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A CONTRIBUTION TO THE CHEMICAL PATHOLOGY  
OF THE LIPOIDS.

by

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## GENERAL INTRODUCTION.

From the biological, and especially medical, point of view no class of substances has in recent years grown so rapidly in importance as the lipoids. The purely chemical investigation of these substances has been carried on by numerous observers since the middle of last Century, but it is only during the last twenty years, and especially the last ten, that any great progress has been made. At the present day we know thoroughly the constitution of several important lipoids and we are able, in a general way, to make a reasonable classification of those which are less well known.

Mayer and Overton's theory of narcosis, the explanation of the pharmacology of many drugs, and the numerous physiological theories dependant upon the physics of a cell membrane have all helped to increase the importance of a thorough knowledge of the lipoids in physiology, and have stimulated numerous researches, but the rôle played by these substances in pathological processes remains almost uninvestigated.

## Part I. THE CHEMISTRY OF NERVE DEGENERATION.

The chemical substances found in normal nerve tissue - as in animal tissues in general - may be separated conveniently into five classes, namely, water, proteins, lipoids and fats, organic water extractives, and inorganic salts. The relative proportions present of these classes of substances vary in different parts of the nervous system. Thus the central nervous system contains a somewhat greater proportion of water than the peripheral nerves, and the grey matter of the brain and spinal cord more than the white matter. Again, the peripheral nerves contain free fat in the meshes of their connective tissue framework, whilst this substance has never been recognised as a normal constituent of the brain (Koch<sup>1</sup>), and it is not found in the spinal cord (of the cat) or is present in such small amount as to be unrecognisable, as will be shown in the experimental section of this essay. It is therefore impossible to give an approximate composition of nerve tissue to cover all parts of the nervous system, but it may be stated that water forms 60 - 80% of all nerve tissue and that lipoids are relatively abundant.

Koch<sup>1</sup>, in his elaborate analysis of the brain, separates the proteins into the following groups:-

(a)/

- (a) Globulins coagulating at 47°- 50°C.
- (b) " " " 70°C.
- (c) Neurostromin.
- (d) Nucleoprotein.
- (e) Neurokeratin.

Each of these was investigated and estimated for the brain and spinal cord. Their bearing on nerve degeneration at present does not warrant a detailed description of their properties.

A large number of "organic water extractives" have been isolated by various authors but many of the substances obtained were undoubtedly due to post-mortem decomposition, or were derived from blood contaminating the tissue under examination. Such are, for example, tyrosin and leucin; formic, acetic, succinic and lactic acids, all of which were described by Thudichum<sup>2</sup>. Of the bodies which are probably not merely accidental impurities the more important are the following:-  
 Members of the purin bases (Levene<sup>3</sup> and Thudichum<sup>2</sup>); creatin (Krause); urea (Gulewitsch<sup>4</sup>); peptones, albumoses, and inositol (Thudichum<sup>2</sup>).

The inorganic constituents are Na, K. (NH<sub>4</sub>), Ca, Fe; Cl, (SO<sub>4</sub>), (PO<sub>4</sub>) and (CO<sub>2</sub>) present partly in organic and partly in inorganic form.

The term lipid is applied to all chloroform soluble substances, including fat and fatty acids by some<sup>authors</sup>, by others not. In the nervous system these compounds/

compounds are found most conspicuously in the myelin sheath. Many of the histological methods for the examination of nerves depend upon the chemical properties of the lipoids. Thus, owing to the presence of unsaturated bonds within the molecule of one or more of the lipoids, osmic acid is reduced by the myelin sheath to form a black precipitate. When a nerve degenerates the changes which are most easily observed, both chemically and histologically, occur among the lipoids and it is for this reason that a study of these substances is so important.

The simplest and most convenient classification of the lipoids is based upon the presence or absence of nitrogen, phosphorus, and sulphur within the molecule. Upon such a basis we have:-

- (a) Cholesterin and its esters in which all the above-mentioned elements are absent.
- (b) The phosphatides, containing phosphorus and nitrogen.
- (c) The cerebrosides, containing nitrogen but no phosphorus or sulphur. They yield galactose when boiled with dilute hydrochloric acid.
- (d) Protagon, the only lipid which contains sulphur.

CHOLESTERIN the best investigated member of the lipoids, is a colourless, odourless compound, fatty to the touch. It/



from the other lipoids is, however, a matter of such difficulty that it is almost certain that many of the bodies described are decomposition products. This applies especially to the substances which have been obtained by the use of strong reagents. Thudichum prepared and described a number of such compounds. As a natural result the literature of the subject is difficult and often contradictory, and the difficulty is increased by the frequency with which the same compound is described under two or more different names, or different compounds are labelled by the same name. An analysis of the literature of the subject with a chemical classification is given by Cramer in the article on lipoids in Abderhalden's Handbuch der Biochemische Arbeits Methoden. Here it is necessary only to describe the general properties of the phosphatides, and to describe shortly the best known members of the group, viz., lecithin and kephalin.

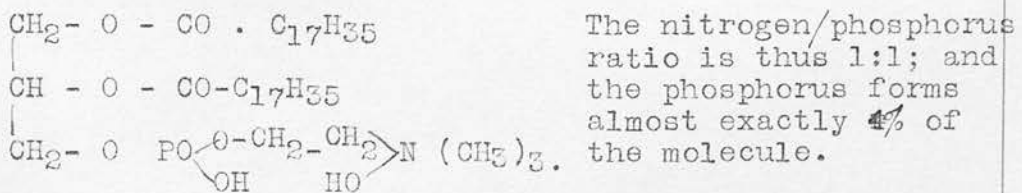
General Properties.

The phosphatides, as a rule, cannot be crystallised. When dry, they form a wax-like mass which cannot generally be powdered. They melt with decomposition. When pure they are colourless, odourless and tasteless and are, especially the unsaturated members, hygroscopic, becoming liquid in damp air. They develop a brown colour when exposed to light and moisture, and give off in this impure state an odour resembling that of/

of butyric acid. The phosphatides form colloidal solutions in water from which they are precipitated by neutral salts. In alcohol, chloroform, ether and benzol they are soluble. Acids and alkalia rapidly hydrolyse them.

In considering the purity of a phosphatide the ratio of nitrogen to phosphorus is conveniently employed as a guide, owing to the comparative ease with which these elements can be determined.

Lecithin forms an orange yellow semisolid mass which, in the dry state, feels sticky and cannot be completely reduced to a powder. A fresh preparation has a faint peculiar odour. It is easily soluble in ordinary organic solvents except acetone and methyl acetate. A watery solution of alkaline bile acids dissolves lecithin; with water it swells up and forms a turbid liquid. It melts at  $60^{\circ}$ ; at  $110^{\circ}$  it becomes brown and decomposes. At ordinary temperatures a slow decomposition occurs. Lecithin splits off choline quantitatively when heated for one hour with 10%  $H_2SO_4$ . Alkalis, including baryta, rapidly cause hydrolysis. When exposed to the air lecithin takes up oxygen. Its probable chemical structure is:-



Kephalin/

Kephalin is a resinous, easily powdered, hygroscopic almost colourless substance. Its solubilities are very similar to those of lecithin. It forms a colloidal solution with water. It melts at about  $100^{\circ}$  with decomposition. Kephalin, in ethereal solution rapidly absorbs atmospheric oxygen. It is rapidly hydrolysed by acids and alkalis. It contains nearly 4% of Phosphorus.

#### The Cerebrosides.

Included under this term are a number of substances which possess in common the power of yielding galactose when hydrolysed by dilute acids and of giving a purplish colour when treated with concentrated sulphuric acid. As has been indicated they contain nitrogen but no phosphorus and no sulphur.

The classification of the cerebrosides is a matter of very great difficulty for reasons similar to those given under the phosphatides.

Only those compounds which have well marked chemical and physical properties will be described here. They are cerebrone, cerebrin and homocerebrin.

Levene<sup>7</sup> in a recent paper has suggested that these three cerebrosides are optical isomerides.

Cerebrone was first prepared by Gamgee in 1880 and named by him pseudo-cerebrin; later, in 1900, it was prepared and named cerebrone by Thierfelder. In 1902 Koch described the same substance under the name cerebrin./

cerebrin. It is best prepared by Thierfelder's method<sup>8</sup>. It is soluble in warm alcohol, in benzol and in chloroform; difficultly soluble in acetone. It melts at 209°- 212°. When heated with methyl alcoholic baryta it is not changed.

Cerebrin and Homocerebrin swell up when added to water, they are not decomposed by boiling with water but are hydrolysed by prolonged treatment with boiling baryta. Cerebrin separates from alcohol in friable masses; it is difficultly soluble in ether and acetone, easily soluble in other organic solvents; it melts at 176°C. Homocerebrin has very similar properties but it is more soluble in absolute alcohol. When dry it forms a wax-like mass which cannot be powdered; it melts at 150°C.

#### Protagon.

Concerning the chemical individuality of this substance there has been considerable controversy. It is unnecessary here to analyse the literature of the subject, but it may be stated that, apart from protagon, it has not been possible to prepare a lipid which contains sulphur\* and that pure protagon possesses well defined physical and chemical properties. Koch<sup>10</sup> & <sup>11</sup> convinced himself that lipid sulphur plays a very important part in nerve metabolism and endeavoured, without/

\*Lately such a compound has been described. See P. 9

without success, to separate the sulphur of protagon in the form of a simpler "lipoid". He found that when protagon is boiled for several hours with dilute hydrochloric acid, the sulphur is split off as sulphate<sup>11</sup>.

When dry protagon is a white non-hygroscopic powder; it forms an emulsion with water and is coagulated by boiling with concentrated neutral salt solutions. It is easily soluble in hot methyl - and ethyl - alcohol, and in glacial acetic acid. In boiling ether and acetone it is sparingly soluble but in ether containing cholesterolin or kephalin it is soluble in the cold. It melts at 200°C. When hydrolysed with dilute acids galactose is split off; with methyl alcoholic baryta protagon yields a cerebroside (Liebreich<sup>12</sup>) glycerophosphoric acid, a fatty acid and choline. Rosenheim and Tebb<sup>13</sup> claim that sphingosin also is split off. The simplest explanation of these properties is that protagon is a complex of a cerebroside, a phosphatide, and an unidentified substance containing sulphur.

Levene<sup>14</sup> in a recent paper claims to have prepared a sulphatide from the brain which contains neither nitrogen nor phosphorus. He has not yet given the methods by which the substance was prepared and only gives one analysis.

#### Preparation of Material

and

#### Methods of Analysis.

When it is found necessary to analyse grey matter apart/

apart from the white thin slices are shaved with a razor from the surface of a brain which has been freed from its membranes. These slices inevitably contain a small proportion of nerve fibres, but by cutting parallel to the surface of the brain and using the knife with care, one can separate all white matter visible to the naked eye.

The corpus callosum affords an abundant supply of ~~fine~~ white matter.

An average of the composition of the grey and white matter is obtained by mincing one half of the brain or the cord.

The tissues thus obtained must be dried before being treated with lipoid solvents. A large number of methods have been devised, and perhaps the simplest is to allow the part to be analysed to lie in 10% formalin for 24 hours and then to mince it with a fine meat-mincer, or in the case of small amounts of tissue to divide it with a knife. The material is then allowed to dry in the air for 24 hours and afterwards in vacuo over sulphuric acid until a constant weight is reached. When such small pieces of tissue as, e.g., a cat's spinal cord, are being dealt with, it is easy to hasten the process of drying by grinding the fragments in a mortar after they have stood 24 hours over sulphuric acid in vacuo. It is generally difficult, however, /

however, to powder nerve tissue, but the difficulty is overcome if the tissue is treated with absolute alcohol after formalin. There is a disadvantage in this since the alcohol dissolves out some of the cholesterol, and though the solvent is allowed to evaporate from the vessel a possibility of slight loss is introduced.

Of other methods which have been introduced the following are the more important.

Baumstark's Method<sup>15</sup> for the brain consists in drying the finely divided tissue by means of ether. The small pieces are tied up in fine-meshed linen gauze and placed on a shelf in a large vacuum desiccator. Ether is poured in until the brain is covered by several centimetres of the liquid. Water flows from the tissue and accumulates in the bottom of the desiccator. This watery solution is pipetted off every eight days and fresh ether added. At the end of three months the process is complete. Altogether, though the method is of great value in the chemical examination of the brain, for purposes such as those which led to the experimental work of this thesis, it is unsuitable.

In the method of Erlandsen<sup>16</sup> the brain is, as before, cut into small pieces which are spread out on plates; a stream of warm air is blown over the plates, the pieces of tissue being frequently turned. In twelve hours the bulk of the water is evaporated. The pieces are now more finely divided and placed in a desiccator/

desiccator over calcium chloride; the desiccator is evacuated and maintained at a temperature of  $37^{\circ}$  by being placed in water heated to the necessary temperature. In four to six hours the material can be powdered. The product is further dried in vacuo over sulphuric acid and is finally obtained as fine, gray, hygroscopic easily-oxidised powder.

Büntz<sup>17</sup> mixed the finely divided brain with about half its weight of anhydrous sodium sulphate and ground the mixture to a powder. He extracted the lipoids directly from the powder.

When small quantities of tissue are being handled the following method may be used. The finely divided material is placed in a weighed vessel and covered with acetone which is allowed to evaporate in the open air. The beaker is then kept in vacuo over sulphuric acid until a constant weight is obtained.

#### Extraction of the Lipoids.

The solvent used varies with the lipoids it is desired to extract. Chloroform is the best solvent to employ when one wishes to obtain the total lipoids. A rough separation of cholesterol and fats from phosphatides and from protagon and cerebrone, can be effected by the use of cold acetone, while cold ether separates phosphatides from the remaining lipoids.

The Soxhlet extraction apparatus is usually employed/

employed when it is of no moment to keep the temperature of the solvent as low as that of the laboratory; on the other hand a mechanical "shaker" is used if it is desired to extract in the cold. This latter method is very tedious and besides being laborious is not adapted to quantitative work. The author has therefore contrived to adapt the Soxhlet apparatus for both purposes.

The Soxhlet employed has ground-glass joints to obviate the use of corks and rubber "bungs" which swell and crack from exposure to hot chloroform vapour, and is fitted with a spiral-glass tube which fits in the cylinder containing the material to be extracted.

The spiral is connected to a reservoir of water by means of rubber tubing which passes down the inner tube of the condenser. It is easy by this means to regulate the temperature of the extracting liquid to within five degrees between the limits of 18°C and 55°C.

The flask of the apparatus is weighed before the extraction is begun, and again after it is completed and the solvent had been evaporated. The difference gives the weight of lipoids dissolved out. In order to avoid oxidation the solvent is evaporated in a stream of coal gas. The total lipoids are redissolved in chloroform (or other solvent) and the solution is made up to a known volume - 100 c.c. generally - and aliquot/

aliquot portions are removed for analysis.

Estimation of Cholesterin.

Windaus<sup>18</sup> has described an easy and exact means of estimating cholesterin which has displaced all other methods. An excess of a 1% solution of **digitonin** in 90% ethyl alcohol is added to an alcoholic solution of cholesterin. The mixture is warmed on the water bath and then allowed to stand at room temperature for 24 hours. The crystalline precipitate which separates out is insoluble in water, alcohol, chloroform and ether. In order to test whether an excess of the digitonin solution has been added or not, about one-fifth of a cubic centimetre of the clear supernatant liquid is pipetted off and added to a 1% alcoholic solution of cholesterin. If a copious precipitate comes down within an hour an excess of digitonin has been added. If not, then more digitonin must be run in and the mixture again placed aside for 24 hours. When sufficient digitonin has been added and precipitation is complete, the granular precipitate is filtered through a Gooch crucible, washed with water, alcohol, and finally with chloroform, dried at 110°C and weighed.

The cholesterin-digitonate which separates out contains <sup>one</sup> quarter its weight of cholesterin and from this the percentage of cholesterin in the lipoids may be/

be estimated.

#### Estimation of Cholesterin Esters.

The filtrate obtained after separation of the cholesterin-digitonin compound is evaporated to dryness on the water bath and the residue repeatedly extracted with ether. The ethereal solution thus obtained contains the cholesterin esters; it is evaporated to dryness and alcohol is added to the residue. To the alcoholic mixture an appropriate amount - 15c.cs. for every 5 gr. of residue - of a 5% solution of sodium ethylate is added and the mixture heated for one hour on a water bath. The saponification is then complete and the mixture is neutralised with acetic acid and filtered. The filter-paper is washed with hot alcohol to ensure the complete removal of the cholesterin liberated in the hydrolysis. Digitonin solution is added to the clear filtrate and the precipitate formed is filtered, washed and weighed in the manner already described.

#### Estimation of Phosphatides.

Since the complete separation of the individual members of this group from one another has not yet been satisfactorily accomplished, even when large amounts, e.g., a kilogram and more, of dried brain have been used, it is obvious that it is not yet possible to estimate each phosphatide separately.

Koch/

Koch<sup>19</sup>, Koch and Woods<sup>20</sup> and Koch and Goodson<sup>21</sup> introduced methods for the estimation of lecithin and kephalin separately. The following is a description of their final and most satisfactory method. To an alcoholic solution of the phosphatides a sufficient amount - 5 c.c. as a rule - of hot saturated alcoholic solution of lead acetate is added. The mixture is heated on the water bath for ten minutes, 1 c.c. of 50% ammonium hydrate is then added, the whole vigorously shaken and allowed to remain on the water bath five minutes longer. The flask is set aside to cool and the lead compound of kephalin slowly separates out. It is filtered off and the phosphorus is then determined in the precipitate and in the filtrate. By allowing 4% as the proportion of phosphorus in both kephalin and lecithin it is possible to calculate the percentage of each of these substances present.

To obtain a knowledge of the total amount of phosphatide and of the lipid containing both phosphorus and sulphur, Koch determined the total phosphorus and the sulphur by fusing 5 gm of the dried tissue with sodium peroxide or "fusion mixture". The resulting mass was broken up and the sulphate determined in one portion and the phosphorus in another. By allowing one molecule of phosphorus for every two molecules of sulphur, protagon is calculated and the phosphorus/

phosphorus remaining represents the lecithin and kephalin - already estimated - and any other phosphatide not accounted for. By this method it is therefore possible to account for the total phosphorus, protagon, lecithin and kephalin and, by difference, any remaining phosphatide.

The method, however, is long and tedious and the results do not warrant the labour involved. It is usually sufficient, certainly, for all comparative purposes, considering our limited knowledge of the individual lipoids, to estimate the group together by determining the phosphorus content and then calculating the phosphatides on the assumption that 4% represents an average phosphorus value. This percentage is accurate for lecithin and kephalin, but for several other ill-defined, doubtful substances, notably sphingo-myelin, which is said by Rosenheim to be the phosphatide of protagon, it is too high. If sphingo-myelin and these other phosphatides exist in nerve tissue, then the total phosphatide estimations would invariably be too high; and since protagon forms 20% of the total lipoids of a nerve and one third of the protagon molecule is phosphatide, the error introduced would be considerable. There is good reason, however, to doubt the existence of sphingo-myelin as will be shown in the experimental section of this essay.

Neumann's/

Neumann's<sup>22</sup> method of determining the phosphorus of an organic substance is almost invariably employed.

About 0.2 gm. of the material to be analysed is weighed out and placed in a long-necked Kjeldahl flask and covered with 10 c.c. of fuming nitric acid. A brisk reaction ensues, copious fumes of nitrogen peroxide being evolved. After about five minutes the reaction slows down and the flask is then gently heated with a small Bunsen flame until all the brown fumes have disappeared. A mixture of equal parts of concentrated nitric and sulphuric acids is then slowly - 5 to 10 drops to the minute - run in, the flask meanwhile being gently heated. As a rule 5 c.c. of this mixture suffice, and when such an amount has been added, the flask is heated more strongly until white fumes of sulphuric acid appear. If the liquid in the flask remains clear or of a pale yellow tint after cooling, the reaction is complete. Water, about three times the volume of acid used, is now added and the flask again heated for five to ten minutes, allowed to cool, and 50 c.c. of a 50% solution of ammonium nitrate added. Now the mixture is heated until bubbles form (about 80°C) and 40 c.c. of a 10% solution of ammonium molybdate added; the flask is shaken vigorously for two minutes and then allowed to cool. A yellow precipitate separates out; the clear supernatant liquid is poured through an asbestos lined Gooch/

Gooch crucible and the precipitate washed with distilled water until the washings are no longer acid. As much as possible of the insoluble phosphorus compound is retained in the flask. Finally the precipitate which has been caught in the asbestos is washed back into the flask and a sufficient amount of  $\frac{N}{2}$  Na OH added to dissolve the yellow compound and leave an excess of alkali over. Ammonia is liberated in this reaction and it must be got rid of either by boiling or by the addition of neutral formaldehyde when urotropin is formed. The excess of alkali is titrated with standard half normal sulphuric acid. Every cubic centimetre of caustic soda used up is equivalent to 0.554 Milligramme of phosphorus.

#### Estimation of Cerebrosides.

Several methods have been devised but only two will be described here.

1. The method of Lorrain Smith and Mair is adapted to cases where comparatively large amounts e.g. 5 gm. of cerebrone are being estimated. About 1 gramme of the total lipoids is saponified for four hours with one-fifth normal methyl-alcoholic baryta; the mixture is evaporated to dryness and the residue extracted for 24 hours with boiling acetone. On cooling the cerebrosides separate out and are filtered off and weighed.
2. Koch's method<sup>23</sup>. It has been pointed out already that/

that cerebrosides yield galactose when hydrolysed with dilute hydrochloric acid. The method to be described is based upon this reaction.

The lipoids for analysis are gently heated for 20 hours with a 1% solution of HCl in a round bottomed flask. During the process of saponification a good deal of frothing occurs if the liquid be boiled too vigorously. Towards the end of the reaction globules of oil appear on the surface of the mixture. In order to facilitate filtration 20 to 30 c.c. of a saturated solution of sodium sulphate is poured into the flask and the mixture well shaken. By filtration the insoluble residue of organic matter is separated from the solution of galactose. To this clear filtrate, after neutralisation with caustic soda, 20 c.c. of freshly prepared Fehling's solution are added, and the mixture is then boiled.

The liquid should retain a deep blue colour, a tinge of green indicating that an insufficient amount of Fehling's solution has been added. By this process cuprous oxide is thrown down and it is now separated by filtration through a weighed asbestos-lined Gooch crucible. The cuprous oxide is converted to the cupric oxide by heat and the weight of the latter obtained. From this result the amount of cerebrosides hydrolysed is known by reference to Koch's table.

This/

This method has yielded consistently excellent results. Before using it in the experimental work to be described later, it was tested repeatedly with different weights of nerve tissue obtained from the same source and with specimens of protagon and cerebrone. In every case uniform results were obtained. The advantages of the method are:- (1) its simplicity; (2) it can be applied in the presence of all other lipoids; (3) a minimum of time is spent in drying the cupric oxide since the crucible is heated directly with the Bunsen flame. The disadvantage is that the weight of cupric oxide obtained is invariably small - from 6 to 89 milligrammes, generally 20 - being from  $\frac{1}{3}$  to  $\frac{1}{2}$  the weight of the cerebroside it represents; hence the percentage error is increased. But this is compensated for to some extent by very careful weighings and by using a light Gooch crucible, say 10 grammes.

#### Estimation of Protagon.

Noll showed that protagon can be estimated by the method described above for cerebroside. But this, obviously, only applies to pure protagon since the cerebroside also yield a sugar. Hence, in order to obtain the percentage amount of protagon in a tissue it is necessary to estimate the lipid sulphur and to calculate from this on the basis that protagon contains 0.73% of sulphur.

The/

The simplest method of determining the sulphur is to heat the lipid with sodium peroxide or "fusion mixture", by which the carbon is converted to  $\text{CO}_2$ , the hydrogen to  $\text{H}_2\text{O}$ , the phosphorus to phosphate, and the sulphur to sulphate. The resulting mass is dissolved in water and the sulphur precipitated as Barium Sulphate which is filtered off and weighed. From the weight obtained the amount of sulphur is readily calculated.

This method of estimating protagon in a mixture of lipoids depends upon the assumption that no other lipid contains sulphur. If Levene's recent work be subsequently verified this method will need modification. It is possible, however, that Noll's method may eventually be employed since it is by no means certain that any cerebroside, apart from that which is combined as protagon, exists in the nervous system. To decide this point it will be necessary to make a large number of analyses in which the sulphur content of the total lipoids of the brain is compared with the amount of galactose split off by the action of dilute hydrochloric acid.

Review of the Literature of the Chemistry  
of  
Nerve Degeneration.

Gutnikov<sup>25</sup> published in 1897 the first paper dealing with this subject. He studied the composition of the human brain, separating the grey from the white matter, in the foetus and in the adult, and in cases of certain nervous diseases. The methods of chemical examination available at that time were not sufficiently accurate to allow of a systematic estimation of the individual lipoids or other complex constituents of nerve tissue. Gutnikov, therefore, confined his studies to the percentages of water, phosphorus, nitrogen and sulphur in the conditions noted above. A comparison of the figures given in his paper with the more accurate ones obtained by elaborate analytical methods<sup>26,27,28</sup> show that his results are not sufficiently reliable to warrant a detailed description. He found wide variations in the percentages of phosphorus in the brains of foetus and adult, and a great diminution of the same element in such diseases as paralytic dementia, neurasthenia and epilepsy. A priori, it would appear improbable that such a decrease in the phosphorus percentage of the brain occurs in the so called functional diseases in which neither macroscopic nor microscopic/

microscopic changes have been observed. It is in such a disease as General Paralysis of the Insane that we should expect to find the most marked chemical differences from the normal. Reasoning in this way Barratt<sup>29</sup> made observations on the percentages of water and phosphorus in the brains and spinal cords of "normal" persons, and of persons who had died of General Paralysis. The water was determined by drying the tissue in an oven at 100°C. and then at room temperature in vacuo over sulphuric acid; the phosphorus by heating a weighed portion of the dry material with a mixture of caustic potash and potassium nitrate, and precipitating the phosphorus eventually as magnesium pyrophosphate. In these methods there are two sources of error: First, heating the nervous tissue to 100°C may lead to changes in composition with the production of volatile bodies, and therefore loss of substances other than water; Barratt noticed slight charring. Secondly, the method of estimating phosphorus does not give accurate results. The following table is taken from Barrett's paper.

	% of Water.	% of P.
Case I (Healthy Brain)	76.3	1.47
( G. P. I.	78.8-81.9	1.39-1.47
Case II (Healthy Brain)	73.6	1.47
( G. P. I.	74.1-77	1.28-1.47

From these results it appears that in General Paralysis of/

of the Insane there is an increase in the percentage of water and a decrease in that of phosphorus in the brain. The spinal cord showed similar changes.

There is one point which requires notice in connection with the above work: the phosphorus percentages given are for the total dry tissue and not for the lipoids alone; and since in the diseased conditions investigated one of the conspicuous microscopic changes observed is a perivascular lymphocyte infiltration and a general increased activity of connective tissue cells, it is obvious that coincident with these changes an increase of nucleo-proteins must occur. This means chemically that the percentage of phosphorus is raised. On the other hand, if the lipid phosphorus is diminished, as it probably is, we have two opposing factors represented in the above figures for the total phosphorus, and it is impossible therefore to draw any conclusion from them.

Koch<sup>21</sup> was unable to confirm completely Barratt's figures.

Noll<sup>24</sup> investigated the chemical changes in degenerated nerve by experimental means. He divided the sciatic nerves of horses and dogs and estimated in the degenerated and in healthy nerves the percentages of protagon and of phosphorus. His results show a diminution in the percentages of these substances.

Protagon/

Protagon was estimated by the method already described; phosphorus was determined by a method similar to that employed by Barratt. The following table is taken from Noll's paper:-

								Healthy	Protagon Degenerated.	% of Degenerated to Healthy.
Horse-sciatic nerve taken				8	days	after	section.	53.1	51.3	96.61
"	"	"	"	14	"	"	"	91.8	55.9	60.89
Dog	"	"	"	16	"	"	"	61.4	33.0	53.75
"	"	"	"	23	"	"	"	60.0	28.6	47.6
"	"	"	"	25	"	"	"	68.4	-	-

An examination of the two halves (lateral) of a spinal cord taken from a case of cerebral hemiplegia which had been followed by degeneration of the motor tracts of one side, revealed an increase in the percentage of water, an increase in the amount of material extractible by ether and a diminution in the lipid phosphorus on the degenerated side. Histologically, the motor tracts affected were stained by Marchi's method (Mott and Barratt<sup>30 & 31</sup>).

From the foregoing account of the literature up to 1899 it is seen that the main facts which had been brought forward were that in degeneration a nerve becomes more watery, loses some of its lipid phosphorus, and some of its protagon. Mott and Halliburton<sup>32,33,34,35</sup> associated these facts with the chemical constitution of lecithin/

lecithin~~s~~ considered it probable that in the disintegration of the myelin sheath of a nerve the lecithin is split up into glycerophosphoric acid, fats and choline, and that these products pass into the cerebrospinal fluid and the blood. They saw in this hypothesis a possibility of diagnosing nerve degeneration by the detection of one of the products of lecithin disintegration, choline being selected as the most promising substance on account of its basic nature and its well defined character.

Mott and Halliburton proceeded to test this hypothesis<sup>34,35,35B</sup> They studied first the chemical and physiological properties of choline and then proceeded to examine the cerebrospinal fluid and blood of healthy animals, and of animals in which artificial nerve degeneration was occurring.

Their chemical method of detecting choline was as follows:- the blood or cerebrospinal fluid was evaporated to dryness and the residue extracted with absolute alcohol. This was repeated three times in order to separate choline from potassium and sodium salts, which are sparingly soluble in absolute alcohol. The final absolute alcoholic solution was evaporated, the residue was taken up in 30% alcohol and platinum chloride added. In the cases where choline was present a copious precipitate of choline-platino chloride crystals/

crystals formed.

Choline was tested for physiologically by injecting the final solution obtained as described above into the blood stream of anaesthetised cats. When choline is present a fall of blood pressure occurs and this fall is abolished or replaced by a rise, if the animal is first poisoned with atropine.

As a result of numerous experiments Mott and Halliburton came to the conclusion that a positive result obtained by these two methods, chemical and physiological, affords an absolute proof of the presence of choline in the liquid under examination.

Applying these tests to cases of disease in human beings they found choline in 10 c.c. of blood taken from patients suffering with beri-beri, disseminated sclerosis, combined sclerosis and alcoholic neuritis. In each case a comparison was made with 10 c.c. of normal blood which yielded uniformly negative results.

In order to test the method under experimental conditions they severed the sciatic nerves of a series of cats, and tested the blood and cerebrospinal fluid at varying intervals after the operation. At the same time the divided nerves were examined chemically, the total solids and water, and the phosphorus of the lipoids - very roughly separated from the total solids - being estimated. The remarkable results obtained can best/

best be shown by the following table taken from Halliburton's Physiology (1907 edition).

Cat's sciatic nerves.						Percentage of Phosphorus in solids.	Condition of blood.	Condition of nerves.
	Water	Solids.						
Normal . . . . .	65.1	34.9		1.1)	Minimal traces of Choline present.	Nerves irritable and histologically healthy.		
1 to 3 days after section	64.5	35.5		0.9)	Choline more abundant.	Irritability lost; degeneration beginning.		
4 to 6 " " "	69.3	30.7		0.9( )	Choline abundant.	Degeneration well shown by Marchi reaction.		
8 " " "	68.2	31.8		0.5 )	Choline abundant.	Marchi reaction still seen, but absorption of degenerated fat has set in.		
10 " " "	70.7	29.3		0.3( )	Choline abundant.	Absorption of fat practically complete.		
13 " " "	71.3	28.7		0.2 )	Choline abundant.	Return of function nerves regenerated.		
25 to 27 " " "	72.1	27.9		traces )	Choline much less.			
29 " " "	72.5	27.5		0 ( )	Choline almost disappeared.			
44 " " "	72.6	27.4		0 )	Choline almost disappeared.			
100 to 106 " " "	66.2	3.8		0.9 ( )	Choline almost disappeared.			

This table shows that the amount of choline present in the blood under the conditions described was inversely proportionate to the amount of phosphorus in the solids.

The/

The criticism already offered to Barratt's work applies to some extent here also. Although Mott and Halliburton roughly separated the lipid from the non-lipoid phosphorus, the separation was not sufficiently complete to enable them to obtain figures for the phosphorus of lecithin alone; it follows therefore that some of the phosphorus of the nucleo-protein is included in the figures in column 3 of the above table. Considering the activity of the cells of the sheath of Schwann, one would expect an increase in the phosphorus of the nucleo-proteins. The complementary variations of columns 3 and 4 are therefore all the more remarkable.

The same observers found, by the same methods, choline in the blood and cerebrospinal fluid of persons suffering from general paralysis of the insane.

The importance of these observations was soon recognised both by clinicians and laboratory workers. From the standpoint of the clinician the easy identification of choline became of first-rate importance. Mott<sup>36</sup> published the results of a chemical examination of the blood in cases of Potts' disease, tabo-paralysis, disseminated sclerosis, amyotrophic lateral sclerosis, beri-beri, and acute alcoholic poisoning, in all of which choline was found to be present. So delicate was the chemical test proved to be that Mott found 5c.c. of/

of blood sufficient in any case and frequently very much less was required.

Folin and Schaffer<sup>37A</sup> made a large number of careful analyses of urines of healthy persons, and of patients suffering from nervous diseases and came to the conclusion that nothing can be gained from a study of the urine in cases of nerve degeneration.

Criticisms of the "choline-hypothesis," as it was now termed, were initiated by Vincent and Cramer<sup>37,38</sup> in this country, and by Mansfeld<sup>39</sup> on the Continent. It was pointed out by the first named observers that the contentions of Mott and Halliburton were "a priori improbable" and "scarcely conceivable" since choline in the blood is quickly oxidised and the amount liberated from 1 gramme of nerve tissue is extremely small. Further, they showed that the chemical test for choline is not reliable owing to the extreme difficulty of separating potassium, sodium, and ammonium salts from choline. If normal blood be dried and the residue extracted with absolute alcohol in the manner recommended by Mott and Halliburton<sup>34,35</sup> and finally the extract treated with platinum chloride, a precipitate of crystals is obtained which is as copious as that from an equal volume of pathological blood. With regard to the physiological test on the blood pressure of animals, they showed that the healthy blood of the rabbit/

rabbit has a similar and equal effect on blood pressure ~~to~~ that given by Mott and Halliburton for pathological blood.

It may here be noted that in 1896, three years before the publication of the choline hypothesis, Schäfer and Moore<sup>40</sup> noted a temporary lowering of blood pressure when extracts of brain were injected into animals. Schäfer and Vincent<sup>41,42</sup> made similar observations and Osborne and Vincent<sup>43</sup> showed that all parts of the nervous system produced similar effects.

Mansfeld<sup>39</sup> criticised the platino-chloride test for choline and after careful experiments arrived at the conclusion that it is not possible to free choline from potassium, sodium or "ammonium" by means of absolute alcohol.

Allen and French<sup>44</sup> also tested the platinum chloride method of detecting choline. They studied the solubilities of the platino-chlorides of choline, K.Na.NH<sub>4</sub>.Mg.Ca. & Fe. and came to the conclusion that it is impossible to separate choline absolutely from K. & NH<sub>4</sub>. Moreover when they applied the test to normal blood they obtained the crystals in as large amount as from pathological blood.

One point of special interest they brought forward, namely, that if the test be carried out as far as the fourth extraction with absolute alcohol, and the extract then obtained be incinerated so as completely/

completely to get rid of choline, then both with normal and pathological blood crystals of platino-chloride are obtained which are identical in appearance with those figured by Mott and Halliburton as being characteristic for choline from pathological blood. The crystals must necessarily be of inorganic origin and are almost certainly due to Potassium. This result really disposes absolutely of the platino chloride test for choline as applied in Mott and Halliburton's first publication<sup>34,35</sup>.

The following year, 1904, Allen<sup>45</sup> published a new method of testing for choline in cerebrospinal fluid and in blood. 10-20c.c. of the freshly drawn fluid are mixed with 4-6 times the volume of 96% alcohol, allowed to stand for two hours, filtered, and the filtrate allowed to evaporate to dryness at 30-40°C. The residue is extracted three times with 99.6% alcohol and then dialysed in distilled water. The resultant clear solution is evaporated to dryness in a small porcelain crucible and a drop of distilled water is added to the residue, and then, drop by drop, a saturated solution of Iodine in 30% alcohol. If choline be present, a reddish brown precipitation or colouration appears; it quickly disappears on standing or on adding excess of the iodine solution.

When/

When this method was applied to normal blood a negative result was obtained; on adding .001 gm. of choline to 20 c.c. of blood the result was positive. Applied to pathological fluids Allen obtained positive results in two cases of G. P. I. with convulsions; a case of very acute diphtheritic paralysis of four months duration; in a case of cerebral syphilis; one of complete transverse myelitis, and in a case where the brachial plexus had been severed, the blood in the last case being tested 17 and 33 days after the accident.

In the following cases a negative result was obtained in each case:- myesthesia gravis, posterior basal meningitis, transverse myelitis, and a case of doubtful diagnosis, but probably either tumour or meningitis.

With regard to cerebrospinal fluid Gumprecht<sup>46</sup> claimed to have shown by a microchemical test that choline is present in the normal fluid.

The evidence, chemical and physiological, for the "choline hypothesis" had now become conflicting. The supporters of the hypothesis endeavoured to find new tests for choline, and to improve the old ones. Foremost among the workers in this direction were Rosenheim<sup>46A, 46B</sup> and Donath<sup>46</sup>, each of whom claimed to have discovered exceedingly delicate tests. The first named, /

named, after a careful examination of the platino chloride test found that under <sup>the</sup> conditions of Mott and Halliburton's work potassium and sodium platino-chloride compounds were a source of error. He claimed that the three following tests were absolutely reliable.

1. By the addition of iodine (dissolved in potassium iodide solution) to the crystals of choline platino chloride, a per-iodide of choline is formed. This compound exists as doubly refracting crystals, which spontaneously decompose on exposure to the air, and which may be obtained again by the addition of the reagent. Its formation can be watched under the microscope, the appearances presented being, it was claimed, absolutely distinctive. The test was not new; the references are given in Rosenheim's paper.
2. A drop of a 1% solution of choline hydrochloride to 2 or 3 drops of a saturated solution of alloxan give on evaporation on the water bath a beautiful rose violet colour, which becomes deep blue or violet on the addition of caustic soda; mineral acids discharge the colour. Since, however, protein and ammonium salts give the same reaction, the same objection is applicable to this test as to Halliburton's original one. The test was first employed by Paehl<sup>47</sup>.
3. The Bismuth Test. Kraut's modification of Dragendorff's test was employed and found to be sufficiently/

sufficiently sensitive to detect one part of choline in 20,000 parts of water. In this test protein must not be present.

These three tests are each sufficiently delicate to enable one to detect the presence of choline in 20 c.c. of a solution of 1 in 20,000.

Donath's<sup>48</sup> method depends upon the use of the polarising microscope. When a mixture of the crystals of the platino-chlorides of choline, potassium, Na and  $\text{NH}_4$  are examined with the polarising microscope the crystals of the choline platino-chloride compound remain bright in the dark field, while those of Na, K and  $\text{NH}_4$  become invisible. By the application of this test Donath discovered choline in the cerebrospinal fluid of four cases out of six of epilepsy, the fluid being examined immediately after convulsions had occurred. He also found that choline is present in the cerebrospinal fluid in cases of "hystero-epilepsy" and states that in general where there is organic disease of the nervous system choline may be detected in the blood and cerebrospinal fluid, and that there exists "a certain degree of parallelism in the occurrence of lymphocytes, Protein, choline, and phosphoric acid in the cerebrospinal fluid."

In another paper<sup>49</sup> Donath determined the amount of choline in the cerebrospinal fluid of patients. His results/

results indicate the presence of 20 to 40 milligrammes of choline in 100 c.c. of cerebrospinal fluid, and this was in cases, among others, of epilepsy and neurasthenia.

With regard to the application of his test Donath found that K,Na and  $\text{NH}_4$  had no influence on his results. In the latter part of his work he found that it facilitated the detection of choline to add KCL to his solutions.

Rosenheim<sup>46A</sup> applied his more delicate tests (described above) to the investigation of the cerebrospinal fluid in cases of nervous disease. He examined 15 cases of the pathological fluid. In four cases of G.P.I. and in one case of hemorrhage into the internal capsule with softening around, choline was discovered in the cerebrospinal fluid; it was absent in a case of post-basis meningitis, in tubercular meningitis, in three cases of G.P.I., two of melancholia, two of dementia and one of shingles.

In addition to the foregoing he examined the blood in four cases of nervous disease; in each case the blood was removed during life. Choline was not detectable in 20c.c. of blood from a case of dementia with hemiplegia of six months standing nor from cases of G.P.I. in the last stages; it was discovered in the blood of a case of "Organic Dementia" accompanied by seizures."

Rosenheim concludes that this single positive result/

result is sufficient to confirm Mott and Halliburton in their contention that the products of nerve degeneration pass into the blood. It is perhaps unnecessary to point out that Vincent and Cramer and Mansfeld had not expressed any doubt as to the possibility that these products pass in-to the blood, but they pointed out that it is very improbable that the products are detectable in the blood.

In addition to the crystals of choline salts Rosenheim obtained others of a nature which pointed to the existence of other bases besides choline, K, Na, and  $\text{NH}_4$ . He failed to identify these substances owing to the paucity of material.

Another observation, of greater importance, was that in all cases where choline was demonstrated in the cerebrospinal fluid, large amounts of potassium could be obtained from the alcoholic extract; on the other hand where choline was absent potassium was scanty. From this fact he draws the following conclusion "The large total yield of crystals from the blood in such cases stands in striking contrast to the small yield obtained from normal blood, or the blood of patients in which no disintegration of nervous tissues had occurred. In the light of recent work there is no doubt that such crystals do not consist entirely of the choline platino chloride as at first Halliburton and Mott considered to be the case. In some/

some of their preparations Halliburton has found that many of the crystals do not yield the characteristic choline periodide, and so presumably they consist of inorganic and especially potassium platino-chloride. A large yield of crystals is nevertheless characteristic of extensive nervous disintegration."

Finally Rosenheim concludes;- "The methods employed by Halliburton and Mott are not quite so conclusive as those I have introduced; now, however, that we are provided with absolutely trustworthy methods, their original conclusions may be regarded as definitely established."

These results of Rosenheim and Donath, and of Allen in his second paper were regarded by Halliburton as finally establishing the "choline hypothesis." In the Oliver Sharpey Lectures for 1907 <sup>18, 19, 20</sup> ~~(35A)~~ Halliburton reviews the whole question and its experimental foundations. He admits that his first chemical tests were fallacious, but considers that the work of Rosenheim and Donath has placed the matter on a sure foundation. In spite of this belief in Rosenheim's and Donath's work, in his final conclusion he places more reliance upon an increase of potassium salts than upon the presence of choline in cerebrospinal fluid. This is shown in the following quotation:- "We still hold that the obtaining of a large crop of crystals, whether they be those of the choline salt or a mixture of potassium/

potassium and choline salts, is diagnostic of extensive breakdown in nervous tissue. The contrast between such cases and the insignificant yield from normal blood is most striking." This position, however, has been contested by Webster<sup>50</sup> who points out that "if the presence of potassium in the blood leads to the formation of the crystals, it is strange that normal blood serum, which contains a considerable amount of potassium, should give an insignificant yield of crystals." But the central point of the question is this, that the physiological mechanism by which the composition of the blood and other body fluids is kept approximately constant would prevent such an increase of potassium salts as is indicated by the quotation above. As soon as an excess of potassium salts appears in the blood, the kidneys, if active, remove it. It therefore follows that the improbability of pathological blood or cerebrospinal fluids yielding a large excess of potassium platino-chloride crystals, is as great as it was shown to be for the choline crystals; and the experimental work of Webster confirms this. He has been unable to obtain any such difference as that insisted upon by Halliburton, and he shows that if 1 gm. of nerve tissue were suddenly destroyed in man only .0017G. of  $K_2O$  would be added to the .9gm. of  $K_2O$  already in the blood. And this difference/

difference is smaller than that given by Aberhalden in his careful analyses of blood obtained from two animals of the same species. But the sudden destruction of a gramme of nervous tissue, followed by the accumulation of the potassium oxide liberated in the blood and cerebrospinal fluid is not conceivable. We may therefore conclude that no such excess of potassium is demonstrable.

In the same paper Webster finally disposes of the "choline hypothesis" both by experimental work and by a simple calculation. He repeated all the original work of Mott and Halliburton, using in his experiments seven dogs and one cat. The sciatic nerves were cut and a piece removed, and the blood was examined a variable number of days after the operation and tested by the chemical and physiological methods previously described. The results were compared with those obtained from normal blood and it was shown that with the pathological blood there is neither an increase in the yield of platino-chloride crystals nor a progressive increase in the depressor effect on the blood pressure of animals such as was described in the original paper of Mott and Halliburton. Webster did not test the cerebrospinal fluid because choline is found in the healthy fluid and this fact would render the comparison of the normal and pathological a matter of quantitative/

quantitative estimation which is obviously more difficult than the mere qualitative examination of the blood.

The following is Webster's calculation:-

The weight of the central nervous system is 1400gms. the weight of choline contained therein, according to Coriat's observations, is 8.5 gms. Allowing the volume of blood to be 4,700c.c. it follows that the sudden destruction of 1 gm. of brain substance would yield  $\frac{8.5}{1400} \times \frac{20}{4,700} = .000025$  gm. in 20 c.c. of blood, or a dilution of 1 in 800,000. This is obviously beyond the delicacy of any of the methods already mentioned, and moreover it presupposes the sudden destruction of a gramme of brain followed by complete accumulation in the blood, which is inconceivable.

If this method be applied to the results obtained by Halliburton for cerebrospinal fluid, it can be shown that the amount of choline found by this observer corresponds to the sudden destruction of from 3 to 15 grammes of nervous tissue. If the method be applied to Donath's figures<sup>49</sup> one is driven to the conclusion that in several cases of epilepsy and neurasthenia 3 to 7 grammes of the brain were suddenly destroyed.

All this is of course impossible and it is simply a waste of labour to pursue the question further in its details. As a summary it may be said that the experimental/

experimental basis of the belief that choline may be detected in the blood and cerebrospinal fluid in cases of nerve degeneration has been proved to be fallacious, and that the contention that these pathological fluids give an increased yield of platino-chloride crystals has been disproved. The whole controversy, lasting ten years, has not brought to light many new facts and has been of no practical value. The final position of one of the originators of the hypothesis is shown in the following paragraph taken from the 1911 edition of Halliburton's physiology.

The cerebrospinal fluid contains an excess of protein which is mainly nucleo-protein; cholesterin can usually be detected in the fluid "and so also can choline or some similar base which originates from the decomposition of the phosphatides."

"It must be admitted that the tests hitherto devised for the detection of choline are not absolutely conclusive, for sufficient of the base cannot be collected for a complete analysis. The base which is present, if not choline, is a nearly related substance, perhaps a derivative of choline, and according to the latest researches the questionable material is trimethylamine. . . ."

Kaufmann<sup>51</sup> collected a litre of cerebrospinal fluid from cases of nervous disease and was unable to isolate choline.

of/

Of other researches bearing upon the subject of this paper those of Koch rank amongst the most important. This investigator devoted himself especially to the work of clearing up such difficult questions as the composition and characteristics of the cerebrosides, the isolation of sulphatides, and to devising new and more accurate methods of analysis. In connection with his work there is one criticism which may be made, and it is that he aimed at obtaining detailed analytical results far beyond the limits of usefulness, imposed upon us by our present meagre knowledge of the individual lipoids and proteins. His laborious estimations of the proteins, of lecithins and lecithans, and of the sulphur content of very numerous extracts of the brain are valueless owing to our ignorance of the exact chemical relationships of these substances. At present it is not necessary to go beyond group estimations in respect of the phosphorised fats and the galactose-yielding lipoids. Apart from these defects the work of Koch stands out conspicuously in the literature of the lipoids of the last eight years.

Of his many results the following are the most important.

Two analyses of the brain of an epileptic who had died during a fit were made.<sup>52</sup> The following figures, modified/

modified by adding together the values given for each member of the class of phosphatides and of the cerebrosides, are given in the paper.

Chemical composition of the Human Brain.  
(Epileptic)

	Corpus Callosum.	Cortex. (Prefrontal)
Water	67.97	84.15
Proteins	9.6	8.4
Extractive	1.57	1.58
Inorganic Salts	.82	.87
Phosphatides	8.68	3.88
Cerebrosides	4.57	1.55
Cholesterin	4.86	.7
Sulphur Compound	<u>1.40</u> 99.41	<u>1.45</u> 102.56

The value for the sulphur compound is obtained by determining the amount of lipid sulphur and then assuming that the sulphur constitutes 4% of the compound. There are no good grounds for doing this.

It is to be noted that in several instances the values obtained are "by difference."

When these figures are compared <sup>with</sup> ~~to~~ those given by Lorrain Smith and Mair, it will be found that there is a close approximation. Thus for the cholesterin of white matter the figure of the last observers is 4.7; Koch gives 4.86. Against Koch's 4.57 Lorrain Smith and/

and Mair put 4.1 for cerebrosides; for phosphatides the comparison is 8.68 to 7.6. In giving these figures it should be pointed out that Lorrain Smith and Mair do not give the % of water, and to make the comparison it has been assumed that Koch's %, 67.97 is accurate.

Among the final conclusions given in this paper the following is perhaps the most important and the most interesting:- "Grey matter, therefore, has a very simple composition consisting of a mass of proteins, lecithin, and the sulphur compound. This result needs further confirmation by other analyses. . ." The method of arriving at this conclusion is ingenious and is probably fairly accurate. In the general results given in the preceding table it will be noticed that the figures for the cerebrosides of the cortex are low; this has been confirmed by other observers.<sup>26,27,28.</sup> It was shown by Noll that cerebrosides exist only in the myelin sheath; if this be accepted as accurate the % of cerebrosides obtained in an analysis of the grey matter can be allowed to represent the unavoidable admixture of white matter with grey, obtained in cutting thin slices of the cortex. Then calculating on this basis the amounts of cholesterin and phosphatide and other constituents introduced as impurities, and subtracting them from the figures for the cortex, there results/

results the true composition of the grey matter. This method depends upon the observation of Noll which requires confirmation.

However interesting these observations may be, they do not directly throw much light on the chemical changes undergone by a nerve during degeneration. They serve to show that the methods, introduced or modified by Koch, are capable of yielding very reliable figures. But their application to the examination of an epileptic's brain is not of first rate importance, and this was felt by Koch for the following year he, along with Goodson,<sup>53</sup> attempted to trace the chemical changes which take place in the spinal cord and sciatic nerves of a dog after section.

The general results obtained showed that in degeneration a nerve becomes more watery, and that there is a slight decrease in the percentages of phosphorus and cerebrosides. This does not confirm the older observations which all showed a great diminution of these last-mentioned substances.

Koch and Goodson further examined the brains of persons who had died of general paralysis of the insane, and their figures for the percentages of the individual lipoids show a general resemblance to those obtained for the artificially degenerated cords of dogs. In other words they showed that chemically, there/

there are no, or very slight, differences between degeneration of nerve tissue due to disease and degeneration following section.

In recent years several attempts have been made to throw light on the causes of more obscure forms of insanity than general paralysis by chemical means. In two cases of Amaurotic Dementia Mott<sup>54</sup> found a large diminution of the nucleo-proteins and a large increase of the simple proteins of the brain, while Koch<sup>55</sup> found in five cases of primary dementia that in the cortical grey matter of the brain there is a great decrease of lipoid sulphur and a perceptible decrease of lecithins and lecithans.

Before any great progress can be made in the study of pathological degenerations of the nervous system, it is necessary to know definitely the composition of the lipoids of the white and grey matter of the normal brain at different ages. This fundamental knowledge has now been supplied by the researches of Koch and Mann,<sup>56</sup> and Lorrain Smith and Mair.<sup>26,27,28,32</sup> The last named observers have made a large number of analyses by methods which are an improvement in most respects on those of previous workers, and their figures are probably the most accurate yet obtained. It now remains to study by the same means the varied pathological/

pathological material exhibited<sup>ing</sup> ~~ed~~ by degenerative changes, with experimental studies as a comparison. This cannot be carried out until the discrepancies in the experimental work of previous observers have been cleared up. This has been attempted by the author, as will be shown in the next section.

EXPERIMENTAL.Introduction.

The objects of this work were (a) to determine accurately the changes in the composition of the lipoids in nerve degeneration; (b) to study the Marchi histological method; and (c) to examine the clearing points of the doubly refracting substances of the myelin sheath.

In the work dealing with the Marchi reaction I have had the advantage of collaborating with Dr. Cramer and Dr. Feiss, to whom I wish here to express my thanks for their kind permission to make use of some of our joint results.

The material employed was obtained from cats. In most cases one sciatic nerve was divided, the healthy one being used as a control; in two of the animals the spinal cord was divided in the ~~upper~~ dorsal region.

At the appropriate period - generally 18 days - after operation the cat was killed with chloroform and the nerve or cord dissected out and freed, in the one case from connective tissue and fat, and in the other from the membranes. This was carried out as quickly as possible and the moist tissue was rapidly weighed. After drying the tissue was again weighed and the % of water calculated.

The dried material was extracted in the Soxhlet apparatus/

apparatus for seven hours with chloroform at 30°-50°C; during this time the chloroform circulated 35 to 40 times.

In each case the solvent was evaporated in a stream of coal gas to avoid oxidation.

The total lipoids were dissolved in chloroform and the solution made up to 100 c.c. From this aliquot portions were removed for analysis.

Cholesterin was estimated by Windau's<sup>5</sup> method, phosphorus by Neubraun's, and cerebrosides by Koch's.

The tissues were systematically examined by Marchi's method in order to determine the existence or non-existence of degeneration.

The following table shows the results obtained for the normal cord:-

	Water	Lipoids		Cholesterin		Cholesters	Phosphatides		Cerebrosides.	
		% of dry tissue.	% of lip-oids.	% of lip-oids.	% of dry.		% of lip-oids.	% of dry.	% of lip-oids.	% of dry.
Cat 1.	64.2	69.6	24.2	16.8	Trace	57.1	39.7	18.3	12.7	
Cat 2.	-	67.5	25.8	17.4	Trace	57.8	39.0	15.7	12.7	
Mixture of 3 normal cords	-	68.9	24.0	16.9	Trace	57.5	40.6	17.9	12.7	
Average		68.66	24.66	17.0		57.5	39.8	17.3	12.0	

Average % of lipoids accounted for = 99.4

Details of the analysis of the mixture of three cords/

records are given below as an example of the quantities dealt with.

Weight of Dry Tissue = 1.2822 gms.

" " Lipoids Extracted = 0.9036 "

∴ % of lipoids = 68.9. made up to 100c.c.

A. Cholesterin Estimation: (1) 10c.c. gave 0.0866 gm. of cholesterin digitonate.

Hence  $\times 10 \& \div 4 = 0.2165$  gm. of cholesterin.

∴ % of total lipoids =  $\frac{.2165 \times 100}{.9036} = 23.9\%$

% of and/dry tissue =  $\frac{.2165 \times 100}{1.2822} = 16.9\%$

(2) 10 c.c. gave 0.0870 gm.

of cholesterin-digitonate.

By the same calculation we obtain the following percentages:- 24.1 and 16.9.

B. Phosphatides.

(1) 10 c.c. required 3.8 c.c. of  $\frac{N}{2}$  Na OH.

Hence  $3.8 \times 1.268 \times .437 \times \frac{100}{4} \div 10 = .52641$  gm. of Phosphatides.

∴ % of total lipoids = 58.3

and % of dry tissue = 41.1

(2) 10 c.c. used up 3.7 c.c. of  $\frac{N}{2}$  Na OH

This gives 56.7% and 40.0%.

C. Cerebrosides.

50 c.c. of the chloroform solution were hydrolysed with 10 c.c. of 1% hydrochloric acid for 24 hours. The galactose split off was estimated with Fehling's solution/

solution.

Weight cupric oxide obtained = 33.2mgm.

This represents 81 mgms of cerebroside. 109.1 mg Ph.

Hence the weight of cerebroside in 0.9036 gm.

of lipoid = .1620 gm. 2182

the % of cerebrosides in the total lipoid = 17.9

and % " " " " dry tissue = 12.7.

The results obtained for the degenerated cords are shown in the table below:-

	Water.	Lipoids.		Cholesterin.		Cholesters.	Phosphatides.		Cerebrosides.	
		% of dry tissue.	% of lipoids.	% of dry tissue	% of lipoids.		% of dry tissue	% of lipoids.	% of dry tissue	
17 days after Section (lower dorsal)	66.2	74.5	22.8	17.1	0	47.9	35.9	10.9	8.2	
18 days after Section (Upper dorsal)	-	63.8	25.2	16.1	0	50.0	31.9	12.1	7.7	
Average		69.2	24.0	16.6	-	48.95	33.9	11.5	7.95	

\* These figures are too low - see later.

Histologically, both cords showed degeneration.

Details of the analysis of the first degenerated cord:-

Weight of Fresh cord = 3.5800 gms.

" " Dry " = 1.3118 "

∴ % of Water = 66.2

Weight of Lipoids = 0.9820

∴ % of Lipoids = 74.5

A. Cholesterin.

(a)/

(1) 20 c.c. gave 0.1790 gm. of Cholesterin digitonate.

Hence  $x \frac{5}{4} = .22375$  gm. of cholesterin.

∴ the % of total lipoids = 22.8

and " " dry tissue = 17.1

(2) 5 c.c. gave 0.0448 gm. of cholesterin digitonate

which, by calculation, gives the same percentages.

#### B. Phosphatides.

(1) 10 c.c. required 3.4 c.c.  $\frac{N}{2}$  Na OH.

Hence  $3.4 \times 1.268 \times .437 \times \frac{100}{4} \times 10 = 0.4710$  gm.

∴ % of total lipoids = 47.9

and % of dry tissue = 35.9

(2) 10 c.c. used up 3.4 c.c.  $\frac{N}{2}$  Na OH.

#### C. Cerebrosides.

30. c.c. gave 12 mgm. of cupric oxide.

This is equivalent to 32.2 mgm. of cerebrosides

$\times \frac{100}{30} = 0.10733$  gm. of cerebrosides.

∴ the percentages are 10.9 and 8.2.

In this estimation a trace of cuprous oxide formed so fine a suspension in the liquid to be filtered as to enable it to pass through the filter. The result therefore is low.

From the analyses thus detailed it will be observed in the first place, that the whole of the lipoids of the normal spinal cord are accounted for. The only possibility of error in the methods employed lies in the assumption made that the lipid phosphorus forms 4% of the total phosphatides. This is true of lecithin but/

but how far it is true for the remaining phosphatides has not yet been determined. But the results given above form a good indication of the probable accuracy of the assumption.

There are no records in the literature where the total lipoids have been fully accounted for, without the use of a "by difference" result.

In the normal cord, also, it is shown that there is no free fat since this, if present, would be extracted by the chloroform, and that the total extract is made up of the lipoids alone. But in the degenerated cord only 81.6% of the chloroform extract consists of lipoids; it follows that the remaining 18.4% is non-phosphorised fat. It may be due to any of the saturated or unsaturated fats, to the fatty acids, or to organic derivatives - eg. choline oleate - of the fatty acids. There is little doubt, however, as will be shown later, that it consists almost exclusively of fat. We thus arrive at the conclusion that in the process of degeneration there is a production of fat. The relation this bears to Marchi's <sup>histological</sup> method will be dealt with in the next section.

The figures obtained confirm previous observers in the following points; there is a diminution in the percentages of phosphatides and cerebrosides; but the great reduction in these substances given by Gutnikov, Mott and Barratt, and Noll is not confirmed.

In/

In addition it is shown that the relative proportions of the lipoids to one another are well maintained in degeneration; the cerebrosides diminish rather more rapidly than the other lipoids.

Finally, the cholesterin is least of all affected. On the whole the figures point to a slight diminution.

The following tables show the composition of three pairs of healthy and three pairs of degenerated sciatic nerves.

#### I. Healthy Nerves.

	Water.	Lipoids.	Cholesterin.		Phosphatides.		Cerebrosides.	
			% of dry Nerves.	% of lip-oids.	% of dry nerves.	% of lip-oids.	% of dry nerves.	% of lip-oids.
1st pair	58.1	65	10.8	7.0	31.3	20.4	17.3	11.3
2nd pair	62.0	62	10.0	6.2	33.0	20.5	16.0	9.9
3rd pair	60.0	63	11.1	7.0	31.8	20.0	16.9	10.6
Average		63	10.6	6.7	32.0	20.3	16.7	10.6

#### II. Degenerated Nerves.

1st pair (18 days)	70.1	65%	8.3	5.4	27.2	17.7	9.4	6.1
2nd pair (18 days)	72.0	65.8%	8.1	5.3	28.0	18.4	9.1	6.0
3rd pair (18 days)	72.0	64.0%	8.5	5.4	27.6	17.6	10.0	6.4
Average	71.4	64.9	8.3	5.4	27.6	17.9	9.5	6.2

As/

As an example of the weights manipulated, details of the first set of analyses are given below.

I. The normal sciatics are taken first.

Weight of fresh nerves = 1.2490.

" " dry " = .5240 ∴ Water = 58.1%

" " Lipoids = .3410 ∴ Lipoids = 65%

They were made up to 70 c.c.

A. Cholesterin.

10 c.c. gave .0210 gm. of cholesterin digitonate

$\times \frac{7}{4} = .03675$  and % = 10.8 of lipoids

= 7.0 of dry nerves.

B. Phosphatides.

10 c.c. used up 1.1 c.c. of  $\frac{N}{2}$  Na OH.

And  $1.1 \times 1.268 \times .437 \cdot \frac{100}{4} \times u = 106.66733$  mgm of Phosphatides.

Hence % of total lipoids = 31.3

and " " dry nerves = 20.4

C. Cerebrosides.

30 c.c. gave 9 mgm CnO

This is equivalent to 25.3 mgm cerebrosides.

$.0253 \times \frac{7}{3} = .05903$ .

Hence the % of cerebroside in total lipid = 17.3

and " " " " " dry tissue = 11.3

II Degenerated Nerves.

Weight of Fresh Nerves = 2.0950

" " Dry " = .6266 ∴ water = 70.1%

" " Lipoids = .4070 ∴ Lipoids = 65.0%

A./

A. Cholesterin.

10 c.c. gave .0136 gm. of cholesterin digitonate  
 $x \frac{10}{4} = .034$  gm. of cholesterin.

Hence the % of total lipoids = 8.3

and " " " dry tissue = 5.4

B. Phosphatides.

3 analyses gave .9, .7, and .8 c.c.  $\frac{N}{2}$  Na Oh for 10c.c.

Hence  $.8 x .554116 x \frac{100}{4} x 10 = .1108232$  gm of  
 Phosphatides

and the % of total lipoids = 27.2

and " " " dry tissue = 17.7.

C. Cerebrosides.

40c.c. gave 5 mgm of CuO and this is equivalent to  
 15.3 mgm of cerebrosides.

Hence the % of cerebrosides of the total lipoids = 9.4

and " " " " " dry tissue = 6.1.

One's attention is first drawn to the fact that the analysis of the lipoids of a peripheral nerve accounts only for 59.3% of the chloroform extract of the normal and 45.4% of the degenerated nerve. This is due to the large amount of fat which is found in the connective tissue of the peripheral nerves. This unaccounted for portion is increased in degeneration and, as in the case of the spinal cord, the increase is due to a production of fat in the disintegration of the myelin sheath.

It is clearly shown by the table that the nerve becomes more watery and that the phosphorus and cerebrosides/

cerebrosides are diminished, thus confirming Gutnikov, Noll and Barratt. In addition to the new facts are added (a) cholesterol is also diminished and (b) the cerebrosides disappear more rapidly than either cholesterol or the phosphatides.

The results in their general meaning agree with those detailed in the paragraphs dealing with the spinal cord.

By adopting the assumption that the grey matter of the central nervous system is devoid of cerebrin, it is possible, by applying the figures for normal and degenerated sciatic nerves to those of the cord, to calculate the composition of grey and white matter separately. Thus in the normal nerve we arrive at the relative proportions of the lipoids to one another in the myelin sheath, namely:-

Cholesterol 10.8

Phosphatides 31.3

Cerebrosides 17.3.

In the healthy spinal cord the relations are:-

Cholesterol 24.2

Phosphatides 57.1

Cerebrosides 18.3.

Assuming that the whole of the cerebrosides are contained in the myelin sheath, and that the composition of this is the same in the peripheral nerves as in the central nervous system, we have as the relative composition/

composition of the white matter.

Cerebrosides - 18.3

Cholesterin  $\frac{10.8 \times 18.3}{17.3} = 11.4$

Phosphatides  $\frac{31.3 \times 18.3}{17.3} = 33.1.$

Hence the lipoid# of the grey matter is composed of cholesterin and phosphatides in the proportions:-

Cholesterin = 24.2 - 11.4 = 12.8

Phosphatides = 57.1 - 33.1 = 24.0.

To derive corresponding figures for the white and grey matter of the cord is a matter of greater difficulty since variable proportions of the nerve fibres are degenerated. If we assume that one half the fibres remain healthy and one half degenerate after complete section of the cord, we get the following figures:-

	<u>Normal</u> <u>Sciatic.</u>	<u>Degenerated</u> <u>Sciatic.</u>	<u>Degenerated</u> <u>Cord.</u>
Cholesterin	10.8	8.3	22.8
Phosphatides	31.3	27.2	47.9
Cerebrosides	17.3	9.4	10.9

Hence the white matter of the degenerated cord has the relative composition:

Calculated on the assumption that all the nerve fibres are degenerated:-

Cholesterin  $\frac{8.3 \times 10.9}{8.3} = 9.7$

Phosphatide  $\frac{27.2 \times 10.9}{8.3} = 31.5$

Cerebroside - 10.9

Calculated/

Calculated on the assumption that none is degenerated:-

$$\text{Cholesterin} = \frac{10.8 \times 10.9}{17.3} = 6.4$$

$$\text{Phosphatide} = \frac{31.3 \times 10.9}{17.3} = 9.6$$

$$\text{Cerebroside} \quad - \quad 10.9$$

And the average composition of the white matter of

the Degenerated cord is:-

For the Normal cord;-

Cholesterin - 8 11.4

Phosphatide - 25.6 33.1

Cerebroside - 30.9 18.3

The average for the grey matter:-

Cholesterin -  $22.8 - 8 = 14.8$  12.8

Phosphatide -  $47.9 - 25.6 = 22.3$  24.0

Considering the number of assumptions made and especially that relating to the proportion of nerve fibres degenerated in the cord, it is impossible to draw any trustworthy conclusions. It is interesting to note, however, that there is little, if any, alteration in the composition of the lipoids of the cells; if we accept the figures as being accurate enough to found any conclusions upon, we are compelled to believe that the cholesterin is not diminished while the phosphatides are. The grounds are too slender to relate this to the changes of so called "secondary degeneration."

Whilst the work described above was being carried out the micro-chemical and histological investigation of Marchi's reaction was being pursued. It had been discovered/

discovered by Dr. Cramer and Dr. Feiss, a year before, that cold acetone dissolves out of a degenerated nerve the substance which is stained black by osmic acid after prolonged treatment with potassium bichromate. This fact was at once correlated with the observation noted above, that fat is produced in the degenerating myelin sheath, and an attempt was made to isolate the material in question. Since phosphatides and cerebrosides are difficultly soluble in cold acetone, a promising method at once presented itself. The dry nerve tissue was extracted with cold acetone in order to extract the fats and cholesterin. The residue was then extracted with cold ether and finally with hot chloroform and the three extracts were examined separately.

The primary object of the work was not realised but this, in the end, was of little importance owing to results obtained by other means to be described later. The reasons for this failure are that cold acetone, by repeated (eg. 10 times) extraction, removes the cholesterin and fat only partially, and at the same time takes up small amounts of the other lipoids. There arises this difficulty, therefore, that in order to extract the bulk of the fat and cholesterin with cold acetone, it is necessary to shake the tissue with acetone so frequently as to remove an appreciable proportion of cerebrone and lecithin. This result is in accordance with the well known fact that the solubility of any lipid is modified/

modified by the presence of another lipoid.

When equal weights of healthy and degenerated sciatic nerves were shaken ten times under identical conditions, with equal volumes of cold acetone, 43% by weight of the dry degenerated nerves and 33% of the healthy nerves were extracted.

The acidity of the acetone extracts was tested and no difference was found between the normal and abnormal nerves. Hence we may conclude that the fat produced is neutral.

Tested for sulphur the extract of the normal nerves gave a negative result, while that of the degenerated nerve was found to contain sulphur. It becomes evident, therefore, that the protagon of the myelin sheath is broken up in such a way as to yield a simpler lipoid soluble in acetone and containing sulphur.

The nitrogen of the acetone extracts was determined by Kjeldahl's method and no difference was found between the normal and abnormal nerves. From this we may conclude that choline is not retained in the nerve in a form soluble in cold acetone.

With regard to the ether and chloroform extracts, nothing of importance was noted. The ether contained a large proportion of the phosphatides and smaller amounts of cholesterin and cerebrone.

The same methods applied to normal and degenerated spinal/

spinal cords yielded substantially the same results. The only important point of difference was that the acetone extract of the normal cord contained no fat. The amount of fat which may be obtained from a degenerated cord is too small to isolate.

MARCHI'S HISTOLOGICAL METHOD.

By the use of Marchi's method we have a striking demonstration of the altered chemical composition of the degenerated myelin sheath. If a normal or degenerated nerve be treated with osmic acid ( $O_8O_4$ ) the myelin sheath is blackened, uniformly in the case of the healthy nerve, irregularly in the case of the degenerated. If now the nerves be first treated with a solution of potassium dichromate at room temperature for 14 days, or at  $37^{\circ}C$  for 7 days, and then with osmic acid, only the degenerated nerve is blackened.

In Wallerian degeneration there are three essential changes, fragmentation of the axis cylinder; breaking up of the myelin sheath; and multiplication of the nuclei of the sheath of Schwann. The exact condition found depends upon the period of degeneration.

These changes have been studied from the morphological standpoint by numerous observers, but the only researches bearing upon the chemical basis of the Marchi method are those of Lorrain Smith<sup>56A</sup> on Weigert's stain.

This observer introduced a very simple and neat method of studying the reactions between histological reagents and pure substances under conditions similar to those of ordinary histological practice. It consists/



Fig. 1



Fig. 2

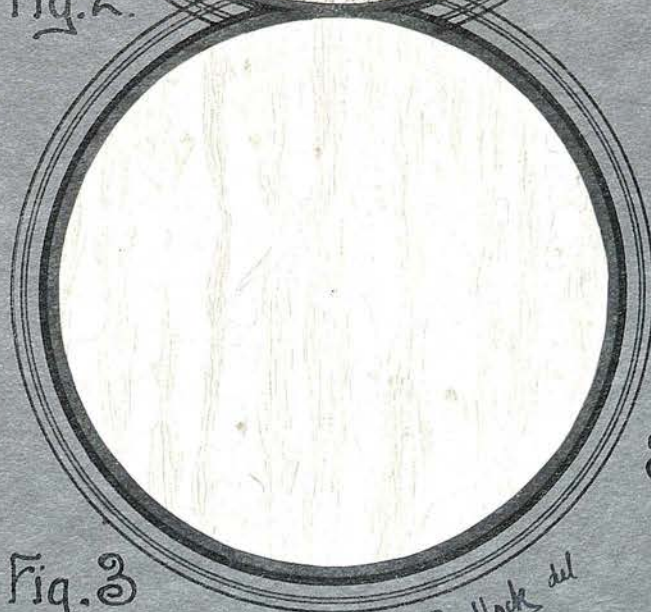


Fig. 3

Preparations to show  
 (a) the shrinkage of the nerve fibres &  
 (b) the increase in acetone-soluble material.

Fig. 1. Teased preparation of normal sciatic nerve of cat.

Fig. 2. Longitudinal section of 7 days degenerated sciatic nerve of cat.

Fig. 3. Longitudinal section of 16 days degenerated sciatic nerve of cat.

In each case the nerve was treated with acetone for 24 hrs. & then stained with osmic acid. The sketches were made with the aid of an Abbe drawing-apparatus.

Scale = X 420

W. E. Bullock del

consists in making a thin smear of the pure substance on to the surface of cigarette-paper and then treating the paper as a section.

This method was employed in the present research. It has already been mentioned that a degenerated nerve treated first with acetone, and then with potassium bichromate and osmic acid is not stained. The conclusion was at once drawn that the acetone dissolves out the specific substance which is responsible for the Marchi reaction. Other explanations were obvious but this was the simplest.

At this point the exact composition of the myelin sheath was not known, and it was hypothesized that the Marchi method is dependent upon the production of cholesterin-esters in the course of degeneration. As the chemical examination proceeded, special attention being paid to the existence of these esters, it became clear that the preliminary hypothesis was wrong. And when the fact that fat is produced in degeneration became established, our attention was fastened on this, since fat is easily soluble in acetone.

The effect of treatment of a normal, a 7 days and a 16 days degenerated nerve with acetone and then with osmic acid is illustrated in the drawings opposite.

The only substances of the normal myelin sheath appreciably soluble in cold acetone are cholesterin and/

and lecithin. To these must be added fat for the degenerated nerve and, since only unsaturated fats are acted upon by osmic acid or react with  $K_2Cr_2O_7$ , the fat required must be olein. Oleic acid was included but it was later shown to be unnecessary.

Weighed quantities of these substances were taken and smeared on to the surface of thin filter paper, and the papers were dropped into the following solutions:- (1) 1% Osmic acid; (2) Müller's fluid, and later 1% osmic acid and Müller's fluid mixed.

In the first series of experiments as much as a centigram of each lipid was used. The results obtained were as follows:-

	<u>1% Osmic Acid.</u>	<u>Marchi.</u>
Cholesterin	Unaffected	Unaffected.
Lecithin	Blackened	Turned Brown.
Olein	Blackened	Blackened.
Oleic Acid	Blackened	Unaffected.

The results show that cholesterin at any rate is not responsible for the Marchi reaction, but the results were indecisive as to Lecithin and Oleic acid, while they showed that Olein might be the required substance.

Now the question arises, Is the action of the Marchi mixture on a single pure substance similar to its action on a mixture of lipoids? In other words, does the presence or absence of one or more of these bodies/

bodies inhibit or facilitate the reaction? Since cholesterol gave negative results in both experiments obviously it was most suitable for a study in this direction.

The following experiments were made:-

Mixtures of equimolecular quantities of cholesterol and lecithin, cholesterol and olein, and cholesterol and oleic acid were treated with Osmic acid and "The Marchi Mixture". The results are shown in the following table:-

		<u>Osmic Acid.</u>	<u>"Marchi"</u>
Cholesterol	Lecithin	Blackened	Turned Brown but less deeply than in previous experiments.
"	Olein	"	Blackened.
"	Oleic Acid	"	"

The results are again indecisive; that is to say, they do not narrow the possibility down to one substance, though the probability that olein is the only substance so far tested to blacken by the Marchi method is at least not seriously shaken. The brown colour with lecithin was puzzling; the fact that Oleic acid gives a positive Marchi in the presence of cholesterol is noteworthy. But since the colour yielded by the Marchi with lecithin is diminished by the presence of cholesterol it was fairly concluded that cholesterol has an inhibitory effect. And it was therefore decided to apply the same methods to mixtures in which the/

the proportions varied from 1 molecule of cholesterin to 5 of each of the other substances, to 5 of cholesterin to 1 of the rest. The general results were that whilst all the mixtures of cholesterin with oleic acid and olein gave positive Marchis, the mixtures of lecithin and cholesterin gave a brown colour when the proportion of cholesterin was low and no colour at all when the cholesterin proportion was 5 to 1.

This again proves that cholesterin has an inhibitory effect upon the lecithin and Marchi mixture. But since the proportion required to prevent any darkening is 5 to 1 and the proportions of these compounds in normal nerve is 1 to 2, these results do not afford an explanation.

All the above experiments were carried out at room temperature and with large amounts (a centigramme) of material, the bichromate solution generally amounting to 3 - 5 c.c.

The effect of temperature was tested by placing similar mixtures in the incubator and in ovens at temperatures varying from 37°C to 55°C. Temperature was found to accelerate the action of the bichromate on the lecithin-cholesterin mixture.

The production of the brown colour with lecithin - not the black, typical of the Marchi - made it impossible to explain why the normal nerve, which contains lecithin, does not give a positive Marchi. But an/

an explanation was obtained by the discovery that the question of weight of lecithin to weight of bichromate is important. The amounts of lecithin previously employed had in every case represented at least one half a sciatic nerve, which is much more than is taken by the histologist when only 3-5 c.c. of Müller's solution are employed. Further, in the myelin sheath the lecithin is spread out in an extremely - a microscopic - thin sheet and is thus far better exposed to the action of the bichromate than can be imitated in test tube or cigarette paper experiments.

The next set of experiments therefore were made with very small amounts of material. The molecular proportions of the last experiments, where cholesterin was mixed with the remaining substances, were observed but in each case only 1 milligramme of the mixture, or pure substance when cholesterin was not used, was acted upon by the reagents. The following tables show the results obtained:-

	<u>Osmic Acid.</u>	<u>Marchi.</u>
1. Lecithin	Blackened	No visible change.
2. Olein	"	Blackened.
3. Oleic Acid	"	Unaffected.

	<u>Osmic Acid.</u>	<u>Marchi.</u>
1. Lecithin & Cholesterin in proportions 1 to 5, 1 to 4, 1 to 3, 1 to 2, 1 to 1, 2 to 1, 3 to 1, 4 to 1, 5 to 1.	Blackened.	No visible change in any case.
2. Olein & Cholesterin	"	Blackened.
3. Oleic Acid & Cholesterin	"	"

These experiments prove that the only constituent of a degenerated nerve which is blackened by the Marchi method is olein. Oleic acid is blackened in the presence of cholesterin but for the following reasons we can exclude this compound as a chemical basis of the Marchi reaction as applied to degenerated nervous tissue; (1) In degeneration there is no increase in acidity; (2) The Nile blue method of staining does not reveal any acid fat. On a priori grounds it is improbable because oleic acid is an irritant and not likely to remain unneutralised in the tissues.

We have now arrived at the conclusion that the Marchi stain is specific for olein and for oleic acid in the presence of cholesterin.

It will be noticed that in the course of the above work only those substances which are appreciably soluble in cold acetone were examined.

The reason for this has been given. But the question of the degree of solubility arises. Cerebrone is almost insoluble in cold acetone and it is therefore very unlikely that this substance can be extracted from a nerve simply by allowing the tissue to lie in the solvent for 24 hours. Moreover it is not certain that the cerebrone exists as such in the myelin sheath; it is possibly present in the still more insoluble combination, protagon. Hence cerebrone can safely be neglected. But cholesterin esters, while soluble only to/

to a slight extent in acetone, are sufficiently soluble to require investigation by the methods adopted above. In the first place it was decided to select a compound of cholesterin with a saturated fatty acid - palmitic acid for example - and another with the unsaturated oleic acid. This covers the general requirements.

These cholesterin esters had to be prepared in the laboratory and for the preparation of the palmitate the method of Abderhalden and Kartzch<sup>57</sup> was employed. Equimolecular proportions of palmityl chloride and dry cholesterin are mixed in dry chloroform at room temperature. When the brisk reaction has subsided the cholesterin palmitate is precipitated with methyl alcohol and separated by filtration. It is purified by redissolving and reprecipitating until the melting point is sharp. The ester thus prepared is a beautiful crystalline product melting at  $78.5^{\circ} - 79.5^{\circ}\text{C}$ . It forms doubly refracting liquid crystals when melted, which clear, that is become perfectly liquid, at  $84^{\circ}\text{C}$ .

A careful search through the literature showed that hitherto cholesterin oleate had not been prepared in a state of purity. The methods which had been employed for its preparation are long and tedious and give a small yield of impure ester. It was therefore resolved to apply Abderhalden and Kartzch's method to this case also. There resulted a brown oily liquid which/

which was solidified with difficulty in the following way. The crude product was dissolved in ether and sufficient acetone added to cause a permanent milkiness, which was caused to just disappear by adding a few drops of ether. The solution was placed aside and allowed to stand until most of the ether had evaporated. Cholesterin oleate is precipitated as a brown solid which can be purified by dissolving in ether and precipitating with acetone.

The pure product crystallises in plates which melt sharply at  $46^{\circ}$  -  $47^{\circ}$  to form doubly refracting liquid crystals. These disappear at  $57\frac{1}{2}^{\circ}\text{C}$  and reappear at  $52\frac{1}{2}^{\circ}\text{C}$ .

The esters thus prepared were treated with osmic acid and Marchi's mixture in the manner previously described. The results are given below:-

	<u>Osmic Acid.</u>	<u>Marchi.</u>
Cholesterin palmitate	Negative	Negative.
" oleate	Blackened	"

The same results were obtained when cholesterin was mixed with each ester.

It follows from this that cholesterin palmitate is not detectable by either osmic acid or Marchi's mixture, and that cholesterin oleate, though blackened by osmic acid alone, is unaffected by this reagent after the use of  $\text{K}_2\text{Cr}_2\text{O}_7$ .

An/

An interesting point may be noticed here; cholesterin and oleic acid, when chemically united, give a negative result with Marchi, whilst a mechanical mixture of these two compounds give a positive result. The explanation of this is difficult to see.

The following table summarises the results obtained:-

	Solubility in		Osmic	Marchi.	Double
	Acetone.	Alcohol.	Acid.		Refraction.
1. Cholesterin	+	+	-	-	-
2. Olein	+	+	+	+	-
3. Oleic Acid	+	+	+	-	-
4. Oleic " & Cholesterin			+	+	-
5. Chol. palmitate	-	-	-	-	+
6. " Oleate	-	-	+	-	+
7. Lecithin	-	+	+	-	+

A study of the above table will show how it is possible to detect, and differentiate between, olein, lecithin, and the cholesterin esters in the tissues. Oleic acid and cholesterin may be placed aside. The tissue to be examined is fixed in formalin and frozen sections are cut. One section is treated with osmic acid, a second with Marchi's method, a third with acetone and then with osmic acid; a fourth is examined with crossed Nicols for doubly refracting crystals, and a fifth similarly after treatment with alcohol. If the/

the section blackens with osmic acid olein, cholesterin and oleate, or lecithin together or alone may be present. The section treated with acetone and then with osmic acid distinguishes between olein and the other two; if olein alone is present there will be no blackening after acetone; if either of the others is present blackening will occur. The Marchi section carries the distinction further since only olein will give a positive result and thus its presence or absence is definitely decided. To decide between lecithin and the cholesterin ester we note whether any doubly refracting crystals are present or not; if any are present the section is washed with alcohol and again examined. Alcohol removes lecithin only and hence if the double refracting crystals disappear we may conclude that they are due to lecithin.

When all the substances are present we can distinguish olein definitely by the Marchi, and the other two are detected by the alteration in quantities of stainable or doubly refracting material when the sections are tested as described.

This scheme was applied to the case of the kidney, the suprarenal bodies, the testis and several other organs. As the method is being developed to include other lipoids and is being applied to most of the tissues of the body, and since it bears only indirectly on the subject of this thesis, reference will be made to/

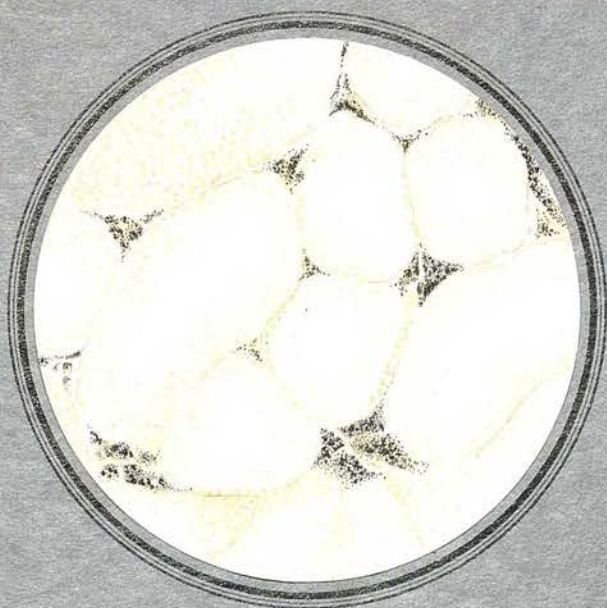


Fig. 1



Fig. 2

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Sections of cat's testis.

Fig. 1 stained by Marchi's method

Fig. 2 stained with Sudan III

Both stains show the presence of fat in the same areas. The sketches were made with the aid of an Abbe drawing-apparatus.

Scale = X 80

to the kidney and suprarenal alone.

	Osmic Acid before Acetone.	Osmic Acid after Acetone.	Marchi.	Double refraction before Alcohol.	Double refraction after Alcohol.
Suprarenal of Cat	+	-?	+	+	+
Kidney of Cat	+	-	+	-	-

It is obvious from these results that the suprarenal body contains cholesterin oleate and olein and no lecithin, and that the kidney contains of the three substances fat only. These substances are present within the parenchymatous cells of the organs.

In order to verify the results in so far as they show the existence of fat sections were stained with Nile blue and with Sudan III and identical portions of the sections compared with osmic acid and Marchi preparations. Degenerated and healthy sciatic nerves and spinal cords, as well as the various organs mentioned above, were stained and in each case a correspondence between the methods was observed. Thus, whilst the normal spinal cord is unblackened by Marchi's stain, the degenerated cord is stained in definite areas; and the same areas are stained, though more diffusely, with Nile blue and Sudan III. The same results were observed with the kidney and suprarenal body, testis and thyroid gland.

R E S U L T S.

- (1) The following observations of Noll, Barratt, Mott and Barratt are confirmed:- In degeneration a nerve becomes more watery and loses some of its lipid phosphorus and cerebroside.
- (2) Cholesterin diminishes also, but to a less extent.
- (3) Cerebrosides tend to disappear first among the lipoids of the degenerating myelin sheath.
- (4) Fat is produced in degeneration.
- (5) The normal myelin sheath contains no fat.
- (6) The lipoids of the myelin sheath are fully accounted for by the methods of analysis employed.
- (7) Sulphur is present in the cold acetone extract of a degenerated nerve; it is not present in the extract of a normal nerve.
- (8) Cold acetone removes the whole of the fat from the degenerated nerve or spinal cord.
- (9) The Marchi method is specific for unsaturated fat.
- (10) A method for the histological detection of (and discrimination between) unsaturated fat, lecithin and cholesterin esters is shown and the result of its application to two organs is given. By the use of other stains, e.g., Nile blue and Sudan III, it is possible to extend the scheme to include saturated fat.
- (11) Cholesterin eleate is here prepared in a state of purity and its chief characters are noted.
- (12)/

- (12) A normal nerve when examined with the polarising microscope shows doubly refracting crystals uniformly distributed through its substance. A degenerated nerve shows doubly refracting crystals but only in patches separated by fatty globules.
- (13) A nerve, normal or degenerated, shows no doubly refracting crystals after treatment with alcohol.
- (14) The relative proportions of the lipoids of the myelin sheath are not greatly disturbed in the process of degeneration.

#### CONCLUSIONS.

The chemical changes occurring among the lipoids of a degenerating nerve affect the most complex substances chiefly. Thus, cerebrosides diminish more rapidly than phosphatides, and the latter more rapidly than cholesterin. Protagon, which is still more complex, probably breaks up first but this cannot be definitely decided until the relationship of protagon to cerebrosides has been cleared up. It is shown, however, that a sulphatide simpler than protagon is formed in degenerating myelin.

In the process of disintegration fat is produced, and this is derived either from the lecithin or the phosphatide portion of protagon. What becomes of the other products is not known; they are probably rapidly/

rapidly removed and oxidised.

The proof that the Marchi method is specific for unsaturated fat displaces the view, commonly held, that it is specific for degenerating myelin and, combined with the method of histo-chemical analysis already indicated, opens up a new field of investigation.

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Part II. The Lipoids of certain Innocent  
and Malignant Tumours.

With the exception of a paper by Robertson and Burnett in the Journal of Experimental Medicine for March 1 of this year, there is no literature bearing directly on the subject of the relation of lipoids to tumour growth. These authors have investigated the effects on the rate of growth of experimental tumours of injections of cholesterin and lecithin. Emulsions of the lipoids were made and small quantities injected directly into the tumours. The general results obtained show that cholesterin causes a marked acceleration in growth of both primary tumour and metastases; lecithin, on the other hand, diminishes the tendency to form metastases, retards metastatic growths and in some cases the primary tumour.

The work is open to criticism. It is simply an empirical attempt to discover a cancer cure. In order to gain knowledge of the part played by lipoids in the metabolism of new growths it is at least necessary to make analyses of a series of tumours under various conditions.

A./

A. An Analysis of the lipoids of a subcutaneous  
Xanthoma Tuberosum Multiplex.

An account of the pathology and clinical features of this tumour is given by Dr. Cranston Low in the British Journal of Dermatology for April, 1910.

I am indebted to Mr Stiles and Professor Schäfer for a sufficient amount of the tumour for analysis.

Weight of dried tissue = 3.60 gm.

" " Lipoids extracted = 1.50 "

% of Lipoids = 41.7%.

	% of Lipoids.	% of Dry Tissue.
i Free Cholesterin	4.43	1.8
ii Cholesterin if the form of Esters	26.4	11.0
iii Phosphorus	Trace	
iv Nitrogen	0.93	
v Cerebrosides	50	20.85
Total	80.8	33.65

If the fatty acids with which the cholesterin is united be calculated on the assumption that they are present in equimolecular proportions we obtain 20 as the percentage of oleic, palmitic and stearic acids, and this brings the total percentage of the first column/

column up to 100.8.

The details of the analysis are given below:-

A. Cholesterin: (1) 10 c.c. gave .0248 gm. of  
cholesterin digitonate.

(2) 20 c.c. gave .057 gm. of  
cholesterin digitonate.

The percentages corresponding to these are

(1) 4.1 (2) 4.75.

B. Cholesterin Esters: From 20 c.c. 0.3170 gm. of  
cholesterin digitonate was  
obtained.

$$\text{Hence } \frac{0.3170 \times 5}{4} = 0.39625 \text{ gm.}$$

$$\text{and the } \% = \frac{39.625}{1.5} = 26.4$$

C. Phosphorus: 20 c.c. gave a trace of ammonium  
phospho-molybdate.

D. Nitrogen: 20 c.c. required 2c.c. of  $\frac{N}{10}$  NaOH.

That is 20c.c. contained 2.8 mgm of  
Nitrogen.

Hence 100c.c. " 14.0 mgm of  
Nitrogen.

and  $\therefore$  the  $\%$  = 0.93.

E. Cerebroside: 10c.c. gave 31 mgm of cupric oxide.

This corresponds to 75 mgm of cere-  
broside.

$\therefore$  100 c.c. contains 750 mgm of cerebroside

and the  $\%$  = 50.

The lipoids of the Xanthoma consist therefore of  
cerebrosides and cholesterin esters and a small amount  
of free cholesterin.

B./

B. Cholesteatoma.

This tumour was found in the brain.

I am indebted to Dr. James Miller for a small piece of the neoplasm.

Weight of dry tissue = 0.1608

" " lipoids extracted = 0.0610

% of lipoids = 37.9.

Weight of Cholesterin-digitonate obtained from the lipoids = 0.0740.

$$\therefore \% = \frac{.0740}{4} \times \frac{100}{.0610} = \underline{30.3}$$

The filtration from the cholesterin-digitonate was tested for cholesterin esters; none was present.

There was not sufficient material for a complete analysis.

The result shows therefore that a cholesteatoma of the brain contains less lipoid matter than the brain tissue, that the percentage of cholesterin to total lipoids is higher than for the brain, and that, like the brain, the tumour is devoid of cholesterin esters.

The comparison between phosphatides and cerebro-sides of tumour and normal tissue unfortunately could not be obtained.

C./

C. Experimental Sarcoma of Rat.

I am indebted to Dr. Cramer for this tumour.

Weight of tumour = 6.8180 gm.

" " lipoids = 1.2620 "

% " " = 18.5

	% of Lipoid.	% of Dry Tissue.
Free Cholesterin	7.8	1.4
Cholesterin Esters	Absent	
Phosphatides	38.8	7.2
Cerebrosides	10.1	1.9
Total	56.7	10.5

Details of Analysis:-

A. Free Cholesterin:

Weight of cholesterin-digitonate from 10c.c.  
= 0.0394 gm.

Hence  $\times \frac{10}{4} = 0.0985$  gm. and the % =

$$\frac{98.85}{1.262} = 7.8.$$

B. Phosphatides:

20c.c. used up 7.2c.c.  $\frac{N}{2}$  NaOH

Hence  $7.2 \times 1.268 \times 0.43 \times 5 \times \frac{100}{4} = .4987$  gm.

$$\therefore \% = 38.8.$$

C. Cerebrosides.

50c.c. gave 26 mgm CuO = 63.7 mgm Cerebroside

i.e. .1274 gm. in 100c.c. The % = 10.1

It/

It will be observed that the sarcoma, compared with the xanthoma and cholesteatoma, contains a relatively low percentage of chloroform soluble matter, that almost one half of this consists of ordinary non-phospharised fat, and that the relative proportions between the lipoids present are roughly similar to those for connective tissue.

D. Mammary Cancer of Mouse.

Two strains of tumour were obtained, a slow growing and a rapid. A sufficient number of mice were inoculated in the axilla; when the new growth had reached the size of a walnut the mice were killed. The tumours were dissected out and dried. In the case of the rapid growing strain the healthy tissue was separated from the necrotic and each was subsequently analysed.

I am indebted to Dr. Cramer for these tumours. The following table summarises the results of analyses of twenty tumours of each strain,+

	Total Lip-oids.	Cholesterin % of Lip-oids.	% of Dry Tissue.	Choles- terin Esters.	Phosphatides. % of Lip-oids.	% of Dry Tissue.	Cere- bro- sides.	Fats. % of lipoids.
Slowly Growing	13.3	8.8	1.1	0	6.6	0.88	0	84.6
Rapidly " (Healthy Parts)	13.1	Trace		0	9.7	1.3	0	91.3
ditto (Necrotic Parts)	41.1	Trace		0	6.2	2.54	Trace	94.8

Comparing the slowly growing tumour with healthy parts of the rapidly growing we see that (1) the percentage of total lipoids is practically the same; (2) cholesterin forms 1% of the total solids of the slowly growing tumour while it is present only in traces in the rapidly growing; (3) phosphatides form only a small proportion of both tumours, though they are more abundant in the rapidly growing; and (4) that both tumours are devoid of cerebrosides and cholesterin esters.

The change in composition brought about by necrosis is shown by a comparison of the second and third lines of the table. The most conspicuous feature of the change is the increase in total lipoids which are trebled in amount, the increase consisting to a great extent of ordinary fat and to a less extent of cholesterin. This last substance is twice as abundant in the necrotic as in the healthy parts of the same tumours. The fats increase/

increase at a slightly greater rate than the cholesterolin and finally cerebrosides are produced in traces.

I wish here to express my thanks to Dr. Cramer for criticism and advice freely given in every direction during the course of this research.

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