millon's reagent; smooth races react only when the soluble specific substance has been removed.

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3. Under ordinary circumstances of test smooth *Salmonella* and related bacilli and filtrates of broth cultures thereof are more toxic to laboratory rodents than are the corresponding rough bacilli and filtrates; the rough bacilli however possess a potential toxicity, disclosed by treatment with alcohol, approximating to that of the smooth forms.

4. Mucoid growth in *Salmonella* has no relation to smoothness and is not analogous to capsule formation.

5. The serological reactions of rough variants appear to form a useful basis for a natural classification of intestinal bacilli.

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DIFFUSE AND NODULAR FIBROSIS OF ADVENTITIA OF AORTA

(Rheumatic periaortitis)

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(Plates IX. and X.)

This case is of interest because as far as I have been able to discover only one other like it has been described before.

Summary of history.—The patient was a man of 23. As a schoolboy he had growing pains and rheumatism in the left shoulder. At 14 he was treated in hospital for precordial pain and dyspnœa; at 20 he was treated for cardiac failure. On admission to University College Hospital in July 1923 he was pale, thin and looked ill, and showed the signs of gross cardiac failure. He was orthopnœic, the veins of neck were engorged, the lungs congested, the liver enlarged and the ankles œdematous. The heart was greatly enlarged, the apex beat being in the anterior axillary line; a loud to-and-fro murmur was heard over the apex and base and the pulse was water-hammer in type. There were no signs of bacterial endocarditis. The pulse was regular and rapid remaining about 120 per minute, the systolic blood pressure was 120 mm. Hg. The temperature varied between 98·4 and 101° F. He died two days after admission.

Summary of post-mortem.—Pallid man, 6 ft. 2 inches, 6 stone 11 lbs. Lungs: right 27 ozs., left 23 ozs.; brown induration and scattered small hæmorrhages. Liver: 68 ozs., advanced "nutmeg" and moderate amyloid change. Spleen: 8 ozs., firm, purple and congested. Kidneys: 11 ozs., irregular, sunken patches with pitted surfaces over cortex of both amyloid kidneys. Effusions: pleural, 1 pint right; ½ pint left; peritoneal 3 pints.

Heart (40 ozs.).—Fibrous pericardial adhesions bound part of the parietal to the visceral pericardium on the posterior and to a less extent on the anterior surface. There were numerous petechial hæmorrhages in both layers of pericardium. The myocardium was hypertrophied and all chambers were dilated, both the hypertrophy and dilatation being most marked in the left ventricle. The cusps of the aortic valve were thickened, rolled and retracted towards their bases (fig. 1). The flaps of the mitral valve were slightly thickened and adherent at their angles. Pockets had formed in the endocardium of the left ventricle below the damaged aortic valves.

Aorta.

The adventitia of the aorta was everywhere greatly thickened by fibrous tissue measuring 3 mm. near the heart and up to 10 mm. in the lower part of the thoracic aorta. In places this had become condensed in such a way that the aorta appeared to have been bound with thick cord. Fibrous tissue was present between the bands but here it was less dense so that on opening the aorta the cut bands stood out prominently as milky-white nodules. The fibrosis was limited to

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the adventitia and apart from small patches of atheroma both the intima and media appeared normal and together measured 1·5 mm. thick. This change extended from the heart to the bifurcation of the aorta but was most marked in the thoracic region. It was continued to a slight extent round the branches and particularly round the branches of the abdominal aorta, which were surrounded by a somewhat less dense but wide zone of fibrous tissue.

Microscopically no cellular reaction could be found in sections of myocardium, pericardium, endocardium and adventitia of aorta or its branches. The sections showed: in the myocardium patches of perivascular fibrosis, in the valve hyaline fibrous thickening and between the layers of pericardium hyaline fibrous adhesions. Many sections of all parts of the aorta were examined in the hope that a cellular reaction, giving some clue to the nature of the process, might be discovered, but while all showed more or less dense fibrosis of the adventitia only occasional groups of two or three lymphocytes with or without plasma cells were found. The fibrosis of the adventitia consisted of dense collagen fibre corresponding to the bands and similar slightly less dense tissue between. The media of the aorta showed slight myxomatous degeneration; there was no trace of perivascular infiltration, any form of fibrosis or breaking up of elastic tissue. The intima was normal. The vessels in the adventitia were conspicuous, being more numerous than normal and many of them having thickened degenerate walls.

Discussion.

The points in the case which have to be considered are the heart, the aorta and the presence of amyloid in the liver and kidneys. Since no focus of suppuration could be found at post-mortem the amyloid was probably a legacy of the more active stage of the process now represented by fibrous tissue.

As there was no evidence of inflammation in the mediastinum nor in the bodies of the vertebræ it seems improbable that the fibrosis of the adventitia had been initiated in surrounding structures. We are left with the lesions of the aortic and mitral valves, the patchy fibrosis of the myocardium, the pericardial adhesions and the fibrosis of the adventitia as the principal parts in the puzzle, while the diseases calling for consideration are syphilis, periarteritis nodosa and rheumatism.

Syphilis. The appearance of the aortic valves is strongly suggestive of syphilitic endocarditis. They have the typical "sausage" shape and it is not uncommon to find syphilitic endocarditis without scarring of the aortic intima. In syphilitic aortitis the granulation tissue starts in the adventitia and extends like a sleeve round the nutrient arteries into the media, where the elastic fibres of the media are broken up. Apart from the fact that there is no spread to the media it would not be necessary to unduly stretch the imagination in order to conceive

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PERIAORTITIS.

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FIG. 1.—Heart: thickened rolled retracted aortic valves and endocardial pockets. (Seven-ninths original size.)

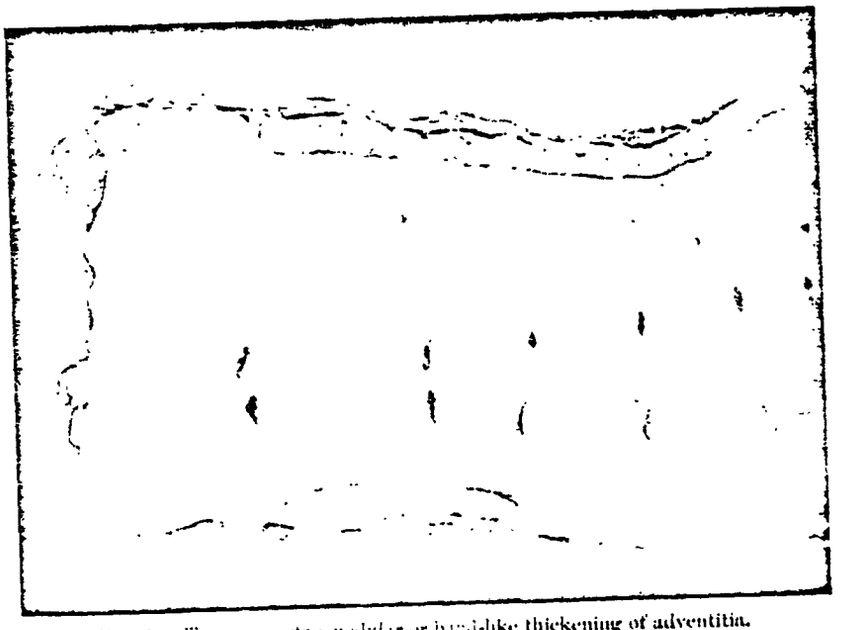


FIG. 2.—Thoracic aorta: nodular or lymph-like thickening of adventitia. (Six-sevenths natural size.)

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PERIAORTITIS.



FIG. 3.—Transverse section of aorta: unaffected media and intima, great fibrous thickening of and conspicuous vessels in adventitia. × 14.

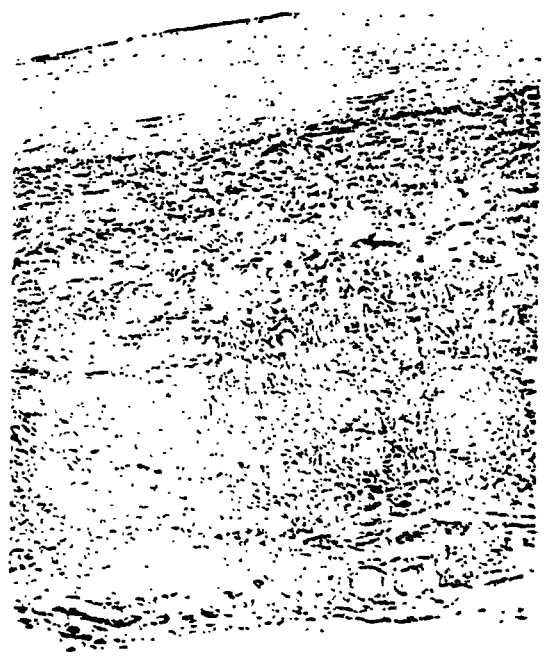


FIG. 4.—Longitudinal section of aorta: nodules of dense and intervening less dense fibrous tissue. × 16.

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a syphilitic aortic endocarditis associated with a completely fibrotic aortitis in which the reaction had been limited to the adventitia. Consideration of the patient's history and age make it very unlikely that this is the true explanation. Syphilitic aortitis is seldom found before 35, and the younger the patient the greater will be the certainty of finding a cellular reaction. This patient was 23 and had had no antisyphilitic treatment. The fact that he had a definite heart lesion at the age of fourteen is also against the possibility of a syphilitic etiology.

Periarteritis nodosa is an active inflammation which, starting in the adventitia of arteries, usually spreads to the media or even the intima. The inflammatory reaction is at first made up of polymorphs, eosinophils, lymphocytes and rarely plasma cells; later fibroblasts proliferate and eventually lay down new fibrous tissue. The media even in the absence of direct invasion often undergoes necrosis and as a result aneurysms are produced. The inflammation is not even, so that macroscopically the arteries appear as a chain of beads each bead corresponding to a nodule of adventitial infiltration, with or without aneurysm formation. If one imagined such a reaction limited to the adventitia and having reached the fibrotic stage it would account for the condition of the aorta and the amyloid in this case but not for the lesions in the heart. There are two further objections. Periarteritis nodosa occurs in arteries of the size of the coronary arteries, or smaller, and though it has been described in the coronary, mesenteric, renal, intra-hepatic, intra-splenic, pleural, peritoneal, small muscular arteries and rarely in arteries of brain and lung, it has never been described in the larger arteries: in this case the smaller arteries were not affected. The other objection is that in the scarred stage of periarteritis nodosa, even if the media escapes, the fibrosis extends beyond the adventitia into the surrounding tissues; in this case it is confined to it.

Rheumatism. The first serious contribution to our knowledge of rheumatic aortitis was made by Klotz in 1912. He divided his cases into groups. Those who died in the first attack showed (*a*) in the *media*—penetration of nutrient arteries beyond the outer third, perivascular œdema and infiltration by lymphocytes and plasma cells, with damage to elastic and muscle fibres in the vicinity: (*b*) in the *adventitia*—connective tissue loose œdematous and diffusely infiltrated with lymphocytes and plasma cells. Those who died from recurrent attacks showed similar changes, except that they tended to be less cellular and there was definite perivascular fibrosis of the media and increase of fibrous tissue in the adventitia. In chronic rheumatism the cellular reaction was still less conspicuous and the fibrosis more dense.

Amongst the most recent are a series of papers by Pappenheimer and von Glahn; their findings may be summarised as follows:

intima: active cellular reaction, cells similar to those forming an Aschoff nodule (1927): *media:* cellular infiltration about the penetrating

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vessels, (1924), localised atrophy and disappearance of muscle, perivascular fibrosis (flame-shaped scars) round vasa vasorum (1926): *adventitia:* cellular infiltration, Aschoff cells, plasma cells and lymphocytes (1924) with little tendency to nodular grouping, slight fibrous thickening of adventitia and obscured demarcation between media and adventitia.

Chiari (1928), in a series of six rheumatic cases, describes reactions varying from Aschoff nodules to fibrosis which were practically confined to the adventitia; the last of the cases he describes is exactly like mine but for the fact that he found cellular reaction as well as fibrosis; he regarded it as a late stage of rheumatic aortitis.

The appearance of the fibrous tissue in the adventitia, in the myocardium, in the pericardium and in the valves suggests that the reaction which preceded it affected all these structures at about the same time and from the history it appears likely that this was about 14, with a possible exacerbation at 20. Of the causes discussed I suggest that what evidence we have supports the rheumatic etiology. When the age and history of the patient are taken into account it seems highly probable that the valvular lesions were due to rheumatism and this is supported by the perivascular fibrosis of the myocardium and the pericardial adhesions. If this is accepted I can think of no valid reason for doubting that the fibrosis of the adventitia was also due to rheumatism. As is well known, rheumatism may affect valves myocardium and pericardium either singly, any two of them or all together. In the same way the coats of the aorta may be affected singly or all together, and I suggest that in this case we are dealing with the scarred results of a rheumatic inflammation of the adventitia of the aorta. The nodular arrangement may be related to the movements of the aorta or to the distribution of blood vessels; in this case there is no clear indication of its cause. A similar nodular fibrosis sometimes occurs in the pericardium, particularly over the auricles in rheumatic affections of the heart.

Summary.

A widespread fibrosis of the adventitia of the aorta is described; the suggestion is made that it is due to rheumatism.

I am indebted to Dr J. W. McNee for permission to use his clinical notes in this case. Throughout this paper "rheumatism" has been used for the specific disease which in its acute form is known as acute rheumatic fever.

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GENERALISED VACCINIA IN RABBITS WITH ESPECIAL REFERENCE TO LESIONS IN THE INTERNAL ORGANS.

S. R. Douglas, Wilson Smith and L. R. W. Price.

From the National Institute for Medical Research, Hampstead, London.

(Plates XI.-XIII.)

During the course of investigations on vaccinia in rabbits a condition of generalised vaccinia occurred, closely resembling human smallpox, in which there was a widespread distribution of macroscopic focal lesions not only in the skin but also in many of the internal organs.

The literature on vaccinia contains numerous references to "generalisation" in rabbits. The majority of authors have used the term to describe simply a widespread skin eruption, in many cases following inoculation into the skin itself. Calmette and Guerin (1901) were the first to record true generalisation; they found that an eruption of typical umbilicated pustules appeared, three days after intravenous inoculation of vaccine, on areas of skin which had been shaved; no lesions were obtained by them on other areas of skin not irritated by shaving. Camus (1917-1918) produced, by intravenous inoculation of dermovaccine, lesions on the lips tongue and genital organs, and showed also that nævi burns and the application of chloroform might determine localisation of lesions.* Levaditi and Nicolau (1923) carried out an extensive and suggestive research, using neurovaccine. Various routes of inoculation were employed, some of which gave rise to generalised lesions on the injured skin. Great stress was laid on the importance of injury as a determinant factor in the production of such lesions, but a few cases were noted in which pocks appeared after intravenous inoculation on skin areas not previously epilated, whilst irritation was found to be unnecessary in the case of the mucosæ. The mammæ were sometimes sprinkled with hæmorrhages and showed microscopically foci of necrosis and polymorphonuclear leucocytic infiltration. Generalisation to the brain followed intratesticular injection. These authors agree with Lipschutz (1922) in placing vaccinia among the *ectodermoses neurotropes*. They state that the virus possesses particular affinity for tissues derived from embryonic ectoderm and no affinity for those derived from the other germinal layers. Occasionally they found small foci of necrosis in liver and lung, but regard these as without definite significance. The very great susceptibility of the gonads to vaccinial infection is explained on the ground that germinal cells contain the potential characteristics of all other tissues including ectoderm. Huon and Placidi (1924) described a condition of generalisation in the rabbits used for the production of "lapine": a number of their animals died from encephalitis about the fourth day after the insertion of virus in skin scarifications: in addition to the lesions found in brain and meninges were pustules on the buccal mucosæ and tongue, but no mention is

* Noguchi (1918) obtained similar results by the intravenous inoculation of testicular virus.

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made of any lesions in the viscera. Nodake (1924) speaks about "generalised vaccinia" in rabbits, but means by this simply a spread of virus from a focus of primary infection in the testis which gives rise to immunity, in spite of early castration: he makes no mention of lesions, either macroscopic or microscopic.

The following protocol gives an outline of the condition of true generalisation including lesions in the internal organs as first met with by us.

Protocol 1.

Rabbit 10 inoculated 10.11.25 by skin scarification with brain "diffusate."* No signs of any reaction were noted until 1.12.25 (21 days) when rabbit became ill with watery discharge from eyes and nose, salivation and rapid breathing. The condition became worse on the following day and the animal was killed.

Post-mortem examination—

Skin . . .	Eruption resembling smallpox over entire surface of trunk, the shaved areas were not more affected than the skin elsewhere.
Lips and tongue .	Pocks on mucosa.
Mammary gland .	Peppered with whitish opalescent spots.
Lungs . . .	Numerous whitish, somewhat translucent spots.
Liver . . .	Small white spots irregular in shape.
Spleen . . .	Enlarged, firm.
Ovaries . . .	Enlarged, firm, paler than normal.

Bacteriological examination—

Heart's blood added to broth. This was incubated both aerobically and anaerobically and remained sterile.

Tests for virus—

The lesions were tested for presence of virus by rubbing the cut surfaces of foci over scarifications in the epilated skin of a normal rabbit:

skin spots	all gave confluent eruptions.
lip pock	
tongue	
mammary gland . .	
lung	
ovary	
liver	produced discrete pocks.
spleen	

Microscopic examination—

Sections of lung, ovary, mammary gland and liver showed foci resembling in essentials vaccinial skin lesions. These sections were kindly examined by Prof. H. M. Turnbull who confirmed our findings (*vide* Turnbull and MacIntosh (1926) *Br. Journ. Exp. Path.*, vii. 210).

* The term "diffusate" is used throughout to describe the clear fluid obtained by dropping a piece of vaccinial tissue into broth or Ringer solution and incubating at 37° C. for varying periods. Such fluids possess the advantages of being practically cell free and of proved bacteriological sterility: their potency is usually such that the intradermal inoculation of 0·2 c.c. of a 1 in 2000 dilution produces a distinct reaction.

The strain of vaccinia virus used throughout our experiments was obtained in the first place from Dr Levaditi. It was carried on by brain or testicular passages in rabbits.

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The post-mortem findings in this case are typical of a true generalised vaccinia, although the absence of primary reactions at the sites of inoculation and the long incubation period are unusual features. Later it was found possible to obtain such generalisation at will by a number of different methods, each of which produced certain special features.

Intravenous inoculation.

Intravenous inoculation is the simplest and most certain method for the production of generalised vaccinia. The severity of the symptoms and the extent of the lesions depend principally upon the nature of the inoculum, the amount injected and idiosyncrasy in the experimental animal. Most of the experiments were carried out on a strain of lop-eared rabbits which appeared to be more susceptible than other breeds especially when young animals were employed. Emulsions of vaccinial brain and testis have been used most frequently and have given rise to the most widespread distribution of lesions, the disease culminating occasionally in death, but diffusates of these organs in either broth or Ringer's solution have been successfully used on several occasions. As a general rule there is a rough proportionality between the amount of virus injected and the degree of resultant generalisation. Emulsions of about 1 part of tissue to 50 parts of saline (by weight) have usually been employed; from 1 c.c. to 5 c.c. of such emulsion rarely fails to produce very widespread generalisation. Idiosyncrasy also plays an important part in the determination of both degree and distribution: if two rabbits of similar weight are inoculated with exactly equal quantities of the same emulsion the resultant conditions may differ widely, as is clearly illustrated by R 447 and R 448 in table I.

The incubation period before the appearance of a skin eruption is usually 4 or 5 days, but has varied in our experiments from 3 to 7 days. There have been no cases of long incubation period following intravenous inoculation like that recorded in protocol 1 (Rabbit 10). The pocks on the skin are irregularly distributed and are particularly frequent round the lips and nares. Those in the hairy skin can be felt better than seen; in the early stages they feel like shot imbedded in the thickness of the dermis. Coincident with the appearance of the skin eruption there is obvious malaise, the rabbit becomes very quiet, sits huddled up in a corner of the cage and refuses food. The extent of the eruption increases for a few days whilst the general condition becomes worse. Frequently there is a watery discharge from the eyes and nose which later may become purulent and is often accompanied by salivation. Loose stools are the rule, sometimes a true diarrhœa sets in. Very occasionally symptoms indicative of an involvement of the central nervous system are met with such as paresis or paralysis of the hind legs, jerky movements of the head, strabismus, etc.; if this occurs the animal dies.

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TABLE I.—

Rabbit No.	Inoculum.	Amount inoculated, c.c.	Interval between inoculation and P.M. in days.	Hairy skin.	Lips or nose mucous.	Tongue.	Conjunctiva.	Mucous.
76	Brain emulsion	4	6	+	+	+	+	...
77	,, ,,	1	6	+	+	+	+	...
160	,, ,,	3	14	+	+	+	...	...
162	,, ,,	3	7	+	+	+	...	...
165	,, ,,	3	5	...	...	...	...	...
166	,, ,,	3	9	+	...	...	...	...
167	,, ,,	3	11	+	+	...	...	...
233	,, ,,	5	7	+	...	...	...	...
295	,, ,,	5	7	+	+	...	...	....
404	,, ,,	5	18	+	+	...	...	...
405	,, ,,	5	11	+	+	+	...	...
406	,, ,,	5	7	+	...	+	...	...
407	,, ,,	5	13	+	...	+	...	...
408	,, ,,	4	28	...	...	...	...	...
412	,, ,,	4	6	+	+	+	...	...
413	,, ,,	4	20	+	...	+	...	...
425	,, ,,	3	12	...	...	+	...	...
436	,, ,,	3	8	+	+	+	...	+
447	,, ,,	5	9	+	+	+	...	+
448	,, ,,	5	9	+	...	...	...	+
453	,, ,,	3	12	+	+	+	...	+
459	Pericardial fluid of R 448	1	15	+	+	+	...	+
464	Brain emulsion	1	13	+	+	+	...	...
465	,, ,,	2	7	+	+	+	...	...
466	,, ,,	5	7	+	+	+	...	...
477	,, ,,	0·04	14	+	+	+	...	...
478	,, ,,	0·1	10	+	+	+	...	...
524	,, ,,	0·5	4	...	...	+	...	...
609	,, ,,	Repeated inoc.	...	+	+	...	...	...
616	,, ,,	,,	...	+	+	...	...	...
757	Testis emulsion	3·5	14	+	+	...	...	...
785	,, ,,	3	7	+	+	+	...	...
786	,, ,,	2	5	+	+	+	...	+
817	,, ,,	2	6	+	+	+	...	...
818	,, ,,	2	6	+	+	+	...	...
830	,, ,,	2	5	...	+	...	...	...
	Total 36 rabbits			31	26	24	2	6

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TABLE I.—

Intravenous inoculation.

Lips or nose inoculated.	Tongue.	Conjunctiva.	Mammæ.	TISSUES WITH MACROSCOPIC LESIONS. Œsophagus.	Lungs.	Liver.	Spleen.	Suprarenals.	Testes.	Ovaries.	Uterus.	Bladder.	Intestine.	Connective tissue.	Muscle.	Heart.	Pericardium.	Peritoneum.
—	+	+	...	...	+	+	+	...	+	...	...	...	...	+	...	...	...	...
+	+	+	...	+	+	+	+	...	...	...	...	...	...	...	...	...	...	...
+	+	...	...	...	+	+	+	...	...	...	...	...	...	...	...	...	...	...
+	+	...	...	...	+	...	+	+	...	+	...	...	...	...	...	...	...	...
...	...	...	...	...	+	+	+	...	+	...	...	...	...	...	...	...	...	...
...	...	...	...	...	+	...	+	...	...	...	...	...	...	...	...	...	...	...
+	...	...	...	...	+	+	+	+	...	...	...	...	...	...	...	...	...	...
...	...	...	...	...	+	+	...	...	...	...	...	...	...	...	...	...	...	...
+	...	...	...	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...
+	...	...	...	...	+	+	...	+	+	...	...	...	...	...	...	...	...	...
—	+	...	...	...	+	+	+	+	+	...	...	...	...	...	...	...	...	...
...	+	...	...	...	+	+	+	+	+	...	...	...	...	...	...	...	...	...
...	+	...	...	...	+	+	+	...	...	+	...	...	...	...	...	...	...	...
...	+	...	...	...	+	+	+	...	...	...	...	...	...	...	...	...	...	...
+	+	...	...	...	...	+	+	+	...	...	...	...	...	...	...	...	...	...
...	+	...	...	...	+	+	...	...	...	...	...	...	...	...	...	...	...	...
...	+	...	...	...	+	+	...	...	...	+	+	...	+	...	...	...	...	...
...	+	...	+	...	+	...	+	+	...	...	...	...	...	...	...	...	...	...
—	+	...	+	...	+	...	+	+	...	...	...	...	...	...	...	...	...	...
...	+	...	+	...	+	...	+	+	...	...	...	...	...	...	...	+	...	...
—	+	...	+	...	+	+	+	...	...	...	...	...	...	...	...	...	...	...
—	+	...	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
—	+	...	...	...	+	+	+	...	+	...	...	...	...	...	...	...	...	...
—	+	...	...	...	+	...	+	+	...	...	...	...	...	...	...	...	...	...
+	+	...	...	...	+	+	+	...	...	...	...	...	...	...	...	...	...	...
—	+	...	...	...	+	+	+	...	+	...	...	...	...	...	...	+	...	...
...	+	...	...	...	+	+	...	...	...	...	...	...	...	...	...	...	...	...
+	...	...	...	...	+	...	+	+	...	...	...	...	...	+	+	+	...	+
—	...	...	...	...	+	...	...	...	...	...	...	+	...	+	...	+	...	...
+	+	...	...	...	+	+	+	+	+	...	...	...	...	...	...	...	...	...
—	+	...	+	...	+	+	...	+	...	+	+	...	...	...	...	...	...	+
+	+	...	...	...	+	+	...	+	...	+	+	...	...	...	...	...	...	...
—	+	...	...	...	+	...	...	+	...	+	...	...	...	...	...	...	...	...
—	...	...	...	...	...	...	...	+	...	+	+	...	...	...	...	...	...	...
25	24	2	6	1	35	24	24	14	7	7	4	1	1	3	2	3	1	3

The period of malaise and eruption is usually followed by gradual recovery, the pocks umbilicate, form black crusts and then fade away in about 3 weeks after their first appearance leaving no visible scars. The disease is succeeded by a complete immunity, even intracerebral inoculation of highly virulent neurovaccine proving entirely innocuous.

If the animal dies or is killed from the 5th to 10th day, a condition similar to that described in protocol 1 is found post-mortem. Some organs show lesions much more frequently than others, the skin and lungs are scarcely ever spared, mammary gland, tongue, liver, and spleen show lesions very frequently, other tissues are affected more rarely, but there is scarcely any tissue which has invariably escaped. Very occasionally the skin eruption is hæmorrhagic, in such cases death is the rule and at post-mortem examination the internal lesions are also found to be hæmorrhagic.

Table I shows the experimental details and the distribution of macroscopic lesions in 36 rabbits inoculated intravenously.

Skin inoculation: skin scarification and intradermal injection.

Generalisation, other than on the skin itself, has not been encountered very frequently as a sequel to the insertion of virus into skin scarifications, although the condition was first seen in such a case. A secondary skin eruption on the shaved area quite distinct from the primary reactions is common. This may be due to the relatively small amount of virus which gains entry in this method of inoculation: Huon and Placidi (1924) found that generalisation and encephalitis occurred only in those animals which had been scarified over a very extensive surface. Furthermore, Ledingham and M'Clean (1928) stated that a testicular strain of virus multiplied to a less extent when introduced by scarification than when inoculated intradermally. In our cases where generalisation did occur the lesions were usually slight, odd foci being found in lungs and liver, but a few exceptions have occurred, notably rabbit 639 which developed lesions in several other organs.

Intradermal inoculation leads to generalisation more frequently, but here also the lesions are seldom abundant or widespread. The local secondary skin eruption however occurs in almost every case and it has been seen even in rabbits which gave no primary reactions at the sites of inoculation; we consider in fact that this local spread in the skin is so common that any attempt to read the results of intradermal inoculation later than the fifth day is futile, especially as the secondary pocks show a tendency to localise in skin areas injured by inoculation of control material known to contain no virus. Table II shows the details of those cases in which true generalisation occurred.

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GENERALISED VACCINIA

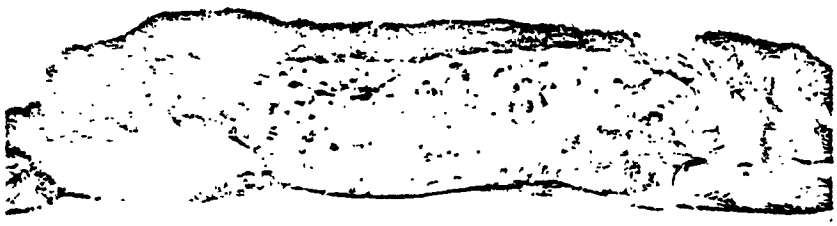


FIG. 1.—Rabbit 405. Typical skin eruption. Fur removed after death. Intravenous neurovaccine, brain emulsion.

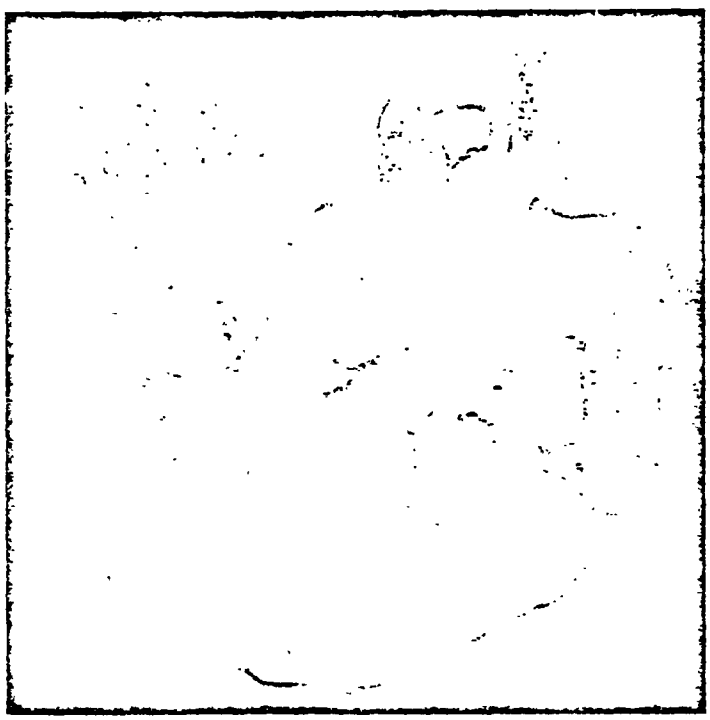


FIG. 2.—Rabbit 502. Lungs: very numerous lesions, areas of consolidation deeply congested, patches of emphysema. Intravenous neurovaccine passaged through testis, testicular emulsion.

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FIG. 3.—Rabbit 407. Liver. Conspicuous round white spots and numerous smaller lesions. Intravenous neurovaccine, brain emulsion.

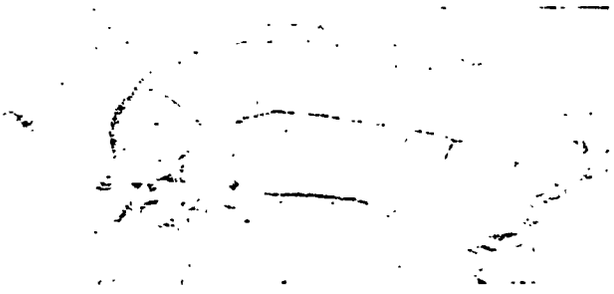


FIG. 4.—Rabbit 770. Appendix: several lesions and dilatation of veins. Intraperitoneal neurovaccine passaged through testis, testicular emulsion.



FIG. 5.—Rabbit 473. Heart with one pock. Spleen. Intravenous neurovaccine, brain emulsion.



FIG. 6.—Rabbit 753. Bladder: numerous large lesions on the peritoneal surface. Intratesticular neurovaccine passaged through testis, testicular emulsion.

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TABLE II.

Skin inoculation.

Rabbit.	Inoculum.	Route.	Interval between inoculation and P.M. in days.	Tissues with macroscopic lesions.											
				Skin.	Lips.	Tongue.	Mucosa.	Lungs.	Liver.	Supra-renals.	Ovaries.	Uterus.	Connective tissue.	Pericardium.	Peritoneum.
10	Diffusate	Scarification	22	+	+	+	+	+	+	...	...	...	...	...	...
173	Various tissues	,,	10	+	...	...	...	+	...	+	...	...	...	...	...
638	Diffusate	Intradermal	17	...	...	...	...	...	+	...	...	...	...	..	...
639	Calf lymph	Scarification	15	+	...	...	...	+	+	...	...	...	+	+	+
761	Diffusate	Intradermal	22	...	...	...	...	...	...	...	...	+	...	...	...
823	,,	,,	9	+	...	+	...	+	+	+	+	+	...	...	...

Intracerebral inoculation.

At least 90 per cent. of rabbits inoculated intracerebrally with neurovaccine die on the 4th or 5th day, even when very small doses are used. Therefore it is not surprising, considering the time relations of generalisation following inoculations by other routes, that the majority of these animals fail to show lesions elsewhere than in the central nervous system. In the few cases which have begun to generalise in this short time the lesions have been few and small. One exception, rabbit 67, which did not die as usual and was killed on the 38th day, showed post-mortem pocks in skin, lips, nose, tongue and soft palate, lungs, spleen, liver and connective tissue, and also congestion of the testes. In rabbit 66, another chronic case, no necrotic lesions were found, but exudates in pleural and pericardial cavities were proved bacteriologically to be sterile and were found by intracerebral and skin scarification tests to contain active virus. At the time it was doubted whether such a reaction could be due to

TABLE III.

Intracerebral inoculation.

Rabbit No.	Inoculum.	Amount c.c.	Interval between inoculation and P.M. in days.	Tissues with macroscopic lesions.										
				Skin.	Lips.	Tongue.	Soft palate.	Lungs.	Liver.	Spleen.	Ovaries.	Connective tissue.	Pericardium.	Pleura.
66	Brain emulsion .	0·2	29	...	...	...	...	...	...	...	...	...	Exu-date	Exu-date
67	Diffusate . .	0·2	38	−	−	−	−	−	−	−	...	+	...	...
467	Brain emulsion .	0·5	5	...	...	−	...	−	−	−	...	...	...	...
541	Diffusate . .	0·5	4	...	...	...	...	−	+	...	..	...	...	...
568	Brain emulsion .	0·5	7	..	...	...	...	+	+	...	...	...	...	...
772	,, ,, .	0·3	5	...	...	...	...	...	−	...	+	...	...	...

vaccinia, but the results of intraperitoneal inoculation dealt with below prove that serous membranes may be extensively affected. Table III shows that lungs and liver are the organs which most frequently develop lesions.

Intraneural inoculation.

Two rabbits were anæsthetised with urethane and ether, the sciatic nerves of both sides were exposed and that on the left severed. After an interval of 14 days the left nerves were again exposed and inoculated with 0·13 c.c. of neurovaccine; in one case the inoculation was into the central end of the nerve, in the other into the peripheral end. The primary object of this experiment was to determine whether the path of vaccinial infection followed the course of the nerves. No symptoms of encephalitis were observed, but in both animals generalisation occurred. Rabbit 193 inoculated peripherally developed typical lesions in skin eyes lungs adrenals and appendix; rabbit 194, inoculated centrally, lesions in skin lungs liver and spleen. The skin pocks first appeared in both cases on the same day after injection and their distribution was quite irregular over the surface of trunk and limbs. There was thus no evidence that the interference with the innervation of a limb modified the distribution of the lesions of generalisation.

TABLE IV.

Intraneural inoculation.

Rabbit.	Interval between inoculation and P.M. Days.	Tissues with macroscopic lesions.								
		Skin.	Lips.	Cornea.	Lungs.	Liver.	Spleen.	Adrenal.	Ovary.	Appendix.
193	15	+	+	+	+	+	...	+	+	+
194	9	+	...	...	+	+	+	...	--	...

Intratesticular inoculation.

Intratesticular inoculations have been performed in most cases with the specific object of obtaining sterile testicular vaccine; in consequence the animals have usually been sacrificed on the 4th or 5th day and therefore have not had time to develop the lesions of generalisation. A few rabbits have shown at autopsy a few scattered white spots in the liver, which on microscopical examination were considered to be vaccinial lesions, and in one a distinct, though small, pock was seen on the skin of the nose at the time it was killed on the 4th day. A few rabbits were spared in the hope that typical generalisation would ensue, but unfortunately they died on the 5th or 6th day.

The most constant and striking feature at autopsy, apart from the condition of the testes themselves, was the presence of a clear, straw-coloured peritoneal exudate of semi-gelatinous consistency which

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set into a firm clot a few minutes after withdrawal; occasionally it was very turbid and tinged with blood. This fluid has been tested repeatedly for bacterial flora, both by aërobic and anaërobic cultures, but has always proved bacteriologically sterile, and we regard it as a definite reaction to the vaccinia virus. It often contains virus in considerable quantity but occasionally fails entirely to produce a reaction on testing it either by intradermal or intratesticular inoculation. Peritoneal exudate was only produced by the inoculation of virus passaged at least three times from testis to testis, and has never followed, in our experiments, the intratesticular inoculation of brain emulsion.

TABLE V.

Intratesticular inoculation.

Rabbit.	Day of death.	Tissues with macroscopic lesions.				
		Skin.	Liver.	Bladder.	Adrenal.	Peritoneal cavity.
610	5	...	+	...	...	...
623	4	...	+	...	...	...
659	4	...	+	...	...	Abundant exudate
699	4	...	+	...	...	Fluid
733	6	...	...	+	...	Abundant clear fluid
760	4	...	...	+	+	Clear yellow fluid
765	8	+	...	...	...	...
768	4	...	...	...	...	Exudate

Intraperitoneal inoculation.

Intraperitoneal inoculations of neurovaccine were stated by Levaditi (1923) to be entirely innocuous. We were led to doubt this, in the first place, while carrying out the series of intratesticular passages. The earlier inoculations produced a testicular reaction only; they were made with emulsions of brain or emulsions of first or second passage testes. After three passages however the local reaction increased, and in addition the peritoneal exudate described in the previous section was found at post-mortem examination. The possibility suggested itself that the virus was not only acquiring increased virulence for testicular tissue but also an affinity for the cells of the peritoneum. Direct inoculation of a potent testicular emulsion into the peritoneal cavity was therefore performed, and resulted in a vaccinial infection with certain unusual features.

Protocol 2.

Rabbit 735 . . . . Inoc. 9.8.28 intraperitoneal with 1 c.c. emulsion of testis of R 724. Died 14.8.28, 5 days after inoculation.

Post-mortem.

Skin and external mucosæ No lesions.

Peritoneum . . . Hard nodule size of bean at site of inoculation. Peritoneum congested and sprinkled all over with punctiform hæmorrhages.

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set into a firm clot a. few minutes after withdrawal; occasionally it was very turbid and tinged with blood. This fluid has been tested repeatedly for bacterial flora, both by aërobic and anaërobic cultures, but has always proved bacteriologically sterile, and we regard it as a definite reaction to the vaccinia virus. It often contains virus in considerable quantity but occasionally fails entirely to produce a reaction on testing it either by intradermal or intratesticular inoculation. Peritoneal exudate was only produced by the inoculation of virus passaged at least three times from testis to testis, and has never followed, in our experiments, the intratesticular inoculation of brain emulsion.

TABLE V.

Intratesticular inoculation.

Rabbit.	Day of death.	Tissues with macroscopic lesions.				
		Skin.	Liver.	Bladder.	Adrenal.	Peritoneal cavity.
610	5	...	+	...	...	...
623	4	...	+	...	...	...
659	4	...	+	...	...	Abundant exudate
699	4	...	+	...	...	Fluid
753	6	...	...	+	...	Abundant clear fluid
760	4	...	...	+	+	Clear yellow fluid
765	8	+	...	...	...	...
768	4	...	...	...	...	Exudate

Intraperitoneal inoculation.

Intraperitoneal inoculations of neurovaccine were stated by Levaditi (1923) to be entirely innocuous. We were led to doubt this, in the first place, while carrying out the series of intratesticular passages. The earlier inoculations produced a testicular reaction only; they were made with emulsions of brain or emulsions of first or second passage testes. After three passages however the local reaction increased, and in addition the peritoneal exudate described in the previous section was found at post-mortem examination. The possibility suggested itself that the virus was not only acquiring increased virulence for testicular tissue but also an affinity for the cells of the peritoneum. Direct inoculation of a potent testicular emulsion into the peritoneal cavity was therefore performed, and resulted in a vaccinial infection with certain unusual features.

Protocol 2.

Rabbit 755 . . . . Inoc. 9.8.28 intraperitoneal with 1 c.c. emulsion of testis of R 724. Died 14.8.28, 5 days after inoculation.

Post-mortem.

Skin and external mucosæ No lesions.

Peritoneum . . . Hard nodule size of bean at site of inoculation. Peritoneum congested and sprinkled all over with punctiform haemorrhages.

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Peritoneal cavity	Full of semi-gelatinous turbid yellow fluid.
Intestines	Matted together with sticky exudate, with fibrinous adhesions; the large gut sprinkled with hæmorrhagic spots.
Liver	Entire surface sprinkled with small, round, white spots.
Spleen	Enlarged. Numerous white spots.
Adrenals	Soft and very friable. Left adrenal showed a pock.
Omentum	Rolled up into sausage-shaped mass, folds stuck together with exudate.
Uterus	Congested, entire surface covered with hæmorrhagic spots.
Bladder	Intense, hæmorrhagic congestion, small hæmorrhagic spots.
Kidneys	Nil.
Lungs	Nil.

Stained smears of the peritoneal exudate and the liver showed no micro-organisms. Cultures, both aërobic and anaërobic, remained bacteriologically sterile.

Sections of the affected organs showed microscopic lesions resembling pocks in the earliest stages.

The peritoneal exudate was tested for virus by inoculation into the testis of a normal rabbit, it produced a typical vaccinial orchitis on the 4th day, together with lesions in the bladder and adrenal.

The appearances of the abdominal cavity and viscera in this rabbit, although different from those hitherto encountered in any vaccinial injection, were undoubtedly due to vaccinia virus. There was however no sign of true generalisation, for only tissues and organs actually covered by the peritoneum, and hence in a position to come in close contact with the original inoculum, showed lesions. Further experiments proved that generalisation could be produced fairly easily by this method of inoculation, the failure of this first case to generalise being due probably to the early death of the animal.

Table VI summarises the post-mortem findings in eleven experiments (seven females and four males). The most striking feature, apart from the peritoneal exudate, was the frequency with which the reproductive organs were attacked. The uterus showed pocks in six cases, the ovaries in three and the mammary tissue in four; the testes were affected in three out of four animals. A further point of especial interest is the frequency with which the bladder showed changes; previously the bladder had been regarded as an organ entirely non-susceptible to vaccinia (fig. 7). The liver escaped in only one experiment, whilst the lungs showed recognisable lesions in only a single case; this is in striking contrast to the distribution of generalisation produced by intravenous inoculations. Peritoneal exudate was very abundant in all except one of the seven females and present, but to a less extent, in three of the males.

Two of the animals were inoculated with very thick emulsions of

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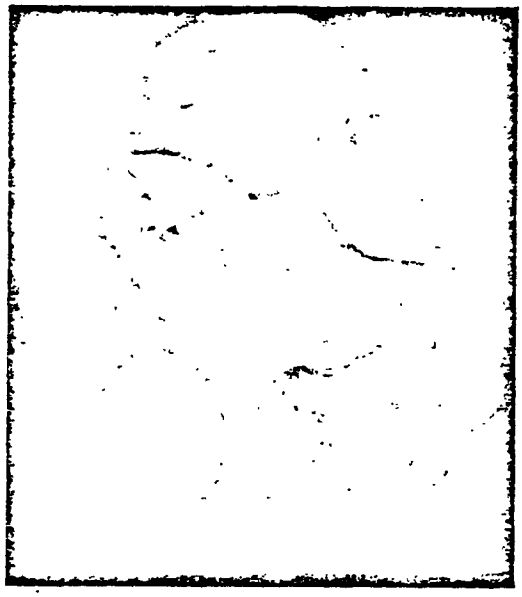


FIG. 7.—Rabbit 302. Uterus and broad ligament. Animal had aborted shortly before death. Left horn: pocks on peritoneal surface. Right horn opened up: inner aspect shows large pocks localised chiefly on placental sites. Broad ligament contains many lesions. Intravenous neurovaccine passaged through testis, testicular emulsion.



FIG. 8.—Rabbit 616. Photomicrograph (X67) of typical lesion in adrenal cortex: central necrosis and peripheral infiltration with mononuclear cells. Intravenous neurovaccine, brain emulsion.

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highly virulent brain in order to test the validity of the hypothesis that modification of neurovaccine by intratesticular passage is necessary to enable the virus to attack the peritoneum. The results proved conclusively that this is not the case, both animals had abundant peritoneal exudate with lesions of the abdominal viscera and also evidence of true generalisation. Nevertheless, in other experiments emulsions of brain failed to infect, and we express the tentative opinion that the testicular passage virus showed some evidence of exalted virulence for peritoneum and the generative organs.

TABLE VI.

Intraperitoneal inoculation.

Rabbit.	Sex.	Interval between inoculation and P.M. in days.	Tissues with macroscopic lesions.																
			Skin.	Tongue.	Lips.	Breast.	Lung.	Liver.	Spleen.	Adrenal.	Ovary.	Uterus.	Testes.	Bladder.	Peritoneum.	Heart.	Intestine.	Peritoneal cavity.	
755	F	5	...	...	...	...	...	+	+	+	...	+	...	+	+	...	+	Exudate	+ +
756	F	7	+	...	...	...	...	+	...	...	+	+	...	...	+	...	...	,,	+ +
766	F	11	...	...	...	+	...	+	...	...	...	+	...	...	...	...	...	,,	+
770	F	6	+	+	+	+	...	+	+	...	...	+	...	+	...	...	+	,,	+ +
771	F	7	+	+	...	+	...	+	+	...	+	+	...	+	...	...	+	,,	+ +
782	F	3	+	...	...	...	+	+	...	...	+	...	...	+	...	...	+	Nil	
783	F	4	...	...	...	+	...	...	...	...	...	+	...	+	...	+	...	Exudate	+ +
790	M	10	+	...	+	...	...	+	...	+	...	...	+	...	+	...	+	Nil	
791	M	11	+	...	...	...	...	+	...	+	...	...	+	+	+	...	...	Exudate	+
792	M	11	...	...	...	...	...	+	...	...	...	...	...	...	...	...	...	,,	+
793	M	11	...	...	...	...	...	+	...	+	...	...	+	...	...	...	...	,,	+

Description of lesions in generalisation.

(a) *Macroscopic.*—The macroscopic appearances of the lesions vary according to the tissues in which they occur and the stage of their development. It is unnecessary to describe separately the appearance of every organ in which evidence of generalisation has been found, for many of them have never presented any special features.

The external pock is best seen on the mucous membrane of lips and nose or on the hairless skin; it commences as a small raised papule which rapidly becomes hard and nodular, and a little later shows vesiculation followed by umbilication. The centre of the pock then assumes a darker colour, becomes necrotic and finally forms a "punched out" ulcer. It heals without leaving any scar (fig. 1).

Liver and spleen lesions are at first round dull white spots. Later they show a semi-translucent greyish zone when the white centre becomes sunken slightly below the surface of the organ. Occasionally on the liver a narrow ring of purple congested tissue forms around the lesion (figs. 2 and 3).

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The lung pocks begin as punctiform hæmorrhagic spots. When fully developed they are glistening greyish nodules with a hæmorrhagic spot in the centre; they do not stand out clearly from the normal tissue and may closely resemble the tubercles of acute tuberculosis before caseation has had time to develop. A peripheral zone of congestion is frequently seen (fig. 4).

The lesions of uterus and bladder are of two types. They may appear as small round white spots sprinkled over the peritoneal covering and quite flush with the surface. On the other hand, they are often distinct protuberant and umbilicated pocks which bear a close resemblance to those in the skin; in these ulceration may occur.

Lesions in other organs conform to one of the above-mentioned types. Those in the suprarenal bodies are not unlike accessory adrenals, except that they are never raised above the surface. In the ovary it may be difficult to distinguish between pocks and Graafian follicles and sometimes it is only by microscopical examination that one can be quite sure that vaccinial lesions are present.

(b) *Microscopic.*—The minute histology of the lesions of generalised vaccinia has not yet been investigated. The essential features of a lesion in any tissue are a central necrosis, consisting of a finely granular acidophilic material enclosing masses of basophilic fragmented nuclei, and a peripheral zone of cellular infiltration. The majority of these cells are mononuclear macrophages, but a number of polymorphonuclear leucocytes are also seen (fig. 8). Lesions of the skin, mucosæ and peritoneum may show ulceration, the edges of the ulcer are ragged and often undermined, the base is formed of necrotic debris. In the later stages fibrosis may be seen starting at the periphery.

Concomitant blood changes.

Blood counts, total and differential, together with estimation of hæmoglobin were carried out on two rabbits inoculated intravenously which showed the signs of generalisation (charts 1 to 4).

The number of erythrocytes commences to fall on the 2nd day after infection. This fall continues until the 7th day on which the red cell count attains its minimum, being approximately half the normal. From this time onwards there is a steady increase until the 15th or 16th day when the normal level is regained. Normoblasts appear in the blood stream particularly during the first week following injection (often to a much greater degree than in the cases illustrated here). There is a corresponding variation in the haemoglobin content of the blood; this is not strictly proportional to the red cell variation and a rise of the colour index accompanies the erythropenia.

The total white cell count decreases for 2 or 3 days, then shows a great increase attaining a maximum about the 9th day. The large

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nagic spots. When with a hæmorrhagic y from the normal acute tuberculosis peripheral zone of

types. They may over the peritoneal ne other hand, they pocks which bear a ese, ulceration may

ne above-mentioned t unlike accessory he surface. In the pocks and Graafian l examination that nt.

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with estimation of lated intravenously o 4).

n the 2nd day after which the red cell half the normal until the 15th or moblasts appear in following injection s illustrated here). bin content of the ell variation and a

days, then shows h day. The large

mononuclear cells show the greatest increase; with these we have included the so-called intermediate cells which possess the same characteristics as the large mononuclear cells except as regards size. The polymorphonuclear leucocytes also increase in number, but to a

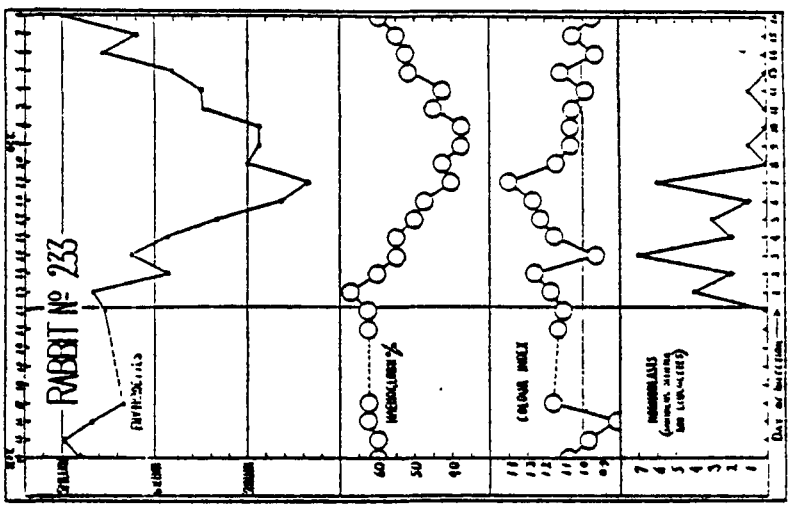


CHART 2.

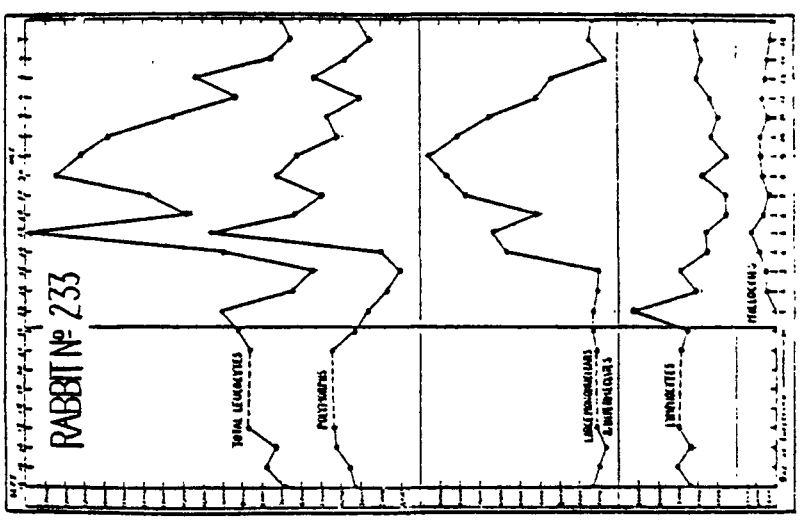


CHART 1.

less extent, while the lymphocytes show a decrease. Myelocytes appear in the blood from the start of the infection.

These findings differ considerably from those reported by Hine (1926) as occurring in rabbits infected by the method of skin scarification, especially as regards the polymorphonuclear leucocyte counts.

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Tissue susceptibility.

Our experiments support to some extent the opinion of Levaditi and Nicolau that neurovaccine displays particular affinity for tissues derived from embryonic ectoderm. But an analysis of tables I to VI makes it abundantly clear that almost all the tissues of the body are

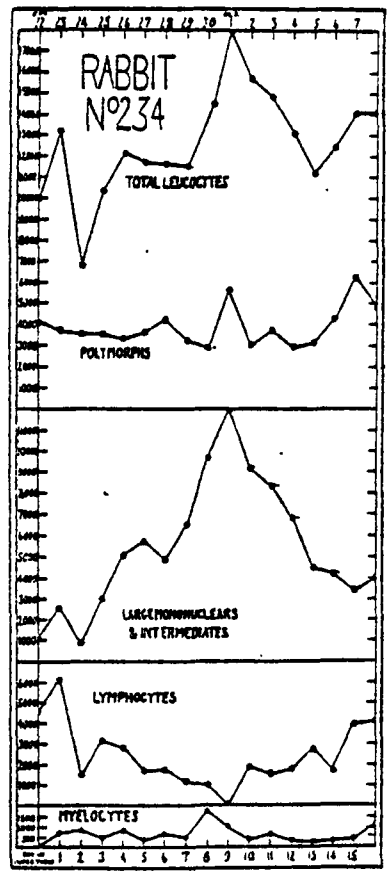


CHART 3.

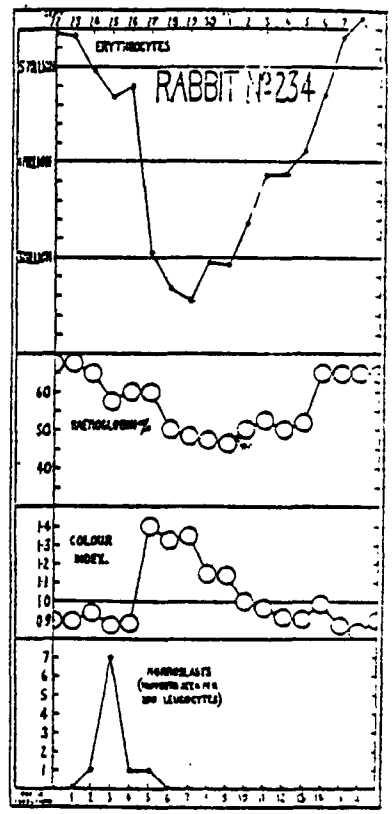


CHART 4.

capable, under certain conditions, of reacting to vaccinia virus by the formation of specific lesions macroscopically visible. The lungs, derived from endoderm, are affected in almost 100 per cent. of the cases of generalisation following intravenous inoculation. Tissues derived from mesoderm, although not so frequently affected, are by no means immune. The kidney is an exception; we have never seen it affected in any of our experiments. Waldhard (1927) however succeeded in cultivating vaccinia virus in the kidneys of rabbits by inoculating lymph directly into the organ and found that histological

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changes ensued which probably represented a specific reaction; generalisation to the injured cornea and skin occurred. He also recovered active virus from striped muscle in a series of six intramuscular passages.

In dealing with the question of tissue susceptibility only those rabbits infected by the intravenous route will be considered, because in no other mode of infection is there the same possibility of all the tissues being brought into contact with virus at the same time. In skin inoculations direct spread of virus probably plays a part, for secondary skin lesions are particularly abundant round the primary foci. Table VII shows the relative frequency with which lesions of various tissues have been found.

TABLE VII.

Susceptibility of tissues.

Total number of animals inoculated = 36. (Sex unrecorded.)

Lungs affected in	35	Suprarenals affected in	14
Hairy skin "	31	Ovaries "	7
Mucosæ "	26	Uterus "	4
Tongue "	24	Testes "	7
Spleen "	24	Mammæ "	6 (all females)
Liver "	24	Other organs "	less than 6

The lungs are therefore extremely susceptible, as much so as is the skin though it must be borne in mind that in intravenous inoculations the lungs probably receive and fix a large proportion of the virus before the remainder of the inoculum reaches the left heart to be distributed to the tissues served by the systemic circulation. Tables II, III and IV however offer additional evidence as to the great susceptibility of lung tissue. Unfortunately a record of sex was not kept, so that we are unable to record the precise frequency of lesions in the respective gonads.

Vaccinial infection as a cause of abortion.

In view of the fact that abortion occurs in almost all pregnant women suffering from smallpox, we induced generalised vaccinia in pregnant rabbits to see if any particular localisation would occur on the uterus and give rise to abortion. The results were positive.

Protocol 3.

Rabbit 302 . .	A pregnant female, expected to litter on 22.9.28.
13.9.28 . . .	Inoculated intravenously with 2 c.c. emulsion of vaccinial testis.
17.9.28 . . .	Aborted. Pocks appeared on lips.
18.9.28 . . .	Very ill, large crop of pocks round lips and nose.
20.9.28 . . .	Pocks developed in hairy skin and several in ears.
22.9.28 . . .	Died.

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Post-mortem examination—

External	Pocks on skin, lips, nose, ears, tongue.
Mammæ	Congested : distinct pocks.
Connective tissue	Pocks.
Peritoneum	Fluid in peritoneal cavity ; pocks on the membrane.
Uterus	External surface covered all over with large hæmorrhagic pocks with necrotic centres. The interior was full of a necrotic debris. Internal surface covered with large, protuberant pocks as large as small peas with ulceration of the necrotic central portions.
Ovaries	Masses of pocks.
Liver	Pale and soft : surface covered with lesions.
Spleen	Enlarged, dark purple, patches of white spots.
Adrenals	Covered with pocks.
Bladder	Very congested : masses of pocks on external surface.
Kidneys	Nothing abnormal.
Large intestine	A patch on one loop sprinkled with small white spots.
Diaphragm	White spots.
Lungs	Entire surface of both lungs covered with well-developed pocks.
Heart muscle	A small patch with white spots.
Pericardium	Cavity contained excess of clear fluid.
Brain	Congestion : no macroscopic lesions.

Sections of organs with lesions showed typical necrosis with the usual peripheral cellular infiltration.

This experiment was repeated on two other pregnant rabbits. In both of these abortion occurred, and the post-mortem findings were similar to the above description. Death ensued in all three cases. In another rabbit killed on the 4th day, before abortion had taken place, emulsions of placenta and of fœtus were found to be highly infective.

Tissue infectivity.

A few workers have been able to recover virus from the tissues of an injected animal after a period sufficiently long for the development of immunity. Levaditi and Nicolau claim to have demonstrated the presence of virus in testis and ovary by the skin scarification method up to 21 days after infection, but they appear to have inoculated a second dose of virus during this interval as an immunity test. They state explicitly that sterility of the organism is total after this lapse of time. Ohtawara (1922) used intratesticular inoculations as the method of test and succeeded in recovering virus from many organs up to the 8th day.

A series of experiments was carried out in order to determine how soon after intravenous inoculation the various tissues became infective, and how long they remained infective in cases of generalised vaccinia.

Eight rabbits (4 of each sex) were inoculated intravenously with 3 c.c. of an emulsion of vaccinial brain. They were killed at various intervals after inoculation. Pieces of the organs and tissues to be

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tested were removed with aseptic precautions and rubbed over linear scarifications in the epilated skins of normal rabbits (table VIII).

TABLE VIII.

Infectivity of tissues.

Rabbit No.	163	164	165	162	166	167	160
Day after inoculation on which animal was killed	2	3	5	7	9	11	14
Blood	?	?	...	...	...	...	...
Lung	+	+	+	+	0	+	+
Liver	0	+	...	+	0	0	+
Spleen	+	+	+	+	0	+	+
Bone marrow	+	+	?	+	...	+	+
Adrenal	...	...	...	+	0	+	+
Testis	...	...	+	...	...	+	+
Ovary	+	0	...	+	...	...	...
Lymphatic gland	...	...	...	...	...	+	...
Brain	0	0	0	+	0	...	+
Kidney	...	...	...	...	...	...	0
Skin	...	...	...	+	...	...	...
Tongue	...	...	...	+	...	...	...

The failure of any of the tissues of R 166 (killed on the 9th day) to give a reaction was possibly due to a certain grade of natural immunity in the test animal. One rabbit of the series (killed on the 38th day) has not been included in table VIII because many of the tissues gave very doubtful reactions.

In view of the more definite results obtained by intradermal inoculation as compared with skin scarification it was decided to repeat this investigation using the former test method. Three rabbits were inoculated intravenously with approximately 3 c.c. of lightly centrifuged emulsion of vaccinial testis. All the animals showed the signs and symptoms of generalisation with widespread skin eruptions. The first was killed on the 14th day when the skin pocks were in the early stages of retrogression: at autopsy it presented the typical picture of generalisation described above. The second animal was killed on the 28th day when practically all the lesions had healed; the third was killed on the 41st day. Tissues for test were removed aseptically and emulsified by grinding with sterile saline and sand in sterile mortars: 0·2 c.c. of each emulsion was inoculated intradermally into the epilated skin of a normal rabbit. Reactions were read on the 4th day (table IX).

Tables VIII and IX show that virus may remain latent in the animal tissues for a considerable time (up to 41 days in these experiments) after injection. The distribution of this "latent" virus is irregular. Of no organ can we be quite certain that it will contain virus in all cases after a given length of time. The organs particularly prone to harbour virus are brain adrenals skin and tongue, perhaps also the generative organs. The brains of such "carrier" animals have

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always shown a complete absence of lesions, both macroscopic and microscopic, whereas the other organs mentioned here have usually been the site of pathological changes.

TABLE IX.

Infectivity of tissues.

Rabbit No.	737	738	779
Day after inoculation when animal was killed	14	28	41
Blood	0	...	...
Skin	+	+	0
Tongue	+	+	+
Lymph gland	+	0	...
Lung	0	0	0
Liver	0	0	0
Spleen	0	0	0
Bone marrow	0	0	...
Adrenals	+	+	—
Ovaries or testes	0	+	+
Uterus	0	0	..
Brain	?	—	—
Kidney	0	0	0
Heart	+	...	0
Skeletal muscle	0	...	0
Bladder	0	0	0

Immunity following intravenous inoculation is usually so well established by the 10th day that even intracerebral injection of large doses of virulent brain emulsion fails to produce any symptoms. Therefore in the above series of animals there can be little doubt that those allowed to live more than 10 days were immune. Further investigations are being carried out to settle this point.

Virus in the blood stream.

In the earlier experiments, in which tests were performed by means of skin scarification, virus was never demonstrated in blood samples withdrawn later than the second day after injection. The distribution of the lesions of generalised vaccinia however strongly suggests a circulatory spread of the disease. Moreover Ohtawara (1922) reported that he obtained specific reactions by intratesticular inoculations of blood withdrawn up to the 15th day and his work has recently been largely confirmed by Gildemeister and Heuer (1928). Hoen Tschertkow and Zipp (1926) and Goldman (1928) produced cutaneous reactions with 4th and 5th day samples only by the use of a special technique in which they attempted blockade of the reticulo-endothelial system.

A series of experiments was carried out in which daily blood samples were withdrawn after intravenous injection and tested by intradermal inoculation into the epilated skins of normal rabbits,

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in the hope that during the process of generalisation the virus content of the blood would increase to a point at which it would be readily demonstrable. A reaction was obtained in one case on the 6th day, but the results set forth in table X show great irregularity. A similar irregularity was reported by all the workers cited above.

TABLE X.

Infectivity of blood.

Day of sampling after inoculation.	R 796.	R 797.	R 817.	R 818.
1	+ +	+ +	...	...
2	+	0	+	0
3	+	+	0	0
4	+	0	0	+
5	0	0	+	+
6	0	0	0	+
7	0	0	...	...
8	0	0	...	...
9	0	0	...	...
10	0	0	...	...

All the rabbits showing positive reactions were proved subsequently to be immune.

Discussion.

The experiments recorded here show that vaccinia virus is capable of producing in the rabbit a severe generalised disease which very closely resembles alastrim or uncomplicated smallpox in man. Although large doses of virus have been used in most of our experiments generalisation has often followed the inoculation of very small amounts. The lesions produced are very widely distributed both over the external surface and throughout the internal organs. They present a distinctive appearance macroscopically and microscopically, and are unlike any other lesions with which we are conversant. The internal lesions are always bacteriologically sterile and contain the virus in a condition capable of reproducing the disease. Those in the skin always heal without leaving visible scars, a fact which brings this experimental disease into closer relation with alastrim than with true virulent smallpox.

The study of the minute histology, which has not been attempted by us, should throw further light on the pathology of smallpox in man. In fatal cases of this disease lesions of the internal organs have nearly always been found to contain streptococci. Histologists have been undecided whether to ascribe the main rôle in their production to the streptococcal infection or to the virus of variola. The suggestion presents itself that the difference between alastrim and virulent smallpox may be that the former is a disease due solely to virus infection whilst the latter is an infection with the same virus com-

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plicated by the presence of pyogenic cocci. The ease with which both factors of such a mixed virus-bacterial infection may be transmitted during epidemics is exemplified by such diseases as measles and dog distemper.

The ability to produce generalisation is apparently not possessed by all strains of the virus. Our experiments strongly suggest that Levaditi's neurovaccine is capable of undergoing considerable modification. In the first place, we found that intradermal reactions became much more constant and easier to elicit with the neurovaccine strain obtained from him after it had been passaged through rabbits' testes. Secondly, the testicular passage induced an increased virulence for peritoneum and bladder. Thirdly, testicular passage virus induced generalisation with greater constancy, no matter by which route it was introduced.

We can offer no satisfactory explanation for the few cases of very *prolonged incubation* period. Rabbit 10 showed no signs or symptoms whatsoever until 21 days after skin scarification, then a very widespread generalisation occurred which would probably have proved fatal had not the animal been killed. Evidently virus may remain latent under certain circumstances in susceptible as well as in immune animals. Rabbit 67 also showed generalisation 38 days after intracerebral inoculation, but in this case a chronic infection produced cerebral symptoms from the 2nd day until it was killed.

Infection by the intraperitoneal route produces a veritable *peritonitis*. The constantly negative bacteriological examinations exclude the possibility of secondary infection as a cause of the condition. The exudate has been found very highly virulent in some cases, absolutely non-infective in others. Whether this disparity is due to the early outpouring of antibodies in the latter cases we are at present unable to say: further experiments are being carried out to settle this point.

The tables show that the particular strain of virus used by us, when inoculated by any given route, possesses varying degrees of *affinity for different tissues*. General statements on tissue susceptibility to vaccinia virus should however be received with caution. It is certain that commercial calf lymph possesses particular affinity for the skin and the original neurovaccine of Levaditi probably showed selective affinity for tissues of ectodermic origin, but the facts recorded here suggest that such "affinities" are merely the result of acclimatisation of virus in a new environment. Given the opportunity, virus might be able to attack any tissue as easily as calf lymph attacks the epidermal cells. This view is supported by the experiments of Walthard (1927) mentioned above.

Several authors have reported *survival of virus* in animals possessed of a certain grade of immunity. Our experiments on tissue infectivity confirm this. Rabbits 737, 738 and 779, containing virus in several

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organs, were doubtless immune at the time they were killed, for we have never known any rabbit to recover from generalised vaccinia and yet fail to show a complete immunity even to subsequent intracerebral inoculations of neurovaccine. How long the virus may remain latent has not been settled, though the point is of obvious importance in connection with the question of convalescent carriers of filterable viruses, such as that of anterior poliomyelitis.

Summary.

1. A condition of experimental generalised vaccinia in rabbits is described which closely resembles human smallpox of the alastrim type.

2. In this generalised disease almost every tissue of the body may be affected with the production of macroscopic lesions; no injury or irritation is necessary. The internal viscera most commonly affected are lungs liver spleen adrenals and the organs of reproduction. The kidneys are exceptional in that they have never shown generalised lesions.

3. The mesodermic origin of an organ does not indicate a non-susceptibility to vaccinia.

4. A strain of testicular passage virus was obtained which was highly infective when introduced by intraperitoneal inoculation; it produced a vaccinial peritonitis and gave rise to pathological lesions in organs hitherto thought to be non-susceptible, for instance uterus, bladder, peritoneum and muscle.

5. Vaccinia virus may remain latent in the tissues of an immune animal for a long period (up to at least 41 days) after infection.

6. Virus was recovered from the blood of an animal 6 days after infection. Infectivity of the blood is irregular and appears to have no correlation with the time incidence of generalisation.

7. Vaccinial infection caused abortion in three pregnant rabbits in all of which there was particular localisation of lesions in the uterus. Both placenta and fœtus contained virus.

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.1.

STANDARDISATION OF ANTI-DYSENTERY SERUM: EFFECT OF INCREASED TEST DOSE OF TOXIN ON THE ACCURACY OF TITRATION

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IN two previous papers published from these laboratories, Sudmersen Runge and O'Brien (1924) have described the standardisation of anti-dysentery serum (Shiga) by the use of intravenous injection of mice. This paper is a further study of the methods described by them. Similar methods were adopted by the Standardisation Sub-Committee of the League of Nations. For fuller details of the methods of numerical treatment used in this work, reference should be made to the following paper by J. W. Trevan, elaborating a statistical method for the presentation of results.

The difficulty in devising an accurate test for the standardisation of anti-dysenteric serum has been commented on by many workers. The error of the present methods, as compared with those in the titration of anti-diphtheritic or anti-tetanic serum, is very great. This appears to depend on two factors, the first of which arises from the great differences in susceptibility to a given dose of Shiga toxin in individual mice, and the second from the difficulty in obtaining a preparation of toxin containing a sufficient number of lethal doses in a volume and in a physical state suitable for intravenous injection of mice.

With regard to the first factor there is little reason to suppose that the rabbit or other animal can be advantageously substituted for the mouse. The same irregularities are found when the figures of the tests on a sufficient number of rabbits are examined as in those obtained by the use of mice (O'Brien and Runge, 1925). After reviewing the results of various workers, a League of Nations sub-committee decided on the use of mice. It might be expected that the individual irregularities in the susceptibility of mice could be partly eliminated by using a specially selected strain of mice: this expectation however has not been realised in our own work where from time to time tests have been put up on carefully chosen mouse populations. The varying susceptibility of mice to Shiga toxin may be seen from the following

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table: with both toxins it requires 4 times the L.D. 50 * to ensure an L.D. 100.

Shiga toxin 3 *A* (*dried bacillary bodies*).

	Number of mice injected.	Percentage mortality.
0·02 mgs.	20	100
0·01 „	20	80
0·005 „	20	45
0·0025 „	20	20

Shiga toxin P.T. III (*precipitated broth toxin*).

	Number of mice injected.	Percentage mortality.
0·2 mgs.	20	90
0·1 „	20	75
0·05 „	40	57·5
0·025 „	20	20
0·0125 „	20	15

The second difficulty is to obtain a toxin with a high "toxicity combining-power ratio." For example a typical high grade toxin consisting of dried bacillary bodies, contains less than 10 minimum lethal doses (*i.e.* L.D. 100) in the test dose as compared with approximately 100 minimum lethal doses in diphtheria, tetanus, botulinus or *œdematiens* toxin.

In 1926 the League of Nations sub-committee, to facilitate comparison of the work done at various laboratories on dysentery standardisation, suggested a test dose of toxin that should be used for the mouse test until further improvements could be devised. This test dose is the amount of toxin which when mixed with 1 unit of Copenhagen standard serum (*i.e.* 0·005 c.c.) and injected intravenously into a group of mice will kill 33 per cent., and with 2/3 unit of standard serum (*i.e.* 0·0033 c.c.) will kill 66 per cent. of mice. In the subsequent discussion this will be referred to as the L.T.D. (League test dose) of a toxin. This was accepted apparently as the largest test dose which could be conveniently used, taking into consideration the physical nature of the toxin and the volume which can be injected into the caudal vein of the mouse.

Most of the toxins chosen for test purposes by workers in connection with the League of Nations sub-committee consist of the dried intact bodies of the Shiga bacillus, and the League test dose in mgs. as well as the toxicity per mg. are all of approximately the same order. The bacillary body toxin 3 A, prepared by O'Brien and Range (1925), is of high potency for this type of preparation. The L.T.D. of 3 A is 0·12 mg. whereas the L.T.D. of 3 similar preparations issued

* As suggested by J. W. Trevan (1927) L.D. 50 is a convenient abbreviation for the dose which kills 50 per cent. of a large group of animals. Analogous symbols L.D. 75 and L.D. 100 indicate doses which kill 75 per cent. and 100 per cent. of animals.

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by the State Serum Institute, Copenhagen, on behalf of the League of Nations sub-committee had test doses of 0·33 mg., 0·45 mg. and 0·36 mg. respectively. Our experiments have shown that one is not necessarily restricted to such a small test dose of toxin as that suggested by the League of Nations sub-committee. For example a test dose of the dried bacillary toxin 3 A, consisting of 5 times the L.T.D. and the equivalent amount of serum, can be used, not only without affecting the specificity of the test but with a definite improvement in its accuracy.

The chart gives the results of the intravenous injection of mice with dried bacillary toxin and Copenhagen standard serum. The ordinates give the percentage survivals of mice and the abscissæ represent the doses of serum in c.cms. plotted on a logarithmic scale. Curve A was constructed by Trevan and O'Brien from observation of several hundred mice injected at various times with one League test dose of dried bacillary toxin and varying amounts of standard serum. We have confirmed the slope of this curve after an interval of more than one year by an experiment where 100 mice at three points were used on the same day and under the same conditions.

The neutralisation characteristic of the highest value serum which we have prepared (K.C.D. 1504) with a value of 6000 units is similar to that obtained with the standard serum (200 units). The results of the experiment using 1 L.T.D. of a dried bacillary toxin 3 A (0·12 mg.) and varying amounts of serum are as follows:—

	Number of mice injected.	Percentage survivals.	Theoretical percentage survivals.
0·000075 c.c. K.C.D. 1504	58	17·2	13
0·0001 „ „	57	26·3	31
0·00015 „ „	57	70·2	70

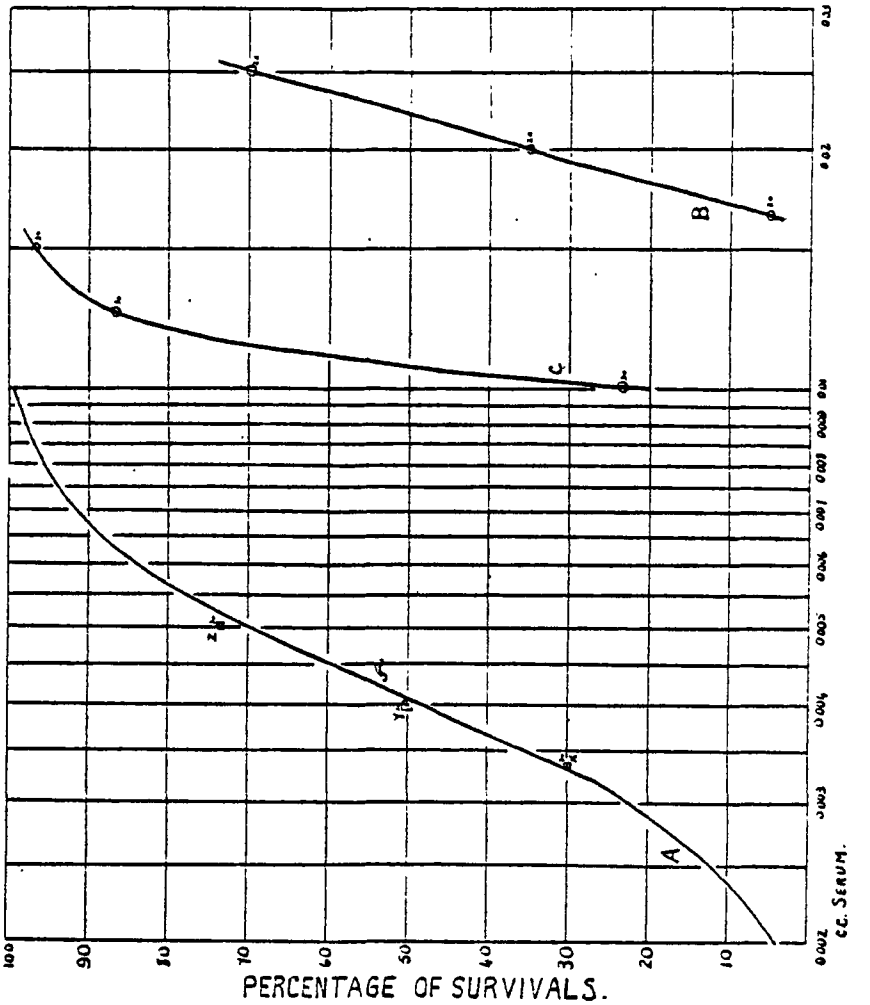
The theoretical percentage survival figures have been obtained by superimposing curve A on a similar curve obtained from the above figures. The differences are not statistically significant. A similar neutralisation curve has also been obtained by using a 2000 unit serum and 60 mice at three different doses of serum.

Curve B is constructed from observation of 20 mice at 3 different points, 5 times the L.T.D. of a dried bacillary toxin 3 A was used and the equivalent amount of serum. Curve B is considerably less sloped than curve A and this difference is shown to be significant in the accompanying paper (below p. 127) by Trevan by the usual statistical method. This effect must be due to the greater ratio of the surplus lethal doses to the neutralised lethal doses in the graded unneutralised mixtures.

A disadvantage in the use of bacillary body toxins is that in saline suspension the toxin is not in an easily diffusible state. Even after hours of standing the supernatant fluid after filtration has little

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toxicity. The toxic material does not diffuse out into the fluid medium but remains almost entirely within the intact bodies of the bacilli and one must imagine that before it can show its toxic effect on animals



Curve A: obtained from hundreds of observations. 1 League test dose dried bacillary toxin *v.* Copenhagen standard serum.
Points X, Y, Z: 30 mice at 3 different doses of standard serum *v.* 1 L.T.D. of toxin P.T. III.
Curve B: 20 mice at 3 different doses of standard serum *v.* 5 L.T.D. of toxin P.T. III.
Curve C: 30 mice at 3 different doses of standard serum *v.* 2½ L.T.D. of toxin P.T. III.

it must be set free from the bacillary bodies by phagocytosis, lysis or other intermediate process which must necessarily involve its own variable and uncontrolled factor. With a view to eliminating this

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complicating factor, attempts have been made to devise a test toxin which will be sufficiently toxic for the purpose of the test and will contain its toxic principle in a colloidal state of dispersion. We have prepared such a toxin (P.T. III) by adding 40 per cent. solid ammonium sulphate to a filtrate of a 14 days' growth of a strain of Shiga's bacillus, and collecting and drying the precipitate. The stability of this toxin has not yet been fully tested over a long period but up to the time of writing the stability seems entirely satisfactory.

The League test dose of P.T. III is 1·75 mgs. and in titration gave a characteristic curve similar to that with 1 L.T.D. of the bacillary body toxin 3 A. The actual results obtained by injecting 30 mice with 1 L.T.D. of toxin P.T. III and 3 different doses of serum are shown by the points X, Y, Z.

The L.T.D. of P.T. III contains 35 L.D. 50 s.
„ „ 3 A „ 24 „ „

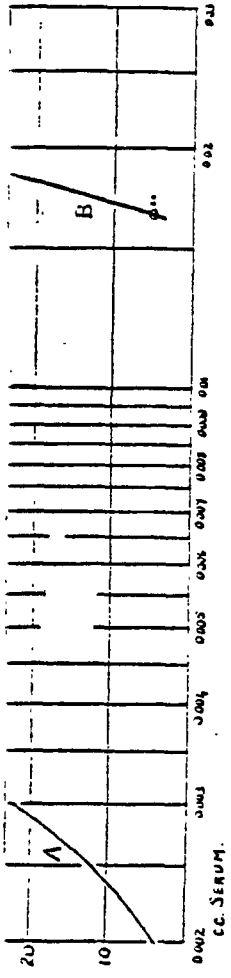
It was found that the largest amount of P.T. III which could be injected intravenously into mice without interference with the specific action of the toxin was 4·375 mgs. *i.e.* 2½ times the L.T.D. of this toxin. This contains approximately 88 L.D. 50 s.

Curve C shows the results of titration of 2½ times the L.T.D. of toxin P.T. III and the Copenhagen standard serum. 30 mice were used at three different points. The increased slope of the characteristic C compared with curve A (*i.e.* the decrease in error of titration) is comparable with the effect of increasing the test dose of 3 A as shown by curve B.

The greater curvature of curve C near the 100 per cent. survival point is due to the death of one out of the 30 mice injected. This was probably an accidental death as the mouse showed no signs of dysenteric paralysis. Owing to the relatively great aberrations in the region of the 100 per cent. survival point due to isolated accidental deaths, this part of the curve cannot be taken as a fair indication of the accuracy of the test.

The results of the experiments may be illustrated as follows. The test dose (L.T.D.) of dried bacillary toxin as recommended by the League of Nations sub-committee, when mixed with 0·005 c.c. of the Copenhagen standard serum, gives 66 per cent. survivals in a group of mice; and with 0·0033 c.c. standard serum gives 33 per cent. survivals *i.e.* a 33 per cent. drop in survival rate corresponds with 34 per cent. drop in serum potency. Interpolating in the curves, it will be seen that by using 5 times the L.T.D. of a dried bacillary toxin, such as 3 A, a 33 per cent. drop in survival rate represents a 10 per cent. drop in serum potency and with 2½ times the L.T.D. of P.T. III a 33 per cent. drop in survival rate represents a drop of 8·5 per cent. in serum potency. This begins to approach the accuracy of anti-diphtheritic and anti-tetanic serum titrations.

The experiments outlined above have been of a preliminary nature



ague test dose dried
r. 1 L.T.D. of toxin
.T.D. of toxin 3 A.
2½ L.T.D. of toxin

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eliminating this

only but we are convinced that the accuracy of anti-dysentery serum standardisation on a limited number of mice can be greatly increased by using a test dose of toxin which contains many more lethal doses than the present League test dose. A precipitated broth toxin such as P.T. III is more suitable as a test toxin than a dried bacillary toxin on account of its greater "solubility." (Professor P. Hartley recently kindly placed at our disposal a sample of a concentrated toxin prepared by him by another method. This toxin seems to possess the same advantage as P.T. III.) Further improvements are still possible in the preparation of a better toxin which will be more soluble and have a higher ratio of toxicity to combining-power.

In conclusion, it seems necessary to ask as in all biological titrations what degree of approximation to exactness should be demanded from estimations of dysentery antitoxin. The degree of accuracy which can be reached by the use of a test dose of precipitated toxin containing 2½ L.T.D. allows one to distinguish a 20 per cent. difference in potency in two sera on two groups of 10 mice. It seems reasonable to think that such a degree of accuracy in titration is more than sufficient to prevent any mistake being made in the estimation of serum which could be detected in therapeutic use.

Summary.

In the testing of dysentery (Shiga) antitoxin greater accuracy can be obtained by increasing the test dose of "bacillary body" toxin 5 times or by employing a toxin precipitated with ammonium sulphate and using 2½ times the present test dose.

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A STATISTICAL NOTE ON THE TESTING OF ANTI-DYSENTERY SERA

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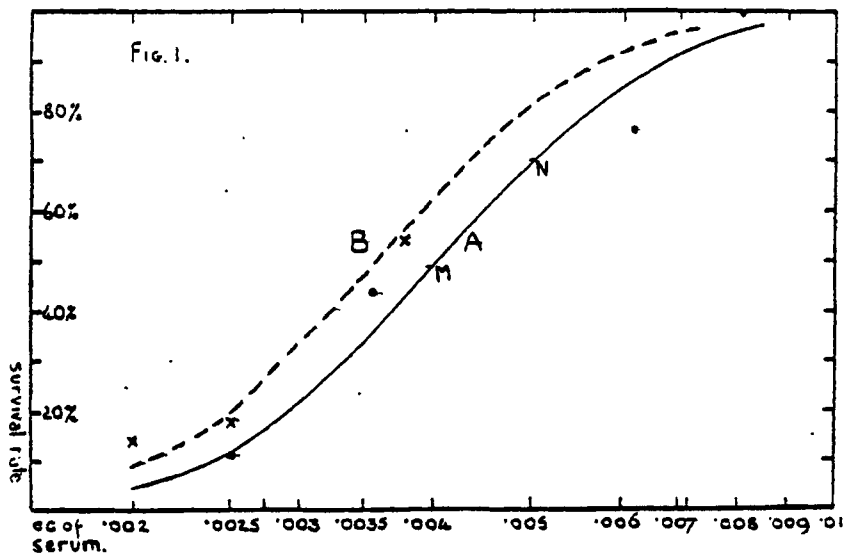
In the determination of the L_+ dose of a serum toxin mixture for any toxin it is usual to take a fixed dose of toxin and add increasing doses of serum until a lethal mixture is found, the addition to which of a small further quantity of serum will produce a non-lethal mixture. With all such mixtures the actual amount of serum necessary to protect some animals against the effect of a standard dose of toxin will fail to protect others. This arises from the fact that the L_+ mixture for one animal contains (or sets free after injection) one lethal dose or more of toxin for that animal. Since the lethal dose varies over wide limits for different individuals of a species the amount of serum which it is necessary to add to a given dose of toxin to leave just one dose of toxin free varies from animal to animal and the final L_+ dose assigned to a serum can only be an average value representing the L_+ dose for an average animal, and giving more or less accurately the average of the L_+ doses for the species used. As has been pointed out before, the accuracy of an estimation of an L_+ dose depends (1) on the proportion of toxoid in the test dose of toxin used, (2) on the number of lethal doses in the test dose of toxin and (3) on the steepness of the curve relating mortality to dose of toxin. The lower the proportion of toxoid, and the larger the number of lethal doses in the test dose and the steeper the curve of toxin mortality, the smaller the differences between the L_+ dose of serum for different animals of the same species and therefore the greater the accuracy attainable in the titration of an antitoxin.

For diphtheria toxin and serum under the conditions standardised, the differences between the individual L_+ doses of serum for different guinea-pigs are so small as to be of little importance, but the individual L_+ doses of anti-dysentery serum for mice vary over a comparatively wide range. In the curve A reproduced in fig. 1 (continuous line), the figures are taken by permission from a large number of titrations made by Dr O'Brien and Miss Runge, by the method described by them and Sudmersen (1924), for the permanent standards commission of the Health Organisation of the League of Nations. The serum doses are plotted on a logarithmic scale as abscissæ and the ordinates represent percentage of mice surviving. The dose of toxin is the same for all doses of serum and is chosen so as to give as nearly as possible a 66 per cent. survival rate with 0·005 c.c. of the International standard

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serum used. A smooth curve, which is a normal percentile frequency curve, has been drawn through the points and can be taken to represent with sufficient accuracy the average relation between dose of serum and survival rate towards which the results actually obtained would approximate as the number of animals injected with each dose of serum increased.

It will be seen that the variation of the response of the mice is such that on the average about 4 per cent. of a group of mice will be protected from the effect of this dose of toxin by 0·002 c.c. of serum,



Titration of antitoxin in anti-dysentery serum. Survival rates plotted against doses of serum, at different dates.

Serum: international standard.

Toxin: 3 A, 0·12 mgm. = 1 "League test dose." 320 animals (crosses) were used for B (by Miss Blake) and 220 for A (Miss Runge). The black circles represent one series of tests on 70 animals for A.

49 per cent. by 0·004 c.c. and 96 per cent. by 0·008 c.c. That is to say 96 per cent. of mice require more than 0·002 c.c. of serum, 51 per cent. require more than 0·004 c.c. and 4·0 per cent. require more than 0·008 c.c. It is very clear that if a small number of mice are used for a test, the dose of serum found necessary in different experiments will vary over a wide range. In addition to the wide range of dose of serum necessary to protect the individuals of any given group of animals Miss Blake has shown that a seasonal variation takes place in the average dose necessary to protect: the results will be described in a subsequent communication. The individual variations from the mean remain proportionally the same at different times as is shown by the two curves in fig. 1. The crosses represent the results with large groups of animals injected at a different time and the curve B

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drawn through them, which fits the points very well, is parallel to curve A. Since the dose scale is a logarithmic one, the parallelism of the two curves indicates that a similar proportionate increase of serum dose for each curve produces the same increment of percentage of survivals. This variation in the mean protecting dose of serum necessitates the use of a standard reference serum for each titration, the potency of a serum being gauged by the comparative survival rate in two groups of animals, one protected with the serum of unknown potency, and one with the standard serum. The estimate of potency of the unknown in terms of the standard can be arrived at most accurately by reading from curve A the ratio of the doses which give the same survival rates as that of the two groups of animals used in the test. Thus if 49 per cent. (point M) are protected by the "unknown" serum and 70 per cent. (point N) by the same dose of the standard, the ratio of the potency of the unknown to that of the standard is that of 0·004 (the dose protecting 49 per cent. = curve A) to 0·005 (the dose protecting 70 per cent. in curve A).

The degree of accuracy of this estimation can be derived by methods already described (Trevan 1927): on p. 501 of the paper cited is a formula for estimating the accuracy of such estimations. That formula is worked for an arithmetical scale of dosage. The corresponding formula for a logarithmic scale of dosage, the geometrical proof of which is similar, is:—

$$\text{Log R} = 200\,A\sqrt{\frac{2pq}{N}}$$

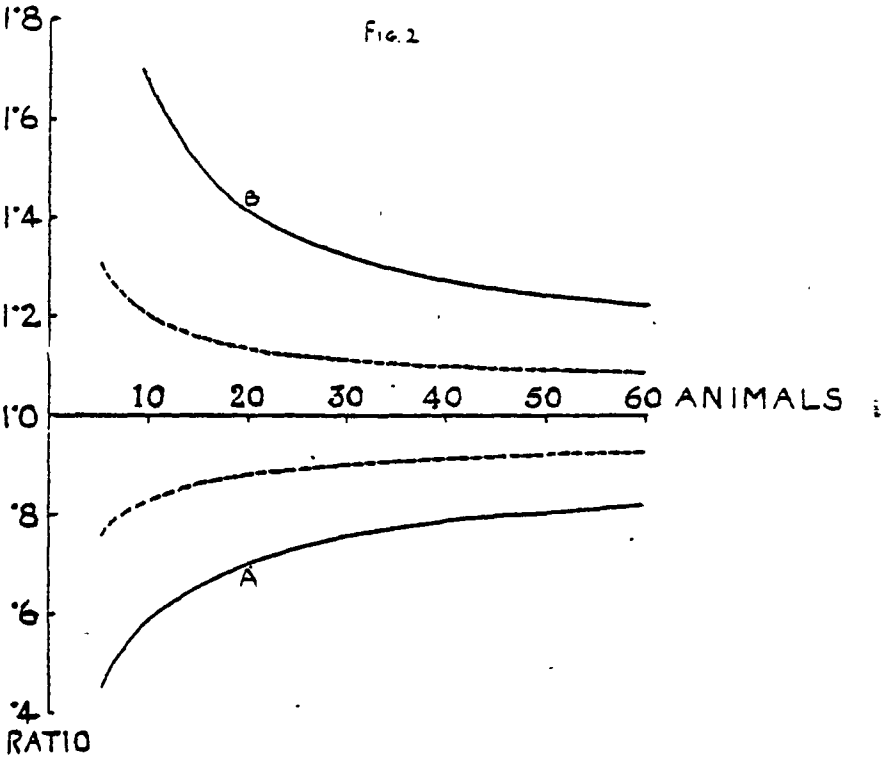
where R is the ratio of the extreme positive variation of the estimated value to the true value and also the ratio of the true value to the extreme negative variation. The extreme variations taken are those outside which only 5 per cent. of estimations will fall. A is the increment of the logarithm of the dose for each addition of 1 per cent. to the survival rate; that is, for example, for an increase in mortality rate from 35 per cent. to 36 per cent. or 71 per cent. to 72 per cent. p is the chance of survival, and q that of death for the dose given, and N is the number of animals in each group.

The value of A to be used is calculated from the average mortality of the two groups. The formula gives much closer results than the one originally derived for characteristic curves on an arithmetical scale of doses, and for the dysentery curve gives almost exact agreement with the more laborious methods as previously described for groups as low as 10 in number and survival rates as high as 90 per cent. In fig. 2, the full line curve gives the limits of the error calculated from this formula for different sized groups of animals. The abscissæ are the numbers of animals in each group, one group injected with standard serum and the fixed dose of toxin, and one injected with serum of which the potency is required and the fixed dose of toxin. The ordinates are the number of units estimated to be

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present for each unit actually present. Thus using 20 animals in each group for each unit of antitoxin actually present the test will give an estimate varying between 0·7 (point A) and 1·4 (point B).

The formula brings out the fact, which is indeed obvious without a formula, that the accuracy of the titration depends on the magnitude of A.

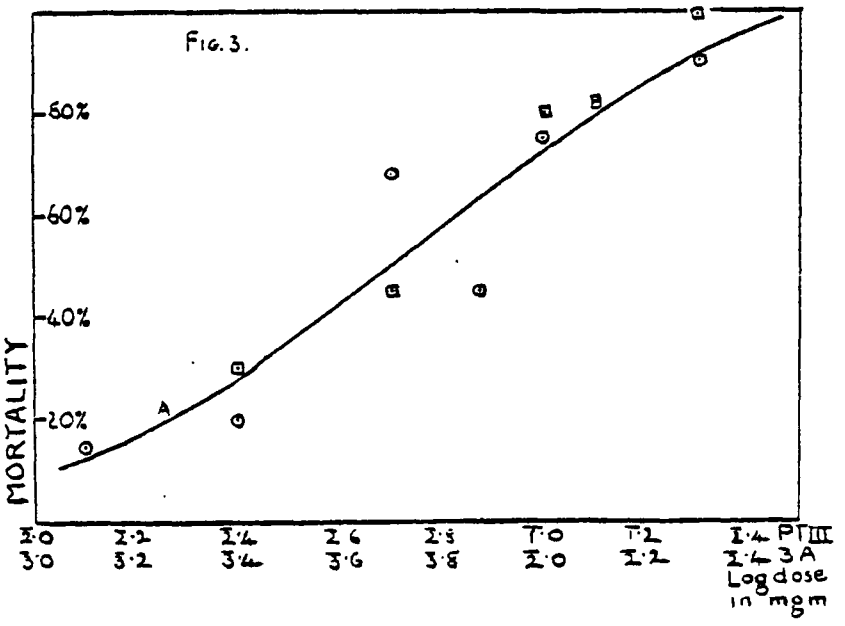


Limits of error outside which 5 per cent. of observations will fall when an unknown serum is compared with a standard. The ordinates marked "ratio" give the number of units estimated for each actual unit present. The abscissæ are the number of animals used; two such groups being injected one with the unknown and one with the standard serum. The continuous lines refer to titrations made with one test dose of 3 A, the broken lines to titrations made with 2½ test doses of P.T. III.

The value of A may be expected to be diminished by use of larger doses of toxin in the titration as is shown by the following argument. The survival rate curve for mixtures of serum and toxin gives a measure of the excess toxin in the mixtures. A survival rate of 80 per cent. using 1 "League test dose" of toxin 3 A corresponds to the presence (or the setting free in the animals) of 0·00182 mgm. of excess toxin in the particular mixture used; as is shown by the toxicity curve (A in fig. 3). This mixture contains 0·00565 c.c. of

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serum. A survival rate of 20 per cent. corresponds to a dose of toxin in excess of 0·0103 (B in fig. 3). The serum dose in the mixture is 0·0029 c.c. If a group of animals only sufficient in number to distinguish between these survival rates is used in a titration of a serum, doses of serum from 0·0029 to 0·00565 c.c. may be confused in successive titrations. The amount of serum necessary to reduce the excess toxin from 0·0103 mgm. to 0·00182 mgm. is 0·00565 − 0·0029 c.c. = 0·00275 c.c. which is 64·7 per cent. of the mean (0·00428 c.c.) of the doses which may be confused.



Toxicity curves for two toxins: 3 A (squares) and P.T. III (circles).
The toxicity of 3 A is ten times that of P.T. III so that two scales of abscissæ are given, one for 3 A and one for P.T. III.

Let us now assume for the sake of argument that further addition of serum to the 80 per cent. mixture produces proportional diminutions in the amount of toxin neutralised. Then if we take a multiple of the test dose, say 5 times, and add serum to it until it gives a mixture which protects 50 per cent. of a large group of animals it will contain 0·0214 c.c., approximately 5 times the dose which protects 50 per cent. when only one test dose is used; and the range of doses not distinguishable using the same small number of animals as before will be from that giving 20 per cent. to that giving 80 per cent. of survivals on a very large group of animals, or mixtures containing 0·0103 to 0·00182 mgm. of unneutralised toxin. If we make the assumption that at this stage of the neutralisation each addition of serum absorbs the same amount of toxin as before, the mixture which will protect 20 per

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cent. of a very large group will contain 0·00275 c.c. of serum less than the mixture which will protect 80 per cent.; this being the amount of serum necessary to reduce the excess toxin from 0·0103 to 0·00182 mgm. The percentage range of the mean dose of serum which this uncertainty in the end-point represents is $\frac{100 \times 0{\cdot}00275}{0{\cdot}0214} = 13$ per cent.—one-fifth of the error for one test dose. Therefore when the serum dose is plotted on a logarithmic scale as in fig. 1 the survival rate curve will be steeper—the value of A will be less.

In actual practice it is likely that the decrease in the value of A will not be in proportion to the multiple of lethal doses used, for when a toxin such as diphtheria toxin is near complete neutralisation further additions of serum take up less and less toxin. The reduction in A for 5 L.T.D. will therefore be less than that calculated above. That the reduction is appreciable is shown by the figures in the preceding paper by Blake and Okell (above p. 125) using multiple test doses of toxin 3 A and of a toxin P.T. III precipitated from a filtered culture. The curves for 3 A in this paper and those given in their paper show that the value of A for different test doses are those calculated in table I. The

TABLE I.

Toxin.	No. of League test doses.	No. of L.D. 50.	Value of A.	Relative number of animals necessary for given degree of accuracy.
3 A . .	1	24	0·00455	6·9
3 A . .	5	120	0·00277	2·4
P.T. III .	1	35	0·00390	4·9
P.T. III .	2·5	88	0·00180	1·0

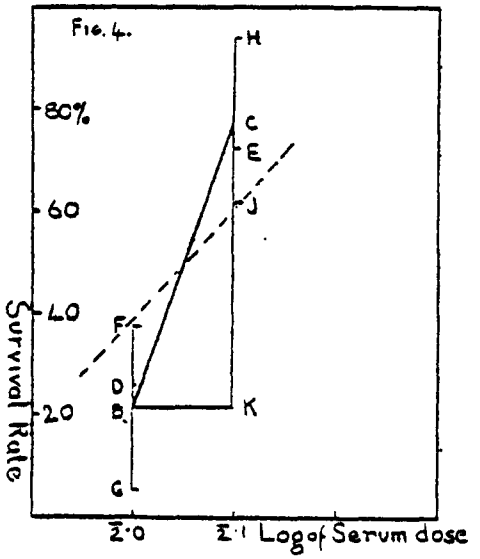
values of A are given for the range between a survival rate of 25 per cent. and 75 per cent. where it is practically constant. The calculation for 2½ League test doses of P.T. III is as follows. An increase of dose from 0·01 to 0·0125 c.c. of serum gave rise to an increase in survival rate of 76·6 per cent. − 21·6 per cent. The difference of the logarithms of 0·01 and 0·0125 is 0·097. A is therefore $\frac{0{\cdot}097}{76{\cdot}6 - 21{\cdot}6} = 0{\cdot}0018$.

The number of L.D. 50 (dose necessary to produce 50 per cent. mortality) refers to the number of lethal doses of toxin in the test dose of toxin and is derived from the mortality curves for toxin alone which is given in fig. 3 from figures obtained by Miss Blake. The shape of the curves is indistinguishable and the L.D. 50 for 3 A is approximately 0·005 mgm. and P.T. III 0·05 mgm.

The relative magnitude of the errors and the relative numbers of animals necessary to obtain the same accuracy are also tabulated, and

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in fig. 4 the accuracy of estimation using 2½ test doses of P.T. III is plotted (dotted lines). The observed value of A is affected by an error which can be estimated in the manner similar to that given for the estimation of the error of K in the formula for arithmetical dose scales (Trevan p. 508), as follows. In fig. 4, BC represents the middle part of the survival curve obtained by Blake and Okell for 2½ L.T.D. of P.T. III and the international standard serum. The dotted line is the slope of curve A fig. 1. B is the survival rate with 0·01 c.c. of serum and 2½ L.T.D. of P.T. III and C that with 0·0125 c.c. The true value D of the survival rate for 0·01 c.c. serum may lie anywhere on the line FG



where FG is the range equal to plus or minus twice the standard deviation outside which only 5 per cent. of such observations would lie. Similarly, HJ represents ± twice the standard deviation for the point C. The difference in survival rates with 0·01 and 0·0125 c.c. of serum, CK, is inversely proportional to A and the standard deviation of this difference is the square root of the sum of the squares of the standard deviations of B(S_B^2) and C(S_C^2).

$$\therefore \text{ true value of } \frac{1}{A} = \frac{CK \pm 200\sqrt{S_B^2 + S_C^2}}{BK}$$

$$= \frac{CK \pm 200\sqrt{\frac{0{\cdot}22 \times 0{\cdot}78}{60} + \frac{0{\cdot}23 \times 0{\cdot}77}{60}}}{BK}$$

$$= \frac{55 \pm 15{\cdot}24}{0{\cdot}097}$$

$\therefore$ A is between $\frac{0{\cdot}097}{70{\cdot}24}$ and $\frac{0{\cdot}097}{39{\cdot}76}$

i.e. between 0·00138 and 0·00244.

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The limits for the value of A for the other doses of toxin are given in table II.

TABLE II.

Toxin.	No. of League test doses.	A observed.	Possible limits of value of A.
3 A . .	1	0·00455	0·00409 to 0·0051
3 A . .	5	0·00277	0·00170 ,, 0·00314
P.T. III .	1	0·00390	0·00262 ,, 0·00750
P.T. III .	2·5	0·00176	0·00133 ,, 0·00244

The possible range is large in all cases, except 1 test dose of 3 A, because of the relatively small number of animals, but it is probable that the number of animals necessary to obtain the same degree of accuracy with 3 A (1 test dose) is not less than three times that necessary with 2½ test doses of P.T. III. The difference between 5 doses of 3 A and 2·5 doses of P.T. III is not significant, but the difference between 1 and 2·5 test doses of P.T. III and the difference between 1 and 5 test doses of 3 A are significant.

The observed values of A for 5 "test doses" of 3 A and 2½ "test doses" of P.T. III suggest that the effect of increasing test doses is greater for P.T. III than for 3 A. This difference, if a real one, is not due to any difference in the distribution of the lethal dose of toxin alone (see fig. 3), but must be due to a difference in neutralising properties, but the number of animals used is not sufficient to establish decisively the advantage of P.T. III over 3 A. The case for using large test doses is, however, quite complete.

Summary.

1. Methods of evaluating the error of determination in toxicity determination are described and applied to the estimation of the potency of anti-dysentery serum.

2. The error of estimation of the potency of Shiga anti-dysentery serum by the method recommended by the League of Nations health committee is calculated. If 40 animals be injected, half with a standard serum and half with an unknown serum, the potency of the known serum in terms of the standard will be not less than 70 per cent. and not more than 140 per cent. of the true value in 95 per cent. of such tests.

3. The use of multiple test doses of toxin as described by Blake and Okell is shown to diminish the limits of error to 88 per cent. and 112 per cent. of the true value for the same number of animals.

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SIDEROSIS OF THE GLOBUS PALLIDUS: ITS RELATION TO BILATERAL NECROSIS.

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(PLATES XIV.-XVII.)

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I. Introduction.

SPATZ has shown that the grey matter of the normal cerebrum and brain-stem with few exceptions reacts positively with the usual histological reagents for detecting free iron, but that the globus pallidus of the lenticular nucleus and the melanin-free portion of the substantia nigra show rapid and precise staining, accurately marking them out from neighbouring nuclei and contrasting strongly with the weaker and more slowly developing colour of the surrounding grey matter. Hurst has shown that this richness of the globus pallidus in iron is very likely related to a gross, and often striking, infiltration of its vessel walls by iron salts, a condition found by him in fifty out of a hundred brains from cases of chronic nervous disease.

In this paper evidence will be brought forward showing that this vascular siderosis is frequently found in the pallidum of normal brains but that under some pathological conditions it is, in all probability, the determining factor in the production of acute bilateral destruction of this nucleus.

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Iron in the brain under physiological conditions.

No iron can be detected histologically in the brain of the fœtus and new-born child but from about the sixth month onward the globus pallidus gives a positive reaction. A free iron reaction then appears in the substantia nigra, the red nucleus, the dentate nucleus of the cerebellum, the corpus Luysii, the putamen and caudate nucleus, roughly in this order. In the adult, in addition to the above situations, a weak iron reaction can be normally obtained in the corpora mamillaria, ganglion habenulæ, optic thalamus, deep layers of the cortex, the claustrum, nucleus amygdalæ and corpora quadrigemina, and a weaker reaction still in the pontine nuclei, the grey matter of the third ventricle, the pineal gland, the pituitary and the nuclei tecti.

The richness of the normal globus pallidus in iron is not necessarily related to its reddish colour for Spatz finds that the reaction can be given by colourless compounds. Furthermore, a similar colour is found in the corpora geniculata, but these nuclei are histologically iron free. In the pallidum and substantia nigra the iron is found in their comparatively large nerve cells and in their neuroglia (Spatz). In the pallidum it may be found normally as particulate granules. The pallidum is rich in fat and lipochrome pigment. In other situations the iron staining is diffuse and particulate granules are absent, no iron being found in the nerve cells.

The brains of some of the mammalia give the reaction, though not so intensely, in those portions which react most strongly in the human brain—the globus pallidus and substantia nigra. Kappers found that the meso- and palæo-striatum of birds gave the strongest reaction.

Spatz believes the iron to be intrinsic and not hæmatogenous, not increased by excessive hæmolysis and in the adult very constant in quantity. Hallevorden and Spatz examined the brain in a case of congenital idiocy with generalised flexor contractures and choreo-athetoid movements. Strictly confined to the globus pallidus and substantia nigra were thickly scattered dark brown pigment granules giving an intense iron reaction. They think this condition is a pathological exaggeration of a condition normal to both the globus pallidus and the substantia nigra and regard it as indicating a close physiological relationship between these structures. They regard the pigment as a product of metabolism and attribute its formation to neuroglial nuclei. Spatz has carried the investigation further by attempting to trace a connection between the globus pallidus and the substantia nigra. He finds the latter present in all mammals and, probably, lower vertebrates but melanotic pigmentation in it is peculiar to man in which it appears at about the fourth year. The compact melanin-containing portion lies in a dorso-mesial position. The reticular portion lies ventro-laterally, is free from melanin but its nerve cells contain iron. It is connected by iron-containing cell-islands to the grey matter of the globus pallidus. This direct connection is denied by Warkavy. Hoff has succeeded in loading the central nervous system with iron, in dogs with an Eck's fistula. Symptoms resembling those found in so-called extra-pyramidal disease were produced when the total iron in the brain reached twice the normal: the highest amount was retained in the globus pallidus. He expresses the view that iron very likely plays a part in the metabolism of the nerve cells of the globus pallidus, substantia nigra and extra-pyramidal system in general.

Iron in the brain under pathological conditions.

There is considerable literature dealing with deposits of ferruginous material in the brain in disease. Weber in 1908 found it encrusting nerve cells near old foci of softening. Clarke confirmed this: incidentally he showed that the so-called calcified tube-casts in the kidney of mercuric chloride poisoning are

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largely composed of iron. Perusini, in addition to describing the encrustation of necrotic nerve cells with iron salts, also described granular ferruginous deposits lying free in damaged nervous tissue and said such deposits were commoner than was previously supposed. He particularly found them in the region of the internal capsule in chronic encephalitic and arterio-sclerotic conditions. Da Fano and Perdrau found nerve cells encrusted with iron salts and a granular deposit of the same material in the brains of two rabbits which had recovered from herpetic encephalitis. Spatz first described a perivascular deposit of iron-containing material in the inflamed cortex of general paralysis and trypanosomiasis and occasionally in the region of a cerebral hæmorrhage or thrombotic softening. In all these instances the material giving the iron reaction is limited to a definitely diseased area, lying free in damaged nervous tissues, encrusting necrotic cells or contained in the bodies of glial adventitial and endothelial phagocytes. It originates, in all probability, in the iron of the inflammatory exudate and is hæmatogenous.

Iron deposits limited to the lenticular nuclei.

Quite apart from the deposits described above there are many records describing amorphous material in the globus pallidus or lenticular nucleus, usually bilateral and not accompanied by signs of encephalitis. In the earlier records the deposits are described as calcareous and frequently as mulberry-shaped. Elischer in 1875 described these in the corpus striatum in chorea. Flechsig and also Jakowenko found them in the globus pallidus in the same disease. Wollenburg found them in the lenticular nuclei in six cases of chorea, and in thirty-two out of forty-six cases of other diseases. Similar deposits were found by Perusini in the region of the internal capsule in chronic encephalitic and arterio-sclerotic conditions and in these he could demonstrate iron but not calcium. It seems very likely from Hurst's observations that the deposits described as calcareous by authors previous to, and by several after, Perusini were actually ferruginous.

Deposits in the vessel walls of the globus pallidus.

Vascular "calcification" in the brain was first described by Aschoff in 1902. It was observed in encephalitis lethargica for the first time by Buzzard and Greenfield in 1919 in a woman of twenty-eight dying four months after a severe and characteristic attack of the disease. The vessel walls of the globus pallidus were heavily infiltrated; a few vessels in the cortex were affected. Dürck found "calcification" in the brain in twelve out of fifteen cases of lethargic encephalitis dying from eight days to five weeks from the onset and at ages varying from thirty to seventy. The "calcification" was found as an encrustation of nerve cells, deposits of globules and flakes and "calcification" in the walls of blood vessels. The encrusted nerve cells were necrotic. The globular deposits were found most often in the white substance of the hemispheres. They had the appearance of cerebral sand normally found in the pineal body. "Calcification" of the walls of the blood vessels was found in the corpus striatum in eight cases, in the dentate nucleus of the cerebellum in three, in the hippocampus major in two, in the centrum semiovale in two, in the optic thalamus in one. The process was often limited to the walls of one artery, its arterioles and capillary field. In the capillaries the deposit was in the outer layers of the adventitia or just external to it. In the arteries it was in the media and the inner portion of the adventitia and was frequently preceded by hyaline degeneration: the intima was rarely affected. McAlpine found "calcification" in the vessel walls and in the form of free granules in the anterior parts of the globus pallidus in six cases of encephalitis lethargica, in one case as early as the fourth day of the disease. The change was also found in a case of Huntington's chorea and in three cases of idiopathic

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paralysis agitans. He emphasises the fact that the deposits in his cases were strictly localised to the anterior part of the pallidum. Preceding the deposit in the vessel wall he describes either a process of hyaline degeneration of the media, or a fatty degeneration of the vessel walls with the escape of fat globules into the Virchow-Robin space. The earliest deposits appear between the adventitia and the media as small granules in one part of the vessel walls later spreading to the media and finally coalescing to form solid plaques entirely surrounding the vessel. In other vessels the Virchow-Robin space appears filled with a deposit. There is rarely any reduction in the lumen of the vessel and no thrombosis. McAlpine found many globules staining with scharlach-R but showing no double-refraction and could trace all transitions from these to those having the appearance of calcium and staining deeply with hæmatoxylin. He assumed that the vascular and extra-vascular deposits were composed of calcium but quotes Wiemann who showed that they give a reaction for iron. As the vessels remain patent in spite of gross infiltration of their walls and he was unable to find any serious nutritional change in the cells of the pallidum he concludes that the infiltration does not play an important part in the production of symptoms of nervous diseases. Urechia and Elekes found "calcification" in the vessels of the lenticular nucleus in cerebral syphilis and believed the deposits to consist of fatty acid, iron and calcium. They emphasised the fact that no symptoms referable to the lenticular nucleus were present during life.

Lhermitte Kraus and McAlpine found deposits giving a positive iron reaction in the vessels of the anterior half of the globus pallidus in encephalitis lethargica: they were unable to demonstrate calcium. Spielmeyer describes the deposition of calcium in the adventitia of cerebral vessels as being a common change met with in chronic diseases and in old atrophic areas and also in acute processes such as carbon monoxide poisoning and in encephalitis lethargica. Dürck and Weingarten found the change in the corpus striatum in cases of malignant malaria and Herzog in three cases of carbon monoxide poisoning dying between the fourth and eleventh day.

In the majority of accounts of the post-encephalitic state some reference is made to "calcification" of the vessels: in some typical cases no sign of the change has been found. Few observers definitely ascribe symptoms to this process: many acknowledge that there seems to be no definite connection between the disease and the change in the vessel walls. Up to 1926 most accounts of the process were based on the examination of diseased brains in which some pathological change in the lenticular nuclei might have been expected. None of these observations seem to have been controlled by the examination of a sufficient number of normal controls.

In 1926 Hurst, working at the National Hospital, Queen Square, published the results of the examination of a hundred brains from patients with many types of nervous disease and came to the conclusion that the so-called "calcification" of the arteries in the anterior half of the globus pallidus which had previously been so frequently described in encephalitis lethargica is "an extremely common occurrence and is found in many diseases affecting various parts of the central nervous system." In his series it was present in 50 per cent. of cases and at all age-periods from twenty to eighty-one. He found that "the process consists in the infiltration of one or more coats of the vessel with iron salts, the application of the usual tests failing to show the presence of calcium." He found the degeneration bodies described by McAlpine in the globus pallidus in 68 per cent. of these brains and regarded the presence of these and the vascular deposits as "an expression of the proclivity of the globus pallidus to undergo degeneration in the second half of life, to be correlated with the high iron content of these parts of the brain." His results are in complete agreement with those of McAlpine as regards the strict anatomical localisation of the

vascular siderosis to the anterior part of the globus pallidus. He found the globus pallidus to be rich in lipoid globules some of which were abnormally insoluble and also pigmented granules of a substance which he regards as a lipochrome. He could not find any clear transition between the lipoid or lipochrome and the iron-containing degeneration bodies. His work leaves one in no doubt that a large part at least of this infiltrating material is iron and not calcium and from his published figures it appears reasonably certain that the change is one likely to be found in a large percentage of otherwise normal brains of people dying in the second half of life. The first part of this paper deals with this aspect of the question.

II. Siderosis of the globus pallidus in the absence of chronic nervous disease.

65 brains have been examined. Of these 22 were from cases of accidental death or from patients dying as a result of disease which had not, in all probability, implicated the central nervous system.

TABLE I.

Age.	Sex.	Cause of death.	Vascular siderosis.	Extra-vascular deposit.	Cerebral arterio sclerosis.	Corpora amylacea.
3/12	F	Asphyxia: overlying	−	−	−	−
11	F	Street accident	−	−	−	−
16	F	Acute otitis: meningitis	−	+	−	−
19	F	Acute rheumatic carditis	−	−	−	−
27	M	Mediastinal tumour	−	+	−	−
35	F	Death under anæsthetic	+ + +	+ +	−	−
35	F	Chronic otitis: cerebral abscess	−	−	−	−
40	M	Lobar pneumonia	−	−	−	−
41	M	Perforated gastric ulcer	−	−	−	−
45	F	Post-traumatic pulmonary thrombosis	+ + +	+ +	−	−
46	M	Acute coal-gas poisoning	−	−	−	−
46	F	Carcinoma stomach	+ + +	+ +	−	−
48	M	Belladonna poisoning	+ + +	+ +	−	−
50	M	Carcinoma stomach	−	+ +	−	−
52	M	Acute bronchitis: cardiac infarction	−	−	+	−
52	F	Carcinoma œsophagus	+	+ +	−	+
54	F	Cerebral hæmorrhage: arterio-sclerosis	−	−	+ +	+
54	M	Lobar pneumonia	+ + +	+ +	−	−
55	M	Street accident	−	−	−	−
56	F	Post-operative pneumonia	−	+	−	+
58	M	Hæmorrhage: gastric ulcer	+ + +	+ +	−	−
60	M	Carcinoma stomach	+ + +	+ +	−	−
62	F	Suppurative cholangitis	−	−	+ +	+
63	M	Leukæmia	−	−	+	+
63	M	Syphilitic aortitis	+ + +	+	+ + +	−
65	F	Traumatic sub-dural hæmorrhage	+	+	+ +	−
65	M	Acute coal-gas poisoning	−	−	+ +	+
67	M	Street accident	−	+	+ + +	+ +
68	F	Cerebral embolism: arterio-sclerosis	+ + +	+ +	+ + +	+
69	M	Carcinoma prostate	−	−	+	+
73	M	Cerebral thrombosis: arterio-sclerosis	+ + +	+	+ + +	−
78	M	Cerebral hæmorrhage	+	+	+ + +	+

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13 were from cases of cerebral arterio-sclerosis, 30 were from cases of chronic mental disease, which were examined in the laboratory of the Bristol Mental Hospital by Dr Barton White, to whom I am indebted for permission to incorporate the results in this paper. In all cases of doubt where there was any suggestion that chronic structural changes might have been present a systematic histological examination of the brain was undertaken.

TABLE II.

No.	Age.	Sex.	Mental state.	Cause of death.	Deposit in pallidus.	
					Vascular.	Extra-vascular.
1	49	F	Dementia	Huntington's chorea	−	−
2	18	M	G. P. I.	G. P. I.	+	+
3	35	F	D. præcox	Tuberculosis	++	+
4	89	M	Dementia	Senility	−	−
5	57	M	Melancholia	Broncho-pneumonia	−	−
6	55	M	Confusion	Prostate and cystitis	++	+
7	77	F	Dementia	Cardio-vascular	++	+
8	27	M	D. præcox	Tuberculosis	−	−
9	77	M	Dementia	Cardio-vascular	++	+
10	69	F	Delusional	Endocarditis	+++	+
11	60	M	Dementia	Lobar pneumonia	−	−
12	64	M	Imbecility	Nephritis	−	−
13	69	M	Dementia	Lobar pneumonia	−	+
14	70	M	Dementia	Influenza	+	−
15	76	F	Dementia	Endocarditis	++	−
16	58	M	Paranoia	Myocarditis	+	−
17	53	M	D. præcox	Influenza	−	−
18	45	M	Epilepsy	Broncho-pneumonia	−	−
19	65	M	Dementia	Pneumonia	++	+
20	27	M	Confusion	Influenza	+	+
21	46	F	Dementia	Pneumonia	++	−
22	51	M	D. præcox	Influenza	+	+
23	55	F	Melancholia	Cardio-vascular	++	+
24	47	M	G. P. I.	G. P. I.	−	−
25	53	M	G. P. I.	G. P. I.	−	−
26	88	M	Dementia	Senility	++	−
27	53	M	Delusional	Influenza	−	−
28	63	M	Imbecility	Pneumonia	−	+
29	30	M	G. P. I.	G. P. I.	+	−
30	63	M	Epilepsy	Influenza	+	+

35 of the 65 brains, or 54 per cent., showed typical vascular siderosis of varying intensity usually affecting the media and adventitia of arteries, the adventitia of arterioles and capillaries and less frequently the veins. The localisation of the change to the pallidum was precise and anatomical and in the majority the anterior half only was affected. The change was bilateral and approximately equal on the two sides: the histological and histo-chemical characters of the deposits were as described by Dürck, McAlpine and Hurst.

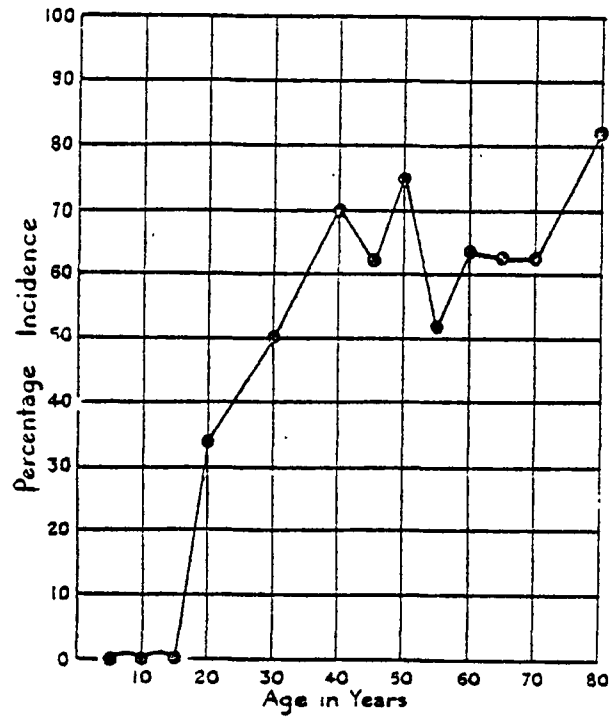
The main features of the cases are shown in tables I and II, from which it will be seen that the percentage incidence of the change is approximately the same in normal brains (51 per cent.), in those from

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cases of chronic nervous disease (50 per cent.) and in those from cases of chronic mental disease (56 per cent.).

The curve shows the age incidence, combining the results found in normal brains with Hurst's published figures. From the age of 30 onwards 67 per cent. of the brains examined showed vascular siderosis. It was found at the age of 18 in a boy who died of juvenile G.P.I. and, by Hurst, at 22 in a patient dying of an intracranial neoplasm. The youngest positive case in which no sign of disease was



Curve showing percentage incidence of siderosis of pallidum from examination of 195 brains.

found in the central nervous system was 35. In normal brains its intensity bears no relation to age.

Severe arterio-sclerosis may be present when siderosis is absent. In several cases of cerebral hæmorrhage siderosis was mild in degree. It was completely absent in a case of senile dementia with high grade cerebral atheroma dying at the age of 89.

Figs. 1 and 4 are taken from sections of the globus pallidus of a woman of 35, who died from an overdose of chloroform given for extraction of teeth. She was a presumably healthy woman. Subsequent inquiries elicited nothing in her history suggesting disease and post-mortem no sign of disease was found in her organs. The

Deposit in pallidus.	
Vascular.	Extra-vascular.
−	−
−	+
− +	+
−	−
−	−
− +	+
− +	+
−	−
− +	+
+ + +	+
−	−
−	−
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brain was examined systematically and no abnormality was discovered. Figs. 2 and 3 are from sections of the globus pallidus of a man of 48 dying 40 minutes after accidentally swallowing 4 ounces of a liniment containing belladonna. Beyond atheroma of a mild grade of the first part of the arch of the aorta there was no evidence of disease in the organs. A systematic histological examination of the brain showed no abnormality.

Discussion.

It appears reasonably certain that siderosis, limited with anatomical precision, to the globus pallidus occurs normally in at least 60 per cent. of individuals over the age of 30, and that the mere fact of its presence cannot be taken as indicating the presence of any disease process, although evidence will be brought forward to show that it does under certain conditions strongly predispose to acute bilateral destruction of these nuclei. The siderosis is in all probability endogenous, the large store of iron held by the nerve cells and neuroglia of the nuclei becoming unmasked and accumulating in a stainable and visible form in the perivascular spaces.

In the intact but siderotic globus pallidus the iron is always extracellular. I have not seen it in the bodies of phagocytes or encrusting nerve cells or their axons, except when, as in cases to be described, there has been acute necrosis. In the intact nucleus it may be that the liberation of iron from its intracellular combination is so slow that phagocytes are not attracted. The presence of iron in nerve cells can be explained only by assuming that it is essential for normal function. Nicholson has shown that there is organically bound iron in the nucleolus, in the oxychromatin of the nucleus and in the chromophilic substance of the cytoplasm of the nerve cells of the hypoglossal nucleus in rats. Degeneration is accompanied by a decrease, regeneration by a reappearance, and necrosis by total disappearance of this iron. The unmasking of iron and its extracellular and perivascular deposition as observed in the pallidum of otherwise normal human brains, quite apart from disease and senility, therefore suggests a retrogressive change of considerable chronicity possibly related to atrophy. From several points of view, it is difficult to escape from the impression that the process is, in some way, related to the phylogenesis of this part of the brain.

The corpus striatum first appears in the telencephalon of the lowest vertebrates as a secondary mass, the palæostriatum corresponding only to the globus pallidus of mammals. The afferents of this mass are from the olfactory system, the efferents are motor. The striate body is added to by the development in reptiles of the archi-striatum, represented in mammals by the amygdaloid nucleus, and the neostriatum represented by the caudate and putamen. Elliot Smith has shown, from its development, that in reptiles it functions as a

substitute for the cortex. In birds cortical growth is chiefly intraventricular, and the striate body appears to carry out those functions which have not yet obtained cortical representation. The cerebral hemispheres and a cerebrospinal motor mechanism gradually evolve as the telencephalon becomes dominant in higher forms, and the primitive motor system of the striate body becomes submerged.

Phylogenetically therefore the pallidum is the oldest part of the primitive motor mechanism of the vertebrate fore brain. Its age in mammals is shown by the early development of its cells and the early myelination of its fibres. Woollard, reviewing the evolution of the striate body, remarks that it is difficult to say what functions may still survive in the human striate body, and remarks that "anatomy offers no support for the various motor functions which have been ascribed to it and were it not for the pathological changes found in it in Wilson's disease and paralysis agitans one might be tempted to regard the whole complex as a vestigial system."

The site of the essential lesion in extrapyramidal motor diseases, such as paralysis agitans, is still *sub judice* and lesions of the substantia nigra and the subthalamic nuclei appear to be more often present than clear-cut lesions of the pallidum. It is probable that siderosis of these nuclei has been interpreted as the pathological basis of the tremor and rigidity found in these conditions, for when specific staining is not employed and the siderosis is gross, the appearances are strongly suggestive of a structural change due to disease.

Against accepting the change as being due to disease are its strict bilateral anatomical localisation, the lack of cellular reaction in the nucleus and its frequent presence in the presumably normal brains of individuals who during life showed no neurological symptoms. For these reasons, combined with the suggestive facts regarding the phylogeny of this part of the brain, it appears probable that *pallidal siderosis is the expression of a slow evolutionary atrophy affecting the nuclei in at least* 60 *per cent. of people over the age of* 30.

III. The association of vascular siderosis with bilateral necrosis of the pallidum.

There is no doubt that under a variety of pathological conditions one or more of the components of the lenticular nuclei are prone to undergo bilateral and symmetrical destruction. Such a change is found in the familial group of diseases included under the term hepato-lenticular degeneration (Hall) including progressive lenticular degeneration of S. A. K. Wilson and pseudo-sclerosis (Westphal-Strümpell). A similar lesion has been described by Paterson and Carmichael in a familial form of cerebral degeneration in children. There are clinical facts which hint at the production of a bilateral lenticular lesion in manganese poisoning (Charles). Acute bilateral

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necrosis has been described in hydrocyanic acid poisoning, and is certainly very frequent in carbon monoxide poisoning especially when the victim survives a fairly heavy dose of gas for a few days. The lesion in carbon monoxide poisoning has been described by von Recklinghausen, Simon, Lesser, and Poelchen. Kolisko claims that some degree of symmetrical encephalo-malacia in the lenticular nucleus is an invariable accompaniment of carbon monoxide poisoning. Emory Hill and Semerak examined 32 brains from cases of carbon monoxide poisoning: gross bilateral lenticular softening was found in 14, and microscopic evidence of damage in the remaining 18; the change was invariably most marked in the globus pallidus. Meyer, describing the lesion in man and animals, observes that "the striking frequency and selectiveness of the pallidum lesion in carbon monoxide poisoning has excited attention for a long time," that "a symmetrical affection of the pallidum is an extremely regular finding in dogs poisoned with carbon monoxide," and that "the vulnerability of the pallidum is greater than any other nucleus." He also mentions that it is the anterior part of the nucleus which is most frequently affected.

I have undertaken the systematic examination of five brains from cases of coal-gas poisoning. In three of these, bilateral and symmetrical areas of softening were found occupying the anterior half of the pallidum, *i.e.* precisely that part of the nucleus which is the site of vascular siderosis in 60 per cent. of normal brains over the age of 30. In all three cases gross vascular siderosis was present. The same naked-eye lesions were found in a case of lobar pneumonia, in which exposure to coal-gas could be excluded, and in which a similar degree of siderosis was present. All the coal-gas cases gave microscopic evidence of varying degrees of degeneration of the cerebral cortex, but in those in which naked-eye pallidal lesions were absent there was no pallidal siderosis.

Case reports.

Case 1.—Male, aged 70. Was found unconscious in a closed room with the gas "full on." He lived three and a half days with a brief lucid interval of a few hours on the second day. There were terminal hypostatic pneumonia and hyperpyrexia but no localising neurological signs.

Post-mortem, the cherry-red colouring of the tissues had largely disappeared. There were many petechial haemorrhages into the white matter of the cerebrum and a small pale focus of acute softening sharply limited to the anterior half of each globus pallidus (fig. 7).

Microscopically, the lesion was clearly demarcated from the surrounding tissue by a zone of marginal cellular infiltration. Its blood vessels showed extensive iron deposits and their walls were disintegrating. Thrombosis and capillary haemorrhage were not prominent. The cellular infiltration at the edge had only penetrated a short distance into the necrotic area in which there was universal death of nerve cells with swelling, indistinctness and feeble staining of the neuroglia. The majority of the necrotic nerve cells gave a diffuse but definite iron reaction and in many of them there were indistinct granules giving

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SIDEROSIS OF NORMAL GLOBUS PALLIDUS.

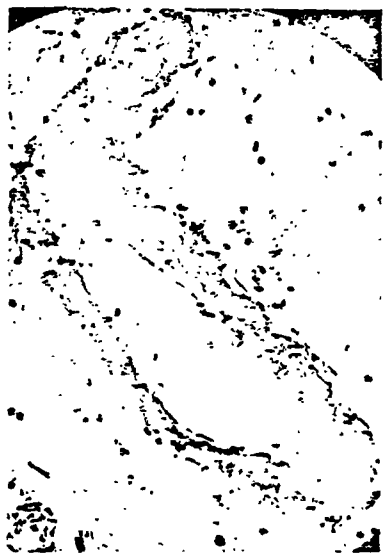


FIG. 1.



FIG. 2.



FIG. 3.

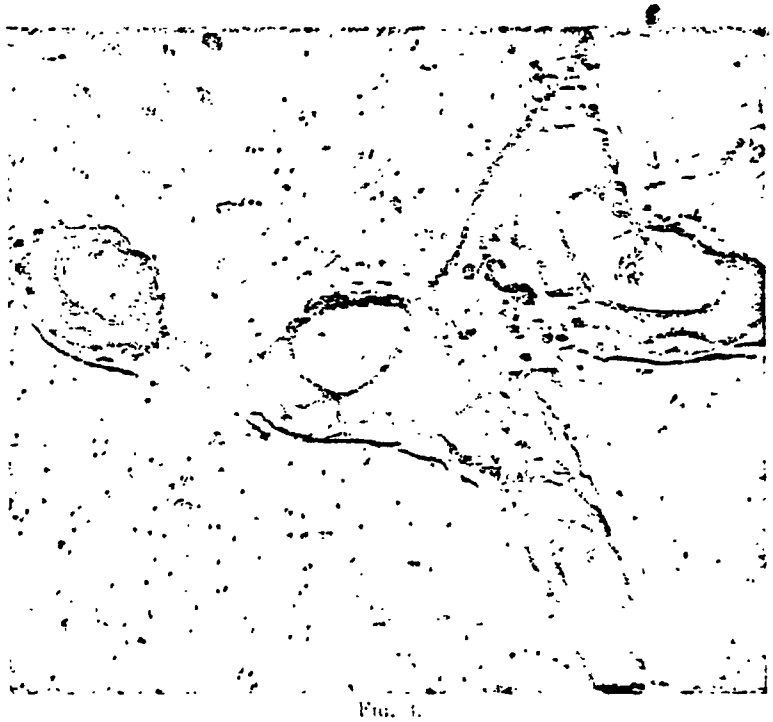


FIG. 4.

FIGS. 1 and 4.—Woman of 35: accidental death. Stained ferrocyanide, neutral red. Vessel walls grossly infiltrated and perivascular spaces fixed.
FIGS. 2 and 3.—Man of 18 dying accidentally. Earlier stage of deposit. In 2 much of media spared.

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SELECTIVE NECROSIS OF PALLIDUM.

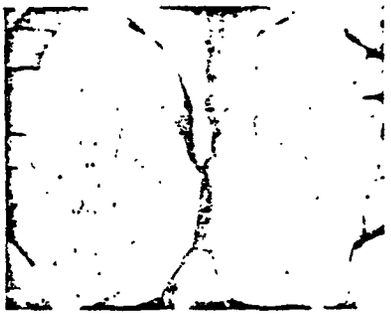


FIG. 5.



FIG. 6.

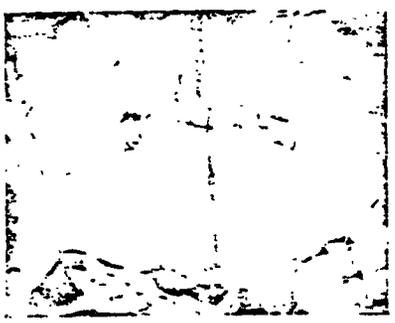


FIG. 7.

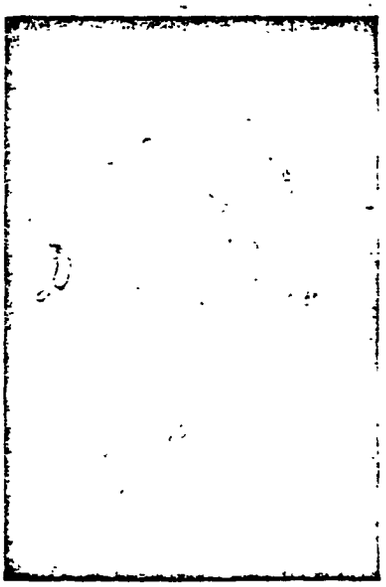


FIG. 8.

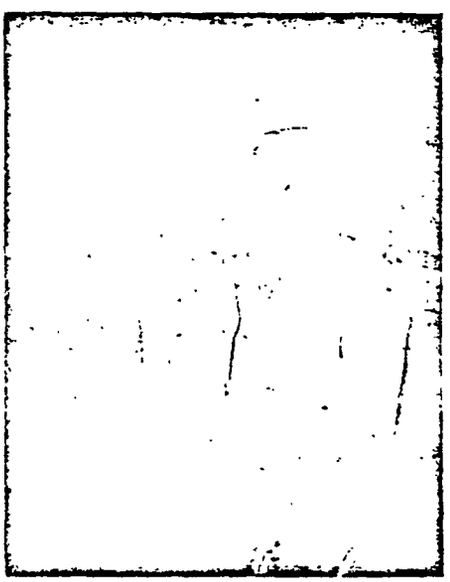


FIG. 9.

FIG. 5.—Coal-gas poisoning: survived 7 days.
FIG. 6.—Lobar pneumonia: 3½ days.
FIG. 7.—Coal-gas poisoning: 3½ days.
FIG. 8.—Low magnification of 5. Clearly demarcated lesion stippled with newly-formed vessels. Walls of large vessels infiltrated with iron, giving the appearance of black rings.
FIG. 9. Low magnification of 6. Outline of intact putamen seen on left. Necrotic focus has shrunken away. Is not vascularised as in 8.

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PALLIDAL NECROSIS IN COAL-GAS POISONING FATAL ON 7TH DAY.

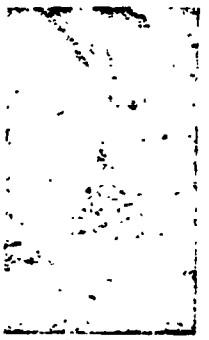


FIG. 10.

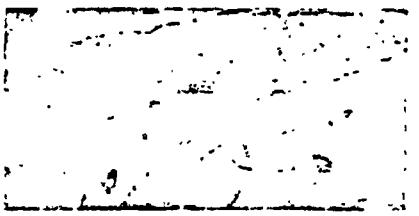


FIG. 11.

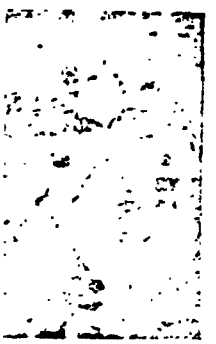


FIG. 12.

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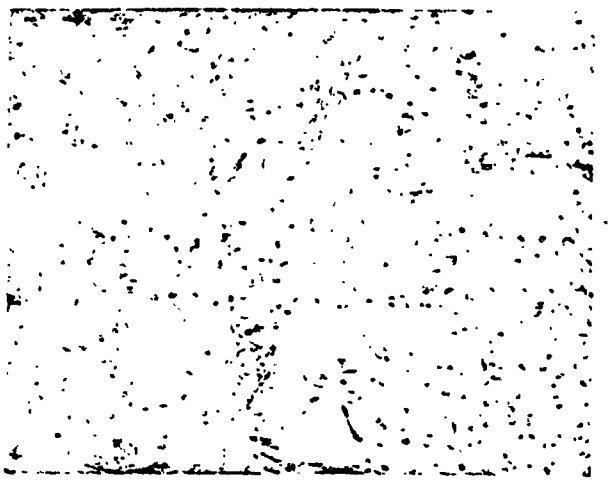


FIG. 13.

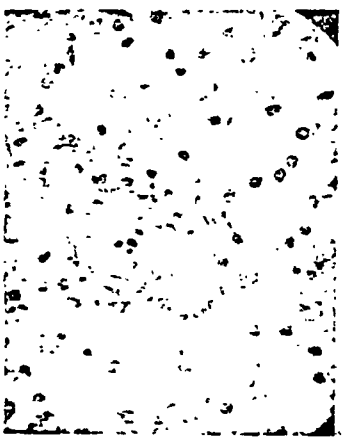


FIG. 14.

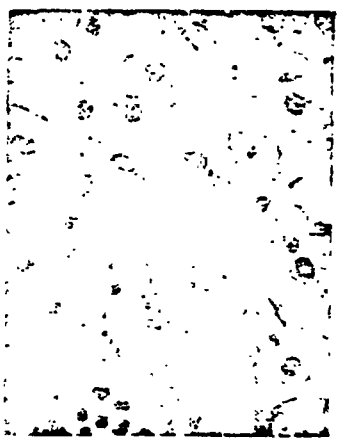


FIG. 15.

FIGS. 10 and 12.—Large necrotic nerve cells encrusted with ferruginous material: axon in fig. 12 also encrusted.

FIG. 11.—Incrustation of nerve fibres with iron.

FIG. 13. Several foci of capillary budding; general infiltration by granule-adipose cells. Nerve fibres encrusted with iron show as fine lines throughout the section.

FIG. 14. Perivascular accumulation of granule-adipose cells.

FIG. 15.—Similar cells concentrated around thrombosed vein.

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PALLIDAL NECROSIS IN COAL-GAS POISONING FATAL ON 7TH DAY.



FIG. 16.



FIG. 17.

FIG. 16. Phagocytosis of iron derived from disintegration of infiltrated walls of a dividing vessel. P1, 2, 3 phagocytes loaded with ferruginous material.
FIG. 17.—Accumulation of myelin phagocytes about walls of another infiltrated blood vessel.

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an iron reaction. Much free ferruginous material was lying free in the nucleus lesion, a considerable part of it obviously derived from the disintegrating vessel walls. In one place near the edge there were a few large cells of the "gitterzellen" type loaded with iron. Apart from this there was no evidence of phagocytosis or repair.

Six blocks were cut from the cortex and patchy degeneration of the cells of the third and fourth layers found. The degree of chromatolysis was definitely greater than could be accounted for by post-mortem autolysis but was not striking except in one section from the left præcentral region. Here there had been moderate capillary hæmorrhage, widespread severe chromatolytic changes in the cells of the third and fourth layers, a mild infiltration by granulo-adipose cells and a fairly brisk endothelial reaction. No free iron was found in the cortex. No other changes were found in the central nervous system: the cells of the substantia nigra appeared normal.

Case 2.—A man of 37 was found unconscious lying against a gas cooking stove in a closed kitchen. It was subsequently proved likely that he fell asleep and accidentally turned on a very loose tap. He died in 4½ days, after an interval of semi-consciousness of 2 hours 12 hours after he was found. The post-mortem examination was delayed and exact histological examination impossible.

Bilateral softening of the pallidum was present affecting its anterior two-thirds and specific staining showed gross siderosis of vessels of all calibres limited to the softened area. No sign of old disease was found in the brain or its vessels. The organs were healthy, except for recent severe hypostatic pneumonia of both bases. The previous history showed that the patient was a strong active man in full muscular work.

Case 3.—A woman of 46, admitted to hospital comatose and cherry coloured; having inhaled a moderately large volume of gas. She remained in coma for 3 days, partially recovered consciousness for 2 days, lapsed into terminal coma and died on the seventh day. There were no localising neurological signs.

Post-mortem, both lungs showed severe hypostatic pneumonia. There were two symmetrically placed foci of softening in the globus pallidus clearly marked off from the surrounding tissue by an incomplete line of separation which became much more accentuated after fixation (fig. 5). The lesions were softer than in the previous case but just as accurately limited to the anterior half of the pallidum. There were no other naked-eye changes in the brain.

Microscopically, in contrast to the previous case, these lesions were infiltrated throughout by granulo-adipose cells and endothelial phagocytes, disposed about the walls of large numbers of recently-formed budding capillary vessels which stippled the whole lesion (fig. 8). Every pre-existing artery and arteriole in the lesion showed gross siderosis: their walls were disintegrating and much free iron-containing material lay scattered in their immediate vicinity (figs. 16, 17). A moderate number of phagocytic cells of the "gitterzellen" type had accumulated about these vessels, their cytoplasm loaded with iron. Granulo-adipose cells were most numerous at the edge of the lesion and were mostly concentrated about the walls of invading capillaries but several more peripherally placed arteries with disintegrating walls showed a heavy perivascular concentration of these cells. The shadows of many necrotic nerve cells were seen, the majority closely packed with fine granules giving an intense iron reaction (figs. 10, 12): in several the axon was seen similarly encrusted (fig. 12). Throughout the whole lesion long straight tracts of closely packed ferruginous granules apparently marked out the course of disintegrating axons (fig. 11). Degeneration in the deeper layers of the cortex was more extensive than in the previous case and

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most marked in the frontal regions. It did not seem to originate so definitely about capillary hæmorrhages but appeared to be a more clearly-cut selective chromatolysis. Where capillary hæmorrhage had occurred there were localised foci of neuroglial and endothelial reaction with capillary budding.

Case 4.—Male, aged 65, living on a farm not supplied by gas, developed a "chill" with backache. After temporary improvement for 36 hours, he was seized with sudden abdominal pains for which he was given morphia. Four hours after this he was admitted to hospital, where he was found to have small pupils and a respiration rate of 35, temperature 102 and pulse 120. He slept soundly for 8 hours and on waking was found to have some weakness in the right arm. After a lucid interval of about an hour, he became unconscious and died 14 hours after admission, or 3½ days after the onset of his illness.

Post-mortem: there was typical lobar consolidation of the central part of the lower lobe of each lung and of the posterior half of the right upper lobe. The spleen pulp was diffluent. A moderate degree of generalised arterio-sclerosis but no renal nor myocardial fibrosis was found.

Two roughly elliptical areas of softening occupied the anterior halves of each globus pallidus (fig. 6). They were isolated from the surrounding tissue by a deep cleft and sharply confined to the pallidum. Their blood vessels showed universal and gross siderosis of their walls. Those of the putamina were free. In each nucleus the walls of the larger vessels were disintegrating and masses of amorphous iron-containing material were seen lying free in the disintegrating nucleus. A feeble endothelial reaction was present in the neighbourhood of these vessels and a few iron-containing phagocytes were present in close proximity to the walls of the more heavily infiltrated vessels. Here and there hæmorrhage had accompanied this disintegration. In some vessels in addition to siderosis there was widespread hyaline change in the media. The nerve cells of both nuclei showed advanced chromatolytic changes. Those of the adjoining putamen, thalamus and caudate nucleus were little altered. The cortical nerve cells were relatively unaffected. Necrosis was confined to the areas of pallidal siderosis.

Discussion.

Several explanations have been given for the striking frequency, selectiveness and bilateral symmetry of the pallidal lesion of CO poisoning. Most observers agree that the basis of the change is circulatory. Kolisko ascribes it to the acutely backward course taken by the long narrow end-arteries of the nuclei. Shellshear lays stress on this anatomical peculiarity, expresses surprise that it has been so rarely described and observes that the course the blood takes is definitely against the current. The acute angle of origin of the lateral striate arteries is apparently due to the great development of the human prosencephalon, but as they supply the other components of the striate body and the adjacent parts of the internal capsule, their angle of origin does not explain the special vulnerability of the pallidum, nor as Meyer points out, why a symmetrical pallidal lesion is so frequent in dogs poisoned with carbon monoxide. Further in cerebral arterio-sclerosis the vessels of the putamen and caudate nuclei are as frequently as and probably more often affected than those of the pallidum.

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Siemerling and Oloff, in discussing the pathology of bilateral pallidal lesions, suggested that the excess of iron in the globus pallidus is an indication of some special biochemical property, and under some conditions of disease might lead to its selective destruction. Meyer considers it "more than accidental" that the anterior part of the globus pallidus, so vulnerable to acute softening in CO poisoning, "should also be the site of predilection for pseudo-calcium deposits on the vessels," and thinks "F. H. Lewy's hypothesis seems plausible—that this peculiar change, which occurs even within the limits of the normal, gives certain indications of a susceptibility on the part of the vessels affected."

In the cases described in this paper necrosis was only found with pre-existing siderosis, and in two cases of carbon monoxide poisoning with no gross pallidal lesion siderosis was absent. From these results it seems reasonable to suppose that vascular siderosis bears a definite causal relationship to bilateral necrosis, and is the only condition which satisfactorily explains its frequency and selective nature in CO poisoning.

Conclusions.

1. The walls of the blood vessels of the healthy globus pallidus are often infiltrated with iron salts which are derived from the nucleus itself.
2. It is likely that the process is one of evolutionary atrophy.
3. It predisposes to the acute bilateral destruction of these nuclei which is frequent in coal-gas poisoning.

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EXPERIMENTS ON THE NATURE OF THE CARCINOGENIC AGENTS IN MINERAL OILS

C. C. Twort and J. D. Fulton.

Manchester.

In our extensive studies on carcinogenic agents one of our main objectives has been to isolate, or at least to gain an insight into the nature of, the chemical substance or group of chemical substances responsible for the malignant action on the skin. The isolation of a single chemical individual with toxic properties we did not hope for in the early stages of our experiments, and therefore endeavoured, as a first step, to concentrate by various means the active agent in the oils. To this end the methods given below have been employed. By testing the extract and the residue of a carcinogenic oil we were able to compare the toxicity of the extract with that of the residue in a parallel series of experiments. In this paper we shall deal only with oils which in our standard experiments were applied twice weekly to the skin on the backs of 100 mice. Details of distillation and the methods of making extracts are given elsewhere (C. C. Twort and H. R. Ing, *Zeit. f. Krebsforsch*, 1928, vol. xxvii. p. 308).

Our experiments with the oils consisted in :—

1. Fractional distillation.
2. Extraction with methyl sulphate.
3. Extraction with picric acid.
4. Extraction with methyl sulphate and picric acid.
5. Extraction with methyl sulphite.
6. Extraction with ethyl alcohol.
7. Treatment with sulphuric acid.
8. Oxidation.
9. Reduction.
10. Silica gel adsorption.
11. Irradiation, etc.

The oils used were :—

1. A blend of a purified american petroleum oil and 10 per cent. sperm.
3. A pure american petroleum oil.
7. A pure refined heavy pennsylvanian petroleum oil.
8. A pure refined shale oil.
9. A pure refined californian petroleum oil.
10. A pure refined texas petroleum oil.
11. A pure refined light pennsylvanian petroleum oil.
17. A medium motor oil.
23. A pure refined anglo-persian petroleum oil.

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Distillation.

In a previous paper we have given details of distillation experiments on two oils which were actually used in the cotton-spinning industry, the distillations being for the most part carried out under reduced pressure, a nitrogen leak being used to prevent oxidation. It will suffice here to give a table of our final results (table I).

TABLE I.

Distillation of two american petroleum oils.

Oil number.	Week of first tumour.		Total number of tumours.		Duration of experiment in weeks.
	Warts.	Epitheliomas.	Warts.	Epitheliomas.	
1. Whole oil . . .	51st	53rd	0	1	73
355–385° at 760 mm. .	...	...	0	0	69
385–420° ,, .	26th	38th	6	4	52
3. Whole oil . . .	35th	59th	1	2	70
400–420° at 760 mm. .	25th	31st	8	2	55
240–300° at 12 mm. .	32nd	...	1	0	52
300–380° ,, .	23rd	18th	10	11	50

These results were encouraging and led us to believe that by distillation alone we had at hand a means whereby we could quickly estimate the relative carcinogenicity of different petroleum oils. The concentration of the toxic components in the higher boiling fractions was most striking. Our subsequent distillation experiments with american petroleum oils left us less sanguine about this method of treatment. Oil 7, a moderately heavy grade of pennsylvanian oil, was distilled under 2-3 mm., five fractions being obtained with a boiling range of from 180-315° C. Each fraction was carefully refractionated once. When applied to the backs of mice the whole oil gave us only two, non-malignant tumours in an experiment lasting over 60 weeks, the first tumour appearing in the 26th week. Contrary to our experience with oils 1 and 3 there was no obvious concentration of the active agent in any of the five fractions of oil 7, a single tumour only being observed among the 500 animals utilised for the experiment. Oil 11, a lighter grade of pennsylvanian oil, was divided into two fractions by distilling under 12 mm. The fraction boiling below 300° and the residue left in the flask both failed to induce tumours in mice, while with the whole oil a solitary tumour was observed in the 49th week of the experiment. Similar distillates were made from oil 9, a californian oil, this oil proved to be more carcinogenic than any of the other refined american oils we had so far tested but distillation did not effect any concentration of the active components of the oil. No very definite results were obtained with distillates of a refined anglo-persian oil.

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Thus our results with distillates of refined american petroleum oils are on the whole too irregular to be of any value, and indicate that distillation cannot be used with certainty for the concentration of the carcinogenic substances in these oils.

Distillation of shale oil. In view of the results we had obtained with the american petroleum oils 1 and 3 we anticipated that we should be able to concentrate the carcinogenic substances in shale oil by simple division of the oil into two fractions. Accordingly oil 8, a refined shale oil, was distilled at 12 mm. pressure, the fraction boiling between 300-360° C. and the residue left in the flask only being used for animal experiments. Table II shows that we have effected no concentration whatever, except possibly in the low boiling fraction which we did not test. The higher boiling fraction will be seen to have a less degree of toxicity than the whole oil, while the asphaltic residue contains still less of the active substances. A series of experiments was also performed with the shale oil under exactly similar conditions to those just mentioned for oil 7. The results given in table II indicate that the majority of the carcinogenic substances present in this oil boil at a fairly low temperature, contrary to what was found with oil 3, and confirm the results obtained with the first experiments where the oil was distilled under 12 mm.

TABLE II.

Fractional distillation of shale oil 8.

Oil fraction.	Week of first tumour.		Total number of tumours.		Percentage of living animals with tumours at 35th week.
	Wart.	Epithelioma.	Warts.	Epitheliomas.	
Whole oil . . .	17th	26th	13	18	42
300-360° at 12 mm. .	22nd	26th	8	10	11
300-360° residue . .	34th	...	7	0	0
140-220° at 3 mm. . .	14th	25th	10	2	44
220-240° ,, .	16th	32nd	20	7	23
240-260° ,, .	26th	31st	7	9	8
260-280° ,, .	16th	...	4	0	12
280-315° ,, .	46th	48th	...	1	0

The duration of each experiment was 50 weeks.

In order to test whether during the process of distillation any decomposition of the carcinogenic components takes place or whether we were manufacturing the substances from previously non-carcinogenic compounds, we have subjected two oils to distillation and utilised the whole combined distillate for control experiments on animals. Our results do not seem to indicate that the distillation processes effect the oils to an appreciable degree. On the whole it seems that possibly distillation renders an oil a little more carcinogenic than it was previously.

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Extraction with methyl sulphate and sulphite and picric acid.

Extraction of oils with methyl sulphate proved of limited use as a method of concentration of the carcinogenic principle. Both the extract and the residual oil, in the single experiment which we have performed, turned out to be less carcinogenic than the original oil from which they were prepared which rather indicates that some at least of the active substances have been rendered inert.

TABLE III.

The concentration of oils by means of methyl sulphite, etc.

Oil number, etc.	Week of first tumour.		Total number of tumours.		Duration of experiment in weeks.	Percentage of animals bearing tumours at 30th week.
	Wart.	Epithelioma.	Warts.	Epitheliomas.		
3. Whole oil .	35th	59th	1	2	70	0
Extract CHR 1	14th	17th	11	1	27	50 (27th week)
7. Whole oil .	28th	...	2	0	60	2
Extract C .	16th	40th	9	0	45	33
Residue C .	...	...	0	0	60	0
9. Whole oil .	17th	26th	13	18	50	50
Extract A .	23rd	35th	13	3	45	0
Residue A .	29th	35th	20	6	50	11
Extract B .	25th	36th	14	6	45	46
Residue B .	...	...	0	0	60	0
Extract C .	11th	30th	7	1	40	50
Residue C .	33rd	46th	5	5	50	0
11. Whole oil .	14th	...	1	0	60	0
Extract B .	...	30th	0	1	38	5
Residue B .	...	...	0	0	60	0
Extract C .	37th	...	3	0	45	0
Residue C .	...	...	0	0	60	0
Extract CR .	10th	31st	8	2	45	40
17. Whole oil .	40th	49th	...	1	60	0
Extract C .	15th	38th	5	4	45	18
Residue C .	..	...	0	0	60	0
23. Whole oil .	...	...	0	0	25	0 (23th week)
Extract CHR .	15th	...	4	0	24	20 (24th week)

Extract A Methyl sulphate extract.
" B Methyl sulphate-picric acid extract.
" C Methyl sulphite extract.
" CR Methyl sulphite removed under reduced pressure.
" CHR Methyl sulphite extraction with heat.

The addition of picric acid to the methyl sulphate enabled us to prepare extracts which were considerably more potent than the original oil, the residual oil being at the same time deprived of most of its carcinogenic activity. However, the majority of our experiments were performed with methyl sulphite, the extract obtained being greater in volume than that obtained by the solvents previously mentioned.

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Here again the extracts were much more, and the residues much less, carcinogenic than the original oils, but we have not yet decided whether this method or the methyl sulphate-picric acid method is the more satisfactory for the routine diagnosis of oil samples which are to be used in industry. One or two of our experiments rather indicate that the latter method is the better. This is seen by the high degree of activity of some of the extracts and the inertness of some of the residual oils. Picric acid in absolute alcoholic solution is now being tested as selective extracting agent, and has given positive results.

The results of these investigations will be best appreciated by referring to table III. Note the difference in the figures relating to residue 8 B and residues 8 A and C. The utilisation of heat and the removal of the methyl sulphite under reduced pressure appear to increase the yield of active substances. This is shown by the number of tumours and their early appearance with extract 11 CR as against the comparative inactivity of extract 11 C, and as we shall see later by the fact that extract 8 C was apparently less potent than extract 8 CHR 2 (table V). In our experiments we have utilised strongly carcinogenic shale oils and the relatively weak american petroleum oils. The ability of a chemical reagent to effect separation of the active substances in oils can be demonstrated whether the oil be weak or strong. In the former case our indication is best obtained from the activity of the extract, and in the latter case from the inactivity of the residue.

Fractional distillation of a methyl sulphite extract of shale oil showed the wide boiling range of the active compounds in the extract (table IV). Thus the distillates of the extract did not act in a manner parallel to that of the distillates of the pure oil.

TABLE IV.

Fractional distillation of a methyl sulphite extract of shale oil at 3 mm.

Oil fraction.	Week of first tumour.		Total number of tumours.		Duration of experiment in weeks.
	Wart.	Epithelioma.	Warts.	Epitheliomas	
Extract C . . .	11th	30th	7	1	40
Residue C . . .	33rd	46th	5	5	50
Extract 140-220° . .	13th	32nd	15	2	40
,, 220-240° . .	12th	30th	11	2	40
,, 240-260° . .	16th	33rd	14	5	40
,, 260-280° . .	12th	20th	19	12	40
,, 280-315° . .	13th	25th	10	4	40

Before we could utilise extracts of oils for the rapid diagnosis of any particular sample, we had to be certain that we were not manufacturing or destroying carcinogenic substances during our chemical processes. The most obvious control was to take an oil which was known to be absolutely devoid of cancer-producing activity, and to test

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the extract obtained. The difficulty was to find a certainly negative oil which would give any yield of extract at all. Ordinary medicinal liquid paraffin has proved to be consistently a negative oil but this oil does not yield a trace of extract when treated with methyl sulphite. In the absence of any lubricating mineral oil free from suspicion as regards carcinogenicity and capable of yielding an extract, there were two possible methods which suggested themselves to us. Firstly, as a control oil we decided to utilise a mineral oil which had been previously treated with sulphuric acid, which treatment, as we have shown (*op. cit.*), purifies a carcinogenic oil of its toxic components in a very thorough manner. The refined american petroleum oil 3 was treated with sulphuric acid in the usual way, and from this purified oil we prepared a methyl sulphite extract. If no residue of carcinogenic substance remains after the acid treatment, and at the same time the methyl sulphite extraction does not introduce fresh carcinogenic compounds, then the resulting extract applied to mice should lead to the development of no tumours. Our second method of control consisted in making an extract of an oil and then re-mixing with the residual oil left over after the extraction had been performed. Such a mixture should act similarly to the original untreated oil if during the methyl sulphite extraction no fresh carcinogenic compounds have been manufactured or if none of the active substances present in the original oil have been destroyed. We selected for this experiment the light pennsylvanian oil 11.

We utilised in this group of control experiments 900 animals. We performed three different series of experiments, 300 animals being used for each series. In each case the extract, the residual oil and a mixture of the two substances in the exact proportion in which they were present in the original oil were used for the applications. Our results indicate that there is no manufacture of fresh carcinogenic substances during the treatment with methyl sulphite. On the other hand, there is quite a possibility that by our extractions we are losing a certain amount of the original active substances present. Additional control experiments with shale oil are being performed in order to test this point.

The methyl sulphite extract of the american petroleum oil 3 which had been previously purified by means of sulphuric acid failed to excite the development of a single tumour, the duration of the experiment being 40 weeks. This is the only first extract of an oil which in our experience has proved to be totally devoid of carcinogenic action. The experiment shows how completely the sulphuric acid treatment had removed the toxic principle of the original oil besides proving that the chemical processes of the making of the extract do not lead to the manufacture of carcinogenic substances. In our two series of experiments with oil 11 both the pure extracts were carcinogenic while both the residual oils and both the mixtures of extract and residual oil were

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completely negative. The controls again indicate an absence of manufacture of carcinogenic substances during our chemical processes. It will be noted that we have spoken above of the first extract of an oil as being the only negative extract we have met with, the reason being that we have found by taking a series of extracts from the residual oil of the first extraction we may finally obtain an extract which has no carcinogenic properties (see table V). Thus we found that our fifth extract of shale oil was completely negative which is an additional proof of the lack of change in the composition of the oil due to the methyl sulphite extraction.

TABLE V.

Fractional methyl sulphite extraction of shale oil.

Extract number, etc.	Week of first tumour.		Total number of tumours.		Duration of experiment in weeks.	Percentage of living animals bearing tumours at 35th week.
	Warts.	Epitheliomas.	Warts.	Epitheliomas.		
Whole oil 5 . .	17th	26th	13	18	50	73
Extract C 1 . .	11th	30th	7	1	40	27
,, CHR 2 .	13th	18th	11	5	31	45
,, ,, 3 .	14th	26th	3	3	40	33
,, ,, 4 .	22nd	..	6	0	40	3
,, ,, 5 .	...	...	0	0	44	0
Residue C 1 . .	33rd	46th	5	5	50	0
,, CHR 5 .	...	...	0	0	50	0

It may be thought that we have utilised an unnecessarily large number of animals as controls. For our part however we were convinced that if we were going to use any method whatever for the quick diagnosis of an oil we were bound to be as certain as possible that we were neither destroying nor manufacturing the active cancer-producing compounds. If we were not reasonably certain that this was the case then no reliance could be placed on the results obtained from each individual test.

Extraction with ethyl alcohol.

Alcohol extracts have been made from six american petroleum oils and one shale oil; details of the extraction having been given elsewhere (*op. cit.*). On the whole, with the petroleum oils we have effected a concentration of the carcinogenic substances in the extract, although our results (table VI) are somewhat irregular. The animals painted with the shale oil responded in a peculiar manner, for while the extract induced but few tumours as compared with the original oil, the residue failed to excite the development of more than a single tumour. We have speculated a good deal upon the results of the shale oil experiments, and the only reasonable conclusion justified at present appears to be that the bulk of the carcinogenic substances have been rejected

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in the second alcohol residue. We are proceeding to test this residue on animals, but shall not be surprised to find that it has no very great carcinogenic activity. We imagine that something more subtle has happened, and are testing the admixture of extract and residue and at the same time we are studying the question along other lines.

TABLE VI.

The extraction of oils with alcohol.

Oil number, etc.	Week of first tumour.		Total number of tumours.		Duration of experiment in weeks.
	Wart.	Epithelioma.	Warts.	Epitheliomas.	
3. Whole oil . . .	35th	53th	1	2	70
Extract . . .	16th	...	3	0	38
7. Whole oil . . .	26th	...	2	0	60
Extract . . .	11th	...	7	0	45
11. Whole oil . . .	49th	...	1	0	60
Extract . . .	...	...	0	0	30
9. Whole oil . . .	19th	33th	7	1	60
Extract . . .	30th	..	2	0	45
Residue . . .	...	...	0	0	40
8. Whole oil . . .	17th	28th	13	18	50
Extract . . .	15th	27th	8	1	43
Residue . . .	43rd	...	1	0	50
10. Whole oil . . .	...	...	0	0	70
Extract . . .	12th	...	3	0	27
23. Whole oil . . .	...	...	0	0	25
Extract . . .	17th	...	2	0	20

We have obtained our highest concentration of the carcinogenic components of shale oil by making an alcoholic extract of a methyl sulphite extract of oil 8. In mice painted with this double extract the first tumour appeared on the sixth week, and 17 weeks after the commencement of the experiment 66 per cent. of the animals alive were bearing tumours.

Purification by means of sulphuric acid.

The oil was shaken up with small successive quantities of concentrated sulphuric acid till the extracted layer was of the same colour as the original. Petroleum ether assists the separation of the oil. Excess acid was removed by successive treatment with alcohol water and ether and the solution dried over potassium carbonate. Our final results with four oils before and after treatment with the acid are given in table VII wherein one can see how thoroughly the oils had been detoxicated (see C. C. Twort and H. R. Ing, *Lancet*, 1928, i. 752). It remained to be seen however whether the oils were entirely freed of their carcinogenic principle. In order to investigate this point we have performed two concentration experiments, one by means of

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distillation and the other by means of methyl sulphite extraction. The latter experiments have already been mentioned when we were dealing with the controls as to the manufacture of carcinogenic substances during the extraction. For the distillation experiments we utilised the same oil, *i.e.* sulphuric acid treated oil 3. The oil was distilled at 12 mm. so

TABLE VII.

The purification of oils by means of sulphuric acid.

Oil number, etc.	Week of first tumour.		Total number of tumours.		Duration of experiment in weeks.
	Wart.	Epithelioma.	Warts.	Epitheliomas.	
3. Heavy fraction . .	23rd	19th	10	11	50
Sulphuric treated .	...	...	0	0	58
8. Shale *a* . . .	17th	26th	13	18	50
Sulphuric treated .	...	...	0	0	70
Shale *b* . . .	16th	35th	12	10	50
Sulphuric treated .	...	..	0	0	70
Heavy fraction . .	22nd	26th	8	10	50
Sulphuric treated .	...	...	0	0	70

Sulphuric acid treated oils gave 0 tumours in 400 animals.
Untreated oils gave 92 tumours in 400 animals.

as to give us two fractions, one consisting of the oils boiling up to 300° C. and the other of the residue. These two concentration experiments have not yielded any tumours, which leaves us assured that at least no more than a trace of carcinogenic substance is left in the oil when it is treated, according to our procedure, with sulphuric acid.

The oxidation of oils.

Oxidation by means of pure oxygen at 150-160° *C.* Oxidation was first attempted by bubbling oxygen through the oils at a temperature of 150-160° C. for 24 hours. We selected for these experiments three oils which we had already shown to be highly carcinogenic, viz. shale oil 8, a methyl sulphite extract of this oil and the high boiling fraction of the american petroleum oil 3. Table VIII shows that the oxidation has rendered highly potent oils almost inert. Shale oil, for example, after treatment resembles in its activity a lowly carcinogenic petroleum oil. The figures relating to the total number of tumours in extract 8 C are lower than they should be owing to the heavy mortality among this particular batch of animals: many other experiments with this extract have shown how very high is its carcinogenic activity. The physical properties of the oil were entirely altered however by the above treatment, and although the method is efficacious in destroying toxic agents experimental conditions for obvious reasons had to be altered. For this reason we have carried out similar experiments at the temperature of the steam bath with shale oil, when little change

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in the character of the oil results. As far as we can judge from the present state of this experiment, oxidation at 100° has effected a change in the carcinogenic potency of the oil, but much less than was the case with oxidation at 150°.

TABLE VIII.

Oxidation and reduction of oils.

Oil number, etc.	Week of first tumour.		Total number of tumours.		Duration of experiment in weeks.	Percentage of living animals bearing tumours at 25th week.
	Wart.	Epithelioma.	Warts.	Epitheliomas.		
8. Whole oil . .	17th	26th	13	13	50	23
Oxygenated 150°	33rd	...	7	0	50	0
8 (2). Whole oil .	9th	22nd	15	4	30	50
Oxygenated 100°	15th	21st	12	3	25	24
Permanganate .	22nd	...	1	0	25	3
Reduced . .	...	...	0	0	10	0
Gel . . .	21st	...	2	0	26	3
,, residue .	16th	...	5	0	25	12
3. 300–380° . .	23rd	15th	10	11	50	5
Oxygenated 150°	...	...	0	0	35	0
8. Extract C . .	11th	30th	7	1	40	15
Oxygenated 150°	24th	...	5	0	40	3

Oil 8 (2) was a second sample of purified shale oil.

As oxidation at 100° C. is not nearly so effective as oxidation at 150° C. in rendering the oils less toxic, we have commenced a series of experiments at the ordinary temperature with certain organic salts as catalysts, of the same nature as driers used in paints, both in the presence and absence of ultra-violet light which is known to be efficient in the promotion of oxygen absorption. Catalysts have also been employed at 100° in further experiments.

Permanganate oxidation of shale oil.—The oil in this case was shaken with slightly alkaline aqueous permanganate for a number of hours when a comparatively small amount of the oxidising agent was reduced. On removal of the precipitated dioxide by sulphur dioxide the oil was taken up in ether, dried and the solvent removed finally *in vacuo*. This method of treatment of the oil has effected a marked change in its carcinogenic activity. There are at present, 25 weeks from the commencement of the applications, 3 per cent. of tumours among the animals treated with the oxidised oil while at the 25th week 50 per cent. of the control animals bore tumours, three out of 15 tumours being at that time malignant.

Reduction of oils.

Up to the present, reduction experiments have only been performed on shale oil. Catalytic reduction of the untreated oil could not be carried out on account of the sulphur compounds present poisoning

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the catalyst but the reduction was effected by the action of sodium on boiling amyl alcohol. The reduced oil was found apparently to be free from carcinogenic properties, there being no tumour formation among the animals painted for 40 weeks. The reduction by catalytic means we feel would have given us a more exact knowledge of the chemical nature of the toxic compounds responsible for the carcinogenic properties of the oils for the changes brought about by this means are limited. We hoped to get rid of the sulphur compounds by means of adsorption by silica gel, but in this we were disappointed and a suitable method for the complete removal of these objectionable bodies has still to be found.

Silica gel adsorption.

It is well known that silica gel on account of its structure possesses certain physical properties whereby, as the result of surface tension effects, unsaturated bodies and certain sulphur compounds can be removed preferentially from a mixture. Percolation of oils through a long column of silica gel was carried out in an endeavour to remove the sulphur present and allow of catalytic reduction subsequently being carried out. In this we were unsuccessful in so far as sulphur still remained in the treated oils. Even after such treatment the oil still contained sulphur bodies possibly of the nature of thiophens which cannot be removed by treatment with bauxite, silica gel or any other adsorbing agent. As the gel in our experiments might possibly have removed unsaturated substances, and indeed our experience had suggested that these were toxic agents, we decided to carry out some experiments with oil so treated. 50 c.c. of shale oil were allowed to percolate through a column of gel 4½ feet high, about 60 per cent. of the oil being retained by the gel at the ordinary temperature. The amount of oil passing through was readily increased by steam jacketing the adsorption tube. 50 c.c. of the oil which had passed through the gel were again run through a similar column before use. The oil which passed was designated "oil 8 gel," while that retained which was collected by repeated extraction of the gel with hot benzene, was designated "residue oil 8 gel." The results of these experiments (table VIII) were at first a little puzzling as both portions of the treated oil proved to be less carcinogenic than the original oil. At the 25th week 50 per cent. of the control animals alive bore tumours while at this time only 12 per cent. of the residue oil 8 gel and 3 per cent. of the oil 8 gel animals bore tumours. A possible explanation of the lack of activity of the two portions of the treated oil is that the silicate has brought about polymerisation of some unsaturated body or bodies in the oil which we believe to have toxic properties, polymerisation of propylenes in this way having recently been demonstrated. It is well known that ultra-violet light can polymerise unsaturated bodies and we have already commenced some experiments making use of this fact.

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Discussion.

Fractional distillation of oils has yielded contradictory results: in some the higher and in others the lower fractions have proved the most toxic. A definite concentration of the carcinogenic agents has however been effected by chemical means. While extraction with absolute alcohol has added little to our knowledge, the use of methyl sulphate is much more valuable as an indication of the nature of the bodies extracted, for example, benzenoid hydrocarbons are known to be removed quantitatively from light spirit by this reagent. Picric acid acts in the same manner to give in many cases definite stable crystalline compounds with the hydrocarbons in question. The combination of the two latter bodies has proved particularly valuable for removing carcinogenic agents from oils. Methyl sulphite may be shown in the same way to remove aromatic hydrocarbons from oils by the formation of solid picrates in alcoholic solution from the extracts. Treatment with sulphuric acid destroys the powerful fluorescence of oils, a phenomenon connected with the presence of the benzene ring. The resulting oils are found to be quite inactive, which fact supports the view that benzenoid hydrocarbons are responsible for the carcinogenic properties. Other experiments with tar and pure organic substances substantiate this view. Unsaturated bodies are usually more active than the corresponding saturated substances and it is probable that removal of such a body takes place in the sludge formed with sulphuric acid. The active carcinogenic agent could equally well combine the characteristics of a benzenoid hydrocarbon and a real unsaturated body by attachment of a side chain with double or triple linkages. Treatment with sulphuric acid, oxidation and reduction could all equally well affect certain sulphur compounds in the oils and experiments have been designed to test this point.

Our experiments on the reduction of oils and tars whereby these substances are rendered inactive gives rise to the belief that unsaturated bodies are the active agents. With oils the real degree of unsaturation is not readily determined by halogen addition as substitution occurs at the same time. Probably the unsaturated bodies present are small in amount but active chemically. Treatment with boiling amyl alcohol and sodium could readily hydrogenate an aromatic nucleus and aromatic bodies will be mostly present in tars which are formed at the temperature employed in their preparation. Unsaturated centres present in open chains would at the same time be saturated. With hydrogen in the presence of palladium the latter type of reduction takes place more readily than hydrogenation of nuclei. The fact that oxygenation exerts so profound a change in oils points to the reactive nature of the toxic substance and encouraged the use of alkaline permanganate for the same purpose. Only a small

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amount of the latter reagent was reduced, which suggests the presence of a small amount of the active agent. Silica gel has in a similar way proved valuable by indicating the possible nature of the toxic bodies and demonstrating a simple (though in the present form not practical) method for removal: experiments to define the limiting amount of gel required are in progress. It is well known that sulphur, besides effecting more profound changes, can remove hydrogen from reduced aromatic nuclei with formation of hydrogen sulphide. Oil treated in this way after reduction at 150-160° C. with sodium and amyl alcohol yields copious streams of the gas and experiments are meanwhile being conducted to find whether bodies rendered inactive can be again activated by this means. If a benzene nucleus is hydrogenated by the above agents then removal of the hydrogen by sulphur would yield the original toxic material and furnish at the same time an indication of the type of reduction previously effected.

Summary.

1. In fractional distillation of carcinogenic oils the active agent is sometimes concentrated in the higher, sometimes in the lower fraction.

2. The active agent is concentrated in extracts made with methyl sulphite or methyl sulphate and picric acid, and the process is useful in testing oils intended for industrial use.

3. There is no evidence that carcinogenic substances are made by fractional distillation or by the processes of extraction used.

4. The carcinogenic activity of an oil is much reduced or completely removed by extraction with sulphuric acid, by oxidation and by reduction.

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NEUROGLIAL TUMOUR OF SPINAL CORD.

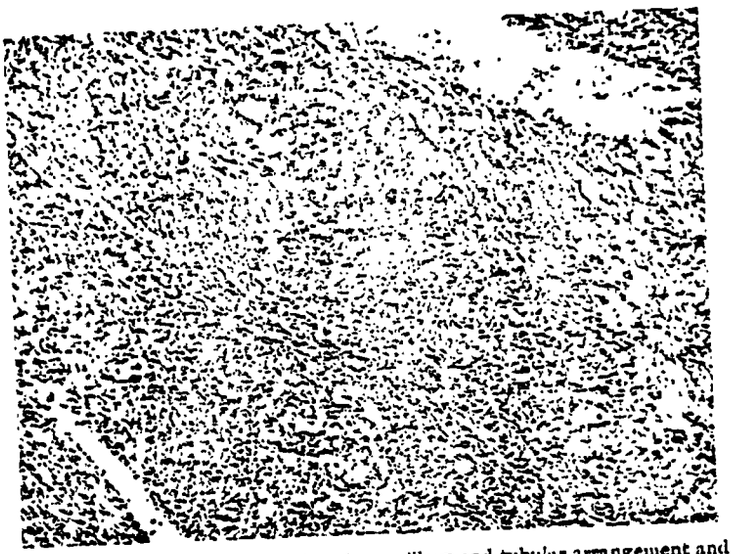


FIG. 1.—A low power view showing the papillary and tubular arrangement and the vacuolated cells.

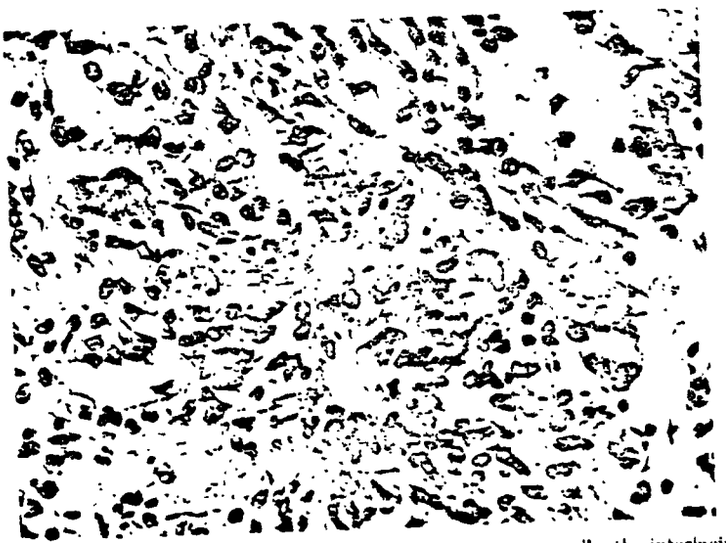
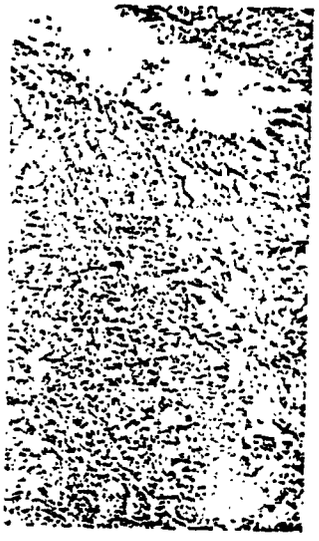


FIG. 2.—A high power view showing the cubical and columnar cells, the interlacing felt-work of fibres and the round and oval nuclei.

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PLATE XVIII

SPINAL CORD.



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and columnar cells, the interlacing
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SHORT ARTICLES.

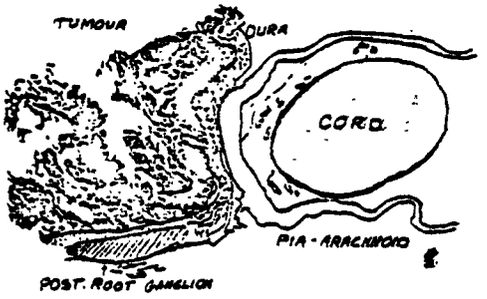
NEUROGLIAL TUMOUR OF SPINAL CORD.

W. G. BARNARD.

From University College Hospital Medical School, London.

(PLATE XVIII.)

This specimen consisting of the spinal cord and a small tumour apparently growing from its membranes was brought to me for diagnosis. It appeared reasonably certain that the tumour had arisen from the cord; the only point that was not clear was whether there was any macroscopic evidence suggesting that the growth had arisen from sympathetic nerves or ganglia and had infiltrated the spinal canal from without. This possibility had not been thought of at the post-mortem examination and so, although nothing was noticed at the time to suggest it, such an origin cannot be excluded.



The tumour formed a plaque (3 × 1.5 × 1.2 cms.) over the posterior surface of the dura in the region of the lower thoracic segments of the spinal cord and infiltrated the laminæ of the corresponding thoracic vertebræ. The inner surface of the dura was free from growth, and though adherent to it the posterior root ganglion was not infiltrated.

Microscopic.—There had been old and recent hæmorrhage into some parts, and other parts of the tumour were necrotic. Elsewhere the conspicuous feature of the tumour was its papillary and tubular arrangement. The cells covering the papillæ and lining the tubules were for the most part cubical or columnar with round or oval nuclei. Most of these cells had a distinct outline but apart from the nucleus appeared to be empty. From many of them slender fibres passed back to ramify with other fibres outside the tubule or in the core of the papilla. Some of the tubules had a definite lumen while others on examination with high powers proved to have a scanty network of fibres passing across the lumen. Apart from the papillæ tubules and pseudo-tubules, the bulk of the tumour was made up of interlacing fibres passing in all directions from cells having oval nuclei similar to those of the cells lining tubules. Both the round nuclei of the vacuolated cells and the oval nuclei had distinct cell outlines and well-marked chromatin nets and nodes.

In the classification of the gliomata by P. Bailey and Harvey Cushing (*Tumours of the glioma group*, 1926) there is a figure number 26 b. p. 34 of what they call a medulloblastoma: this, though not so obviously tubular, bears a fairly close likeness to the tumour here described.

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A SIMPLE METHOD OF PREPARING ACCURATE PEN-AND-INK DRAWINGS OF SPECIMENS.

T. SHENNAN, Aberdeen.

(PLATE XIX.)

In preparing the illustrations for a paper on tumours of mediastinum and lung published in this *Journal* (vol. xxxi., 1928, p. 365) I employed a method which follows out a suggestion made by the late J. G. Adami. The main points of the method are summarised at p. 373 in my paper. I have not been able to identify Adami's paper, but it was one dealing with pathological changes in arteries, and in microphotographs of these he outlined the coats of the vessel in indian ink especially emphasising the internal elastic lamina, and then bleached the prints in 5 per cent. corrosive sublimate. An illustration on similar lines—and almost certainly made in the same way—will be found in his paper on "Arrested dissecting aneurysms" in the *Montreal Medical Journal*, vol. xxiv., 1895-6, p. 945. These illustrations are sufficient for the purposes intended by the author, but I thought that the method might be carried a little further so as to produce slightly more artistic illustrations which might be described as a combination of line and stipple drawing and which would have the advantage of being capable of reproduction as text figures. The results are seen in my illustrations in the tumour paper, but improvements could certainly be made on the method by anyone having artistic capacity and who is an expert in pen-and-ink work. I cannot approach to this ideal, but I wish to show how anyone with a very small knowledge of drawing can produce illustrations which are at least accurate and in which he can, if necessary, emphasise detail.

In the first place an ordinary black and white print is made, preferably on a matt surface paper, as shown in fig. 1. On this print the outlines and details are drawn in with a fine drawing pen, using a good waterproof indian ink. This process proves rather troublesome because the indian ink dries very rapidly at the point of the pen and the point tends to scrape off a very small amount of the gelatine surface. This collects on the point, and, unless frequently wiped off with a piece of folded filter paper or on a piece of old linen, produces thick lines in place of the fine lines which are desirable. Diluting the ink with distilled water helps to make it flow easier, but dilution is not essential. The irregularities and varying shades in the print can be reproduced fairly well by the stipple method, the fine dots being placed closer together in the darker parts and wider apart in the lighter parts of the print. A certain amount of shading can be produced by drawing closely set delicate parallel lines in the parts which are in shadow. For blocking out, a fine camels' hair brush is used.

In fig. 2 my aim was to block out the tumour so as to show its relation both to surrounding structures and to the bronchiectasis which was a prominent feature, but outlining the growth, and stippling in its irregularities would have shown it up almost as clearly.

After the outlining and shading have been completed the next procedure is to discharge the print. Occasionally, one may wish to preserve some indication of it, and if so, it should be treated in "hypo" solution to which sufficient potassium ferricyanide has been added to give a pale yellow colour, or in 5 per cent. aqueous solution of corrosive sublimate. In order to discharge the photograph completely and rapidly, I apply iodine solution which is followed by treatment with *acid* "hypo." The print is treated with 1 per cent. watery solution of iodine until the paper is of a deep blue-black colour. This is then

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Fig. 2.



Fig. 1.

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FIG. 2.



FIG. 1.

rinsed in water and treated with *acid* "hypo," that is, hyposulphite solution containing citric acid.

Formula :—

(*a*)	5 per cent. "hypo"	20 oz.
(*b*)	citric acid	½ oz.
	hot water	1 oz.
(*c*)	sodium sulphite	½ oz.
	hot water	1 oz.

Mix (*b*) and (*c*) and add to (*a*).

This quickly discharges the blue-black colour and reproduces the ivory white colour of the paper on which the indian ink lines and dots stand out very

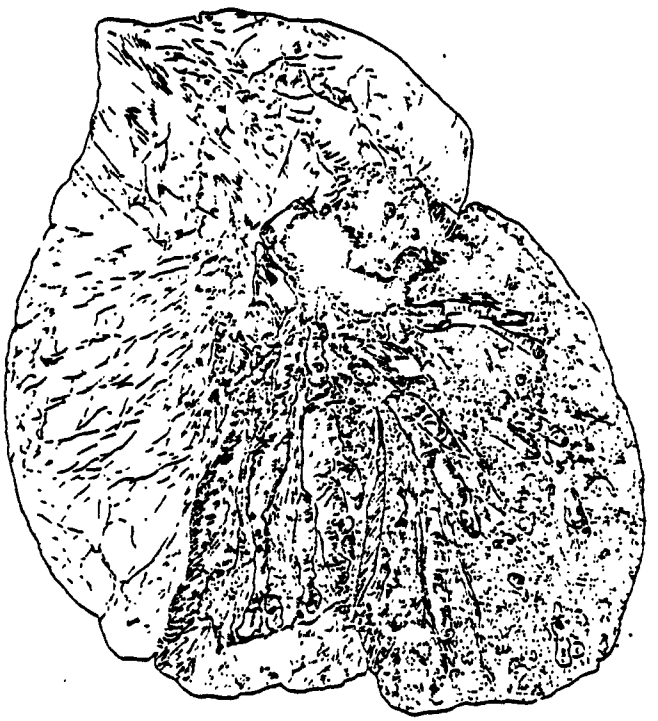


FIG. 3.

distinctly. After washing for about an hour in running water it is allowed to dry. The treatment can be repeated if the print is not completely discharged. One can then compare the drawing with the original preparation and with another print of it, and draw in any part which has been inadvertently omitted or correct or emphasise details. Moreover, any lines which prove to be too heavy can be reduced by scraping with a sharp pointed knife. The result is shown in the text figure (fig. 3). Figs. 2 and 3 have been prepared from the same print, fig. 2 after inking and fig. 3 after the final bleaching.

The method can of course be applied equally well to microphotographs.

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BOOKS RECEIVED

Elementary pathological histology.

By W. G. Barnard. London: H. K. Lewis & Co. Ltd. 1928. Pp. 80, 176 illustrations on 52 plates. 7s. 6d.

Essentially this is a small hand-atlas of elementary pathological histology, with brief pithy descriptions of the more important disease-processes. General principles are illustrated by means of well-chosen particular instances, and the method of approach is on familiar and conventional lines, even if the phraseology of the preface had raised other hopes—or fears. The times are changed, and it is conceivable that students nowadays read prefaces; if so, Dr Barnard's will help to gain him readers. The illustrations are provided by an admirable series of microphotographs and some drawings, with the legends on the pages opposite, a method by which it is possible to have descriptions of adequate length without curtailment of the size of the pictures. The text, which has been reduced to a minimum, deals clearly and simply with the subject-matter. Half the book is devoted to inflammation and the reactions to selected types of infection, one quarter to various regressive and progressive tissue changes and the remainder to tumours. This is a book for use in the practical class room. It can be cordially recommended to students of pathology, and the junior student in particular should find it of the greatest service in studying his class slides. Its price, which is commendably low, places it within the reach of all. There are one or two curious verbal slips (*e.g.* "inspired food stuff" on p. 10) which the author will no doubt rectify in future editions.

Physiology and biochemistry of bacteria.

By R. E. Buchanan and E. I. Fulmer. London: Baillière, Tindall & Cox. 1928. Pp. 516, 78 text figures. 34s.

Everyone will agree with the authors that there is need for the collection and systematisation of the scattered information about the physiology of bacteria yeasts and moulds; they now publish the first volume of their attempt to supply the want. It deals with "growth phases; composition, and biophysical chemistry of bacteria and their environment; and energetics." It is partly good and will be useful, but it falls very far short of what we had hoped for. Instead of an encyclopædic scholarly survey, the authors give us, as they frankly confess in the preface, a revision of the lectures on the physiology of bacteria and on biophysical chemistry given at the Iowa State College to students of bacteriology. The result is patchy and uneven. A great part of the book (roughly 200 pages) is taken up with an account of some aspects of physical chemistry which might have come out of a manual of that subject: it is quite out of place here. Indeed the whole work is absurdly dominated by the physico-chemical outlook. In consequence the physiology is scamped. Instead of describing the facts of anaerobiosis, we have a page of superficial remarks suitable for a schoolbook on general biology and a reference to nine pages on oxidation-reduction potentials. If Prof. Buchanan wants to produce a book up to his usual standard he will have to take it more seriously and not be in such a hurry to throw his lecture

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notes into print: he would also do well to associate himself with the professors of animal and plant physiology rather than the professor of biophysical chemistry. The index (in such a book so essential) is very bad. Anyone who is curious about the foodstuffs of bacteria will find under "hydrogen" only a reference to hydrogen for the hydrogen electrode and will not find "methane" at all.

Filterable viruses.

Edited by T. M. Rivers. London: Baillière, Tindall & Cox. 1928. Pp. 428. 34s.

Knowledge of viruses is moving so fast just now that it would be impracticable to attempt an encyclopædia of all that is known and suspected. Wisely enough this volume does not contemplate any such exhaustive treatment. It consists of ten chapters: on poliomyelitis (by H. L. Amoss), foot-and-mouth disease (P. K. Olitsky), fowl-pox (E. W. Goodpasture), insect diseases (R. W. Glaser), plant viruses (L. O. Kunkel), cytology (E. V. Cowdry), the use of tissue cultures (A. Carrel), filters (S. Mudd): all these are good. The best article is the last—J. J. Bronfenbrenner on the bacteriophage. The worst is the general introduction by the editor, who frankly confesses that the problem of what life is does not interest him, and accordingly makes no attempt to discuss the real nature of viruses. The whole forms a very interesting and useful book and there are abundant bibliographies.

Handbuch der biologischen Arbeitsmethoden.

Edited by E. Abderhalden. Berlin and Vienna: Urban and Schwarzenberg. 1928.

Part 283 (pp. 183: M. 9) contains a long article (153 pages) by K. Saller on the biometry of frogs, small laboratory mammals and birds, a few remarks (8 pages) on racial pathology by A. Plehn and an indifferent account (17 pages) by H. Baum of the methods of injecting lymphatics. In part 268 (pp. 111: M. 6) R. Schnitzer gives an account of chemotherapy in protozoal infections and in part 269 (pp. 109: M. 6) E. Löwenstein discusses toxins and toxoids with a good deal of interesting historical matter. The reasonable price at which this compendium is still sold deserves notice: many german books and periodicals have become impossible luxuries.

Arbeitsmethoden der Mikrobiologie.

By A. Janke and H. Zikes. Dresden and Leipzig: Steinkopff. 1928. Pp. xi + 183. 127 text figures. M. 13.

This book is intended to serve as a complementary volume to Janke's *General technical microbiology* (Dresden and Leipzig 1924). It is essentially a practical handbook and as it is designed for the use of students at technical high schools it does not deal with the medical aspects of bacteriology. It is quite a good compendium of methods in vogue for the study of bacteria and their products, particularly those which are of importance in industrial processes. It covers a wide range of subjects, from the best colour to put on the walls of a laboratory to the details of the methods employed in analysing the chemical changes occurring in the various processes of fermentation. There are chapters on the microscopic examination of organisms, methods of cultivation, sterilisation and enumeration, the study of chemical changes in substrates and the mode of production of these changes and the microbiological examination of air, water, soil, etc. These sections are on the

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whole well done and contain much detailed information in a small compass. The last chapter (20 pages) on the identification of organisms is of much less value. It is interesting to note the complete omission of all reference to serological methods. The illustrations are poor.

Die Bedeutung des Retikuloendothelialsystems für das Streptokokkensepsisproblem.

By N. Louros and H. E. Schryer with the collaboration of A. Schmechel and E. Gaessler. Leipzig: Georg Thieme. 1928 Pp. viii + 101, 13 figures and 4 coloured plates. M. 14.

There are fashions in pathology as there are fashions in clothes and the authors of this volume are certainly not unfashionable in their treatment of the subject of streptococcal infection in mice. They are however perhaps a little behind the times, though not much, in the mystical and almost reverent way in which they use the terms "physical," "chemical," "physico-chemical," "colloid" and "metabolism." In the course of the discussion very great stress is laid on the importance of the reticulo-endothelial system in metabolic processes and on the relationship between success or failure to combat infection with streptococci and the behaviour of this system. The greater part of the work is devoted to the study of the effect of chemical substances, lipoids, proteins, lyes, heavy metals etc., and physical agents, X-rays and light, on the infection and to correlating the results obtained with the effect of these substances on the reticulo-endothelial system. It cannot be said that the discussion is convincing. It is not even clear that the proliferation of the cells of the system described as occurring in certain infected animals is controlled by observation of more than a few normal animals. The alterations in the blood picture are discussed without reference to the total counts. The effects of the various chemical and physical agents employed to influence the course of the experimental disease are not described in such a way as to permit one to compare the experimental results actually obtained with the conclusions drawn from them. As the results are apparently not clean cut but merely consist of a prolongation of life in most instances it is not possible for the reader to judge of their value without much fuller descriptions of the experiments. The case is clearly one for statistical evaluation and none has been made. In a fairly long list of references there is only one to a publication in english.

Recent advances in bacteriology.

By J. H. Dible. London: Churchill. 1929 [1928]. Pp. 363, 22 illustrations. 12s. 6d.

A bright and cheerful account of many of the growing edges of bacteriology —streptococci, roughs and smooths, bacteriophage, experimental epidemics, B. C. G., viruses, pneumococci, spirochætes and so forth. Prof. Dible seems to us to have succeeded quite well in what must have been a difficult job: it would have been easier to do at greater length, and in a small compass he has mentioned most of the things which matter in each topic. A little more dogmatism might be an improvement and the references should be in alphabetical order. It is probably as difficult to decide whether Twort or d'Herelle discovered bacteriophage as to be sure whether the first professor of mathematics in University College, London (1806-1871) or Jonathan Swift (1667-1745) is the only begetter of "little fleas have lesser fleas"; making verses, like scientific discovery, is probably a process, not an event. Publishers in Messrs Churchill's position ought to be above making the pretence that

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their books are more up-to-date than they are by post-dating them. The title page says "1929": a copy reached the editors, by a rather devious route, on December 6, 1928. Two other books published by the same firm this autumn are also dated "1929."

Clinical chemical pathology.

By F. S. Fowweather. London: Churchill. 1929 [1928]. Pp. 216, 18 illustrations. 8s. 6d.

This is a manual for the bedside, not for the laboratory and all descriptions of laboratory technique are purposely omitted. The author's aim is to give the clinician an account of the information that he may obtain from the chemical laboratory, to explain the basis on which the various tests rest, to give help in the interpretation of the results, and last but not least, to make clear the points that the clinician must himself attend to in the collection of specimens. The result is an attractive and distinctly successful volume that collects within a small compass a mass of useful and otherwise scattered information accompanied by much shrewd comment. The tale is told very simply and clearly and the distinction between tests which have proved their worth and those of more doubtful value is drawn with rare judgment. An admirable present from any clinical pathologist to his practising colleagues! The implication (p. 149) that air ever contains as much as 21·07 per cent. of oxygen needs correction.

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PROCEEDINGS OF THE PATHOLOGICAL SOCIETY OF GREAT BRITAIN AND IRELAND

4th and 5th JANUARY 1929

The thirty-eighth meeting was held at St Bartholomew's Hospital, London, on Friday and Saturday, 4th and 5th January 1929.

Communications and demonstrations

(Those marked with an asterisk are abstracted below.)

J. W. M'NEE. The classification of chronic splenomegaly in Britain.

J. S. YOUNG. Further observations on the production of hyperplasia in the alveolar epithelium of the lung of the rabbit.

I. BERENBLUM. Tumour formation in mice following freezing with carbon dioxide snow.

I. BERENBLUM. The modifying influence of mustard gas on the induction of tumours in mice by tar.

H. J. B. FRY. The syphilitic factor in malignant diseases.

J. R. PERDRAU. Acute disseminated encephalomyelitis.

J. G. GREENFIELD. The encephalomyelitis of measles.

D. F. CAPPELL. The fate of intravenously injected iron.

R. D. PASSEY, G. M. BONSER and M. J. STEWART. The phagocytosis of melanin.

C. PRICE-JONES. Variability of mean diameters of red cells.

*F. PARKES WEBER. Generalised osteosclerosis.

W. W. C. TOPLEY, M. GREENWOOD, J. WILSON and E. NEWBOLD. The value of various antigens in prophylactic immunisation against mouse typhoid.

F. C. HAPPOLD. Further experiments upon "heat stable" *Salmonella* precipitinogens: (*a*) group relationships: (*b*) the effect of cultural variations upon the production of the "heat stable" precipitinogens.

A. FELIX and M. RHODES. Antigenic variation in bacteria, illustrated by a problem arising in the serology of typhus fever.

R. MARGARET PITT. The effect of growing smooth and rough cultures of *B. typhosus* in serum.

P. B. WHITE. A factor in normal agglutination.

A. FLEMING. The bactericidal power of human blood to virulent pneumococci.

G. H. EAGLES and D. M'CLEAN. Growth of vaccinia virus in tissue culture.

C. H. ANDREWES. Generalised vaccinia in the rabbit: protective action of immune serum.

J. B. BUXTON. Caseous lymphadenitis in sheep (Preisz-Nocard bacillus).

A. S. STRACHAN. Post-mortem experience in Johannesburg.

R. E. GLOVER. Contagious pustular dermatitis of sheep.

T. DALLING. Lamb dysentery: prophylaxis with formalinised culture.

S. ELLINGWORTH, J. W. M'LEOD and J. GORDON. Further observations on oxidation of dyes of the paraphenylenediamine series by bacteria.

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J. H. DIBLE and C. WEATHERALL. The splitting of æsculin and the production of hæmolysis by fæcal streptococci.

R. ELLIS and C. H. WHITTLE. An epidemic of lobar pneumonia.

D. NABARRO. The present incidence of bacillary dysentery in Great Britain.

V. D. ALLISON and T. H. AYLING. An improved medium for the isolation of *Corynebacterium diphtheriæ*.

H. W. CROWE. The selective affinity of certain streptococci for bone tissue.

*M. J. STEWART and A. C. HADDOW. "Peculiar bodies" of pulmonary asbestosis obtained by lung puncture in the living subject.

H. J. B. FRY. Three cases of chordoma.

D. NABARRO. Congenital syphilis with enlarged thymus and lymphoid tissue hypertrophy.

D. F. CAPPELL. Two cases of myelomatosis: (*a*) plasma cell type; (*b*) unusual type with tumour-like nodules and visceral lesions.

P. B. WHITE. A differential test for smooth and rough bacilli.

M. J. STEWART and S. J. HARTFALL. A case of chronic peptic ulcer of the œsophagus (above p. 9).

G. S. WILLIAMSON, I. H. PEARSE and R. M'CARRISON. Experimental and spontaneous lymphadenoid goitre.

JOAN M. ROSS. A case of sphenomegaly of the Foix and Roemmele type (*Arch. de. med. exp.*, 1912, xxiv. 111).

A. E. BOYCOTT. *Hystrichopsylla talpæ*.

Abstracts.

GENERALISED OSTEOSCLEROSIS.

F. PARKES WEBER.

The specimens are from an Englishman, aged 56 years, who was admitted to hospital in May 1927, with extreme pallor and general debility, after an illness said to have commenced in February 1927. Repeated blood-counts showed severe anæmia of varying degree, with a variable proportion of myelocytes and myeloblasts, and for a time large numbers of nucleated red cells (as in so-called blood-crises). Roentgen ray examination showed a peculiar opaque condition of the bones; the medullary canal of the long bones was badly defined, as in the so-called "Albers-Schönberg Marmor-Knochenerkrankung." No signs of a malignant tumour could be found anywhere; there was no Bence-Jones protein in the urine; the Wassermann reaction was negative. Under repeated blood-transfusion and afterwards liver-diet he improved a little, but in February 1928 he contracted a septic pharyngitis and suppurative otitis media. Staphylococci were cultivated from the circulating blood, and he died on 22nd February.

The *necropsy* showed a condition of generalised osteosclerosis (*endosclerosis*), the medullary canals of the long bones being in great part filled up by hard bone and fibrous tissue. The condition of the spleen, liver and lymph-glands was not typical for any kind of leukæmia. In regard to the classification of the case the following had to be considered:—

(1) The generalised osteosclerosis which has occasionally been noted in association with aleukæmic myelosis and other types of leukæmia.
(2) The constitutional or congenital condition of so-called "marble bones."
(3) Other conditions, especially *secondary* osteosclerotic anæmia, as a reaction towards diffuse miliary carcinomatosis of bones.

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DEMONSTRATION OF THE PECULIAR BODIES OF PULMONARY ASBESTOSIS ("ASBESTOSIS BODIES") IN MATERIAL OBTAINED BY LUNG PUNCTURE AND IN THE SPUTUM.

M. J. Stewart and A. C. Haddow.

Leeds.

The occurrence of highly characteristic golden-yellow bodies in the fibrosed lungs of asbestos workers has now been demonstrated by a number of observers (Stuart M'Donald, *Brit. Med. Journ.*, 1927, ii. 1025; Cooke and Hill, *Journ. R. Micr. Soc.*, 1927, p. 232; Taylor and Grieve, unpublished observation; Simson, *Brit. Med. Journ.*, 1929, i. 865). They have been found in every fatal case of the disease in which they have been looked for, including the original case published by Murray (*Charing Cross Hospital Gazette*, 1900) and recently re-examined by Dr E. L. Middleton of the Home Office (personal communication), and they were present in the lungs of a guinea-pig which had been experimentally exposed to an asbestos dust atmosphere (Marrogordato, quoted by Simson, *op. cit.*). They occurred in large numbers in four fatal Leeds cases of pulmonary asbestosis, in the last of which they were readily demonstrable in smears from the cut surface of the lungs (Stewart, *Brit. Med. Journ.*, 1928, ii. 509). Arising out of this observation it was suggested to one of us by Dr S. A. Henry of the Home Office that exploratory puncture of the lung might yield positive results. So far, this procedure has been attempted in two cases only, and in one of them asbestosis bodies were recovered from the lung.

A married woman of 38, with five children, had worked in an asbestos factory since the age of 16 until a year ago, with intervals totalling about six years. She had a dry, irritating cough with moderate dyspnœa and marked loss of weight. The physical signs were limited to the bases of the lungs, where there was definite dulness with diminished breath sounds and fine crepitations. On 15th December 1928 the left base was explored with a hollow needle and a minute amount of blood-stained material withdrawn. This was smeared on four microscope slides two of which were dried, fixed with heat and mounted in canada balsam; one was treated with hydrochloric acid and potassium ferrocyanide, and one was stained for tubercle bacilli. The material obtained consisted mainly of blood, partly fluid, partly clotted, with numerous carbon-containing macrophages. The peculiar and quite unmistakeable golden-yellow asbestosis bodies were present in considerable numbers, especially within the small blood-clots. They gave a rather feeble prussian blue reaction. Occasionally there was evidence of phagocytosis by large macrophages, some of which had multiple nuclei and contained carbon dust. No tubercle bacilli were found.

In a second very similar case, negative results were obtained, the material withdrawn consisting apparently of blood with an excess of leucocytes.

Following the positive findings in the first case, we attempted to recover the bodies from the sputum. As a rule there is little or no sputum in these cases except during an actual bronchitic attack, and in neither of the cases punctured was any available. About half an ounce of mucopurulent sputum obtained from each of two other patients was dissolved up in an equal quantity of undiluted antiformin, centrifuged, and the deposit washed and examined microscopically. After prolonged search through numerous films two typical asbestosis bodies were found in one of the specimens examined. The other was entirely negative.

This work is being continued, but we thought it well to make a preliminary communication now in the hope that the methods we have adopted might prove of use to other workers.

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