"On the Detection and Localisation of Phosphorus in Animal and Vegetable Tissues." By A. B. MACALLUM, Associate-Professor of Physiology, University of Toronto. Communicated by Professor SHERRINGTON, F.R.S. Received June 15,—Read June 16, 1898.

The distribution of phosphorus, like that of organic iron, in tissues, is a question of considerable importance to the cytologist and it is therefore necessary that the method of detection for this element, should be a satisfactory one. There are difficulties, however, which make the micro-chemical detection of phosphorus less easy than in the case of iron, for there is no precipitate holding phosphorus which, under the microscope, gives as striking a demonstration of its presence as ferrous sulphide does of iron. Ammonium phospho-molybdate is, in the test-tube, a markedly coloured precipitate, but when its constituent crystals are examined under the microscope the colour observed counts for little. When also, as in tissues, the precipitate may be in a much more finely divided form, the canary-yellow colour may be so faint that it is indistinguishable from the yellow produced in the tissue by the action of the nitric acid in the precipitating reagent, although Jolly* holds that the yellow colour of the phospho-molybdate compound in the tissue cannot be simulated by dilute nitric acid.

To get over these difficulties Lilienfeld and Monti† used pyrogallol to reduce the molybdic portion of the compound to the condition of a lower oxide after they had, by washing the preparations in water, removed the uncombined molybdate of ammonia from the tissues. "Pyrogallol gives, in the test-tube with phospho-molybdic acid, an intense colour varying from brown to black, whereby lower oxides of molybdenum arise." ‡ In speaking in another place of the action of pyrogallol on the phospho-molybdate, they state that it gives, in the parts of the preparations rich in phosphorus and according to the quantity of the latter, "a yellow, brown, or black colour."

Raçiborski§ points out that the reaction of pyrogallol with ammonium phospho-molybdate in the test-tube is a green one, while that produced with ammonium molybdate is a brown one. This author further states that the green reaction is obtained in the tissues of *Euphorbia* wherever crystals of ammonium phospho-

* "Contribution à l'histoire biologique des phosphates," 'Comptes Rendus,' vol. 125, p. 538, 1897.

† "Ueber die mikro-chemischen Localisation des Phosphors in den Geweben," 'Zeit. für Physiol. Chemie,' vol. 17, p. 410, 1893.

‡ *Loc. cit.*, p. 411.

§ *Vide* a criticism of Lilienfeld and Monti's observations, 'Bot. Zeit.,' vol. 51, p. 245, 1893.

molybdate occur, but a brown colour in other parts of an intensity which varies according to the length of time during which the preparation is washed, but if it is long and continuously treated with water no brown colour appears. The brown, therefore, would be due to molybdate of ammonium, and is no indication of the presence of the phosphorus compound.

Heine* was unable to confirm Raçiborski's observations regarding the reaction produced by pyrogallol, but he found, using stannous chloride as a reducing agent, that almost invariably a blue reaction appeared, which may pass eventually into a dirty green colour. In the test-tube also the reaction with the reducing agent is, according to the amount of the molybdate present, as well as to the strength of acidity in the fluid, a green, brown, or blue one, whether phosphates are present or not.

Pollacci,† using zinc chloride as a reducing reagent, found the resulting colour range from dark blue to grey.

It is evident from the foregoing that there is error somewhere in the observations which have been made on the action of pyrogallol on ammonium phospho-molybdate, and it is obvious that, if Raçiborski is right in his contention, then the results of the investigations of Lilienfeld and Monti, relying as they did upon the "yellow, brown, or black" reaction to indicate the presence of phosphorus, must be wrong. As a number of observers, including Sherrington,‡ Gourlay,§ and Held,|| have used the same method and the same criteria on special tissue elements, it is therefore important to know the truth concerning the results so obtained.

My observations confirm Raçiborski's on the action of pyrogallol on ammonium phospho-molybdate. When the former, in solution, aqueous or ethereal, is allowed to act on the thoroughly washed phospho-molybdate precipitate, the canary yellow of the latter is invariably turned to green, even in the presence of nitric acid, and this colour is maintained for a couple of hours, after which the precipitate takes up slowly a darker shade, until at the end of twenty-four hours it has a black colour with a faint shade of green in thin layers. The form of the crystals, which are black, is maintained. When the

* "Ueber die Molybdänsäure als mikroskopischer Reagens," 'Zeit. für Physiol. Chemie,' vol. 22, p. 132, 1896–97.

† "Sulla distribuzione del fosforo nei tessuti vegetali," 'Malpighia,' vol. 8, 1894; Abstract in 'Zeit. für Wiss. Mikrosk.,' vol. 11, p. 539.

‡ "Note on some Changes in the Blood of the general Circulation consequent upon certain Inflammations of acute and local character," 'Roy. Soc. Proc.,' vol. 55, p. 161, 1894.

§ "Proteids of the Thyroid and Spleen," 'Journ. of Physiol.,' vol. 16, p. 23, 1894.

|| "Beiträge zur Structur der Nervenzellen und ihrer Fortsätze," 'Arch. für Anat. und Phys.,' Anat. Abthg., 1895, p. 396.

reducing reagent is allowed to act on a nitric acid solution of ammonium molybdate, a brownish-black or black colour is produced, and an amorphous precipitate may be formed, which, under the microscope has a grey blue-black appearance, the fluid itself remaining brown, the colour being due to the oxidised pyrogallol. At the end of twenty-four hours the amorphous elements are black, with or without a brown shade. When, on the other hand, pyrogallol acts on ammonium molybdate in solution the resulting colour is deep brown, very much like that of a saturated solution of Bismarck brown, which is maintained at the end of twenty-four hours, but in no case is a precipitate formed. It must be noted in this case that the colour formed results immediately on the addition of the pyrogallol.

That what happens in the test-tube is what also obtains in tissues may be shown readily. If one impregnates sections of tissue with ammonium molybdate for an hour or more, these, thoroughly washed and then treated with a pyrogallol solution, give a brown colour, which is most marked in the parts of the cells which have an affinity for colouring matters. It is obvious that in the absence of nitric acid there is no phospho-molybdate compound present, and yet the reducing reagent shows that though repeated washings were resorted to, the ammonium molybdate has not been removed. On the other hand, when the tissues are placed in a nitric acid solution of ammonium molybdate the results obtained are strikingly different. One may conveniently demonstrate these by placing fresh *Spirogyra* threads in the solution for from five to ten hours at a temperature of 35—40° C., then washing them quickly in distilled water and putting them in a freshly prepared strong aqueous solution of pyrogallol. In ten minutes the threads may be again washed, dehydrated, cleared in oil of cedar, and mounted in balsam. Wherever in such preparations one expects to find phosphorus, *e.g.*, in the nuclei, it is demonstrated by the green reaction. If the pyrogallol is allowed to act longer than ten minutes it begins to stain the cells and to mark the green more or less with a brown coloration, which distributes itself in them as colouring matters generally do.

Perhaps the most striking way of demonstrating that the phospho-molybdate is turned green and the molybdate brown by the action of pyrogallol, is by impregnating portions of thin strips of writing paper with a solution of sodic phosphate, drying them, and then submitting them to the action of the nitric-molybdate solution, which gives them a yellow colour. On now washing them in distilled water, and submitting them to the pyrogallol solution, the areas which are impregnated with the phospho-molybdate become green in a few seconds, while the parts which contain the molybdate solution alone become brown or yellowish-brown, and the contrast between the two reactions thus appears marked.

The error at the base of the process adopted by Lilienfeld and Monti has been the assumption that it is possible to wash out of tissues all traces of the ammonium molybdate not combined with phosphoric acid. I have found that when the stamens of *Erythronium americanum*, treated for twenty-four hours with the nitric-molybdate solution, were washed with distilled water many times for five months, they gave, at the end of that time, marked evidence of the presence in them of ammonium molybdate. The addition of stannous chloride brought out everywhere in such preparations the appearance of the blue molybdic oxide, whereas, when such preparations were treated with pyrogallol solution, the phospho-molybdate compound was found to be limited in its distribution. From animal tissues also, I have found it impossible to remove by washing the molybdate reagent. Indeed, one may succeed thus in removing the phospho-molybdate compound rather than the other.

Heine* also has specially insisted on the strong affinity that cell substances, those which contain phosphorus as well as those which do not, have for ammonium molybdate, forming with the latter, compounds insoluble in water or in dilute nitric acid solutions. He prepared a quantity of histon, free from phosphorus compounds, which, after treatment with the nitric-molybdate reagent and after frequent washings, gave with stannous chloride abundant evidence of the presence of ammonium molybdate.

One is consequently justified in concluding that the results of Lilienfeld and Monti's observations, based as they are on the "yellow, brown, or black" reaction of the pyrogallol, are incorrect, and that while the reaction may appear in phosphorus-holding elements, it is simply a coincidence, and not an indication of the presence of phosphorus.

The property of pyrogallol to form, in the reduction of the molybdate and the phospho-molybdate compounds, a coloured substance which can be taken by cellular elements just in the same way and to about the same extent that they take up other colouring matters in solution, constitutes an objection to the use of this reducing reagent. It is not possible to be certain in all cases in regard to the length of time during which it is to be allowed to act, and, consequently, a very faint green may be obscured by a light brown reaction, resulting either from the oxidised pyrogallol or from the reduced molybdate in the presence of traces of nitric acid.

In consequence of this objection, I endeavoured to find a reducing reagent which would leave the molybdate compound, in the presence of nitric acid, unaffected, while it would markedly react with the phospho-molybdate, not only in the test-tube, but in tissues. Zinc

* "Ueber die Molybdänsäure als mikroskopisches Reagens," 'Zeit. für Physiol. Chemie,' vol. 22, p. 132, 1896–97.

chloride does this, but in an unsatisfactory way. It is very slow in its action, and feeble in its reducing power. It gives a green reaction with the phospho-molybdate compound, but none with the molybdate in the presence of nitric acid. Stannous chloride reduces both the compounds at once, forming the blue oxide of molybdenum, and therefore it is, for the point in view, valueless. Ferrous sulphate is also very slow in its action, and it has the disadvantage of giving a faint green colour to the tissue, independent of that which may be produced in the phospho-molybdate compound.

The reagent which I found the most valuable from every point of view is phenylhydrazin hydrochloride. This, in an aqueous solution of 1—4 per cent. strength, is certain in its action if it is freshly prepared or not more more than two or three days old. It, in the absence of alcohol or of a caustic alkali, makes a very marked distinction between the molybdate and the phospho-molybdate compounds. It gives with the former, in powder, the brown oxide at once, in solution, a brownish precipitate which may or may not appear immediately, depending on the strength of the solution, but in a solution of the molybdate containing nitric acid, *e.g.*, that used as the reagent for phosphoric acid, it has no apparent effect on the molybdenum compound, although, in a few minutes, a soluble, reddish, aromatic compound may be formed in the solution. On the other hand, with phospho-molybdates, either in the presence or in the absence of ammonium molybdate, or nitric acid, or of both, it gives at once the dark-green oxide of molybdenum. Examined under the microscope, the crystals of the phospho-molybdate alone are found to have the green colour, which, after an hour's action of the phenylhydrazin, is so dark as to suggest, at first sight, black. That this reaction depends upon the presence of phosphoric acid, may be clearly shown by adding to a mixture of the reducing reagent and of the nitric molybdate solution a quantity of phosphoric acid solution. Although the mixture will stand for several minutes without any change other than the formation of a slightly reddish solution, yet on the addition of the acid solution the dark-green reaction appears immediately and markedly, sometimes accompanied by the formation of a blue-violet soluble compound. No other acid exercises a like effect. Solutions of potassium hydrate and sodic hydrate and alcohol, in a certain proportion, will call forth in the mixture a greenish-blue or blue colour, which, in the case of the alcohol preparation, fades out in twenty-four hours. In this latter, the colour would appear to be due to the formation of an aromatic compound, and not directly to an alteration in the molybdate. Nitric acid alone will produce, in a solution of phenylhydrazin, a reddish colour, and rarely also, when ammonium molybdate is present, a blue-violet colour, which appears to be due to a phenylic

compound. What the conditions are, under which this coloured compound is produced, have not been determined, but this reaction cannot interfere with or confuse the results of the action of the reducing reagent on the phospho-molybdate compound.

On the molybdate and phospho-molybdate compounds distributed in animal and vegetable tissues, the phenylhydrazin hydrochloride acts as it does on these in the test-tube. It is not necessary to free the tissue preparations from ammonium molybdate. They may be placed for a minute or two in a dilute solution of nitric acid, after which they are transferred to the reducing solution, which, in less than two minutes, brings out the green colour where the phospho-molybdate compound occurs, but a faint yellow reaction where ammonium molybdate alone is present. Instead of dilute nitric acid, one may use distilled water, but it is not necessary to do even this, for if the preparations are transferred directly to the reducing fluid with but what may adhere to them of the nitric-molybdate solution, the result is the same.

When the reducing fluid has been allowed to act for the proper length of time the preparations are washed in distilled water, then dehydrated, cleared in oil of cedar, and mounted in balsam. Preparations made in this way four months ago are now quite as satisfactory as they were at first.

Reference to the other reagents and methods which have been used is also necessary. The nitric-molybdate reagent was made by dissolving one part of pure molybdic acid ($MoO_3$) in four parts of strong ammonia, and adding thereto, slowly, fifteen parts of nitric acid, sp. gr. 1·2. The proportions indicate weights. The resulting solution had a faint yellow tinge, and, after decantation from the very slight sediment, remained free from a precipitate as long as any of it was unused.

Fresh tissue material was used as well as that which had been hardened in alcohol. The alcohol material is the best, for the nitric acid, before it converts the phosphorus compounds in fresh tissue elements into orthophosphoric acid, must dissolve a portion at least of the phosphorus-holding proteids, and thus the phosphorus when converted may not be distributed as *intra vitam*. I have, however, used fresh material, wherever possible, to compare with that hardened in alcohol. The latter offers advantages in the fixed form of the elements, and in the preparation of thin sections which readily permit the uniform action of the reagent as well as the extraction of lecithin and inorganic phosphates.

The time during which the reagent was allowed to act on the preparations varied from ten minutes to twenty-four, and even, in some cases, forty-eight hours. It was found that a temperature of 35° C. favoured considerably the formation of the phospho-molybdate. The

formation is a progressive one, the extent of the reaction appearing to have some relation to the time employed. The inorganic phosphates are first affected, then lecithin, the organic phosphorus being much more slowly converted into the orthophosphate.

According to Liebermann,* the phosphorus found in such compounds as nuclein and nucleic acid is in the form of monometaphosphate, but Kossel† has thrown doubt on the results on which this view is based, and he claims that the facts point rather to the occurrence of other anhydrous forms of phosphoric acid in these compounds. Jolly‡ has inferred from his experiments that in organic compounds of phosphorus the latter does not occur in the unoxidised metalloid ("metalloïdique non oxydé intégré") form. Milroy§ has found that in the digestion of nuclein compounds with trypsin, some of the phosphorus is set free as orthophosphoric acid, but the greater part (89·08—91·63 per cent.), occurring in an organic form, does not possess the characters of metaphosphoric acid, for its solutions may be boiled a long time without producing an increase in the amount of the ortho compound present.

As the nitric-molybdic reagent reacts only with the ortho form of phosphoric acid, it is obvious that the organic phosphorus in the tissues must be put in the condition of orthophoshoric acid. Lilienfeld and Monti treated the fresh tissues with baryta water or sodic carbonate, in order to set the phosphorus free as phosphate, which was then demonstrated as the phospho-molybdate; but, as Liebermann‖ points out in the case of yeast nuclein, the baryta compound is only after long boiling, or, after heating with acids, converted into the orthophosphate. The action of the baryta must, in part at least, be to change the structure of the elements, and it is not certain, therefore, that in all cases the ortho compound formed should be in the structures where the phosphorus originally existed. This, and the fact that the sodium compound first formed by sodic carbonate, being soluble, may diffuse from its original situation, render this method of doubtful value in localising phosphorus in tissue elements. These observers, however, claim that the nitric acid in the molybdic reagent has the property of gradually converting the phosphorus compounds into the orthophosphate, and they allowed fresh prepara-

* "Nachweis der Metaphosphorsäure im Nuclein der Hefe," 'Arch. für die gesam. Physiol.,' vol. 47, p. 155, 1890.

† "Ueber die Nucleinsäure," 'Verh. Physiol. Gesell. zu Berlin,' 'Arch. für Anat. und Physiol.,' Phys. Abth., 1893, p. 157.

‡ "Recherches sur le phosphore organique," 'Comptes Rendus,' vol. 126, p. 531, 1898.

§ "Ueber die Eiweiss-Verbindungen der Nucleïnsäure und Thyminsäure und ihre Beziehung zu den Nucleïnen und Paranucleïnen," 'Zeit. für Physiol. Chemie,' vol. 22, p. 307, 1896–97.

‖ *Loc. cit.*

tions to remain a long time in this fluid for this purpose. I have, as already stated, found that the long continued action of the reagent has this result, and that the conversion is more marked if the reagent is allowed to act at a slightly increased temperature. One cannot be absolutely certain that the anhydrous forms of phosphoric acid when liberated, and before being converted into orthophosphoric acid, do not diffuse through the tissue elements, but in a number of experiments made to decide this point, I ascertained that if such diffusion did occur, it was in such minute amounts as to be unobservable. A risk of diffusion is incurred when a tissue, very rich in orthophosphates, is acted on by the reagent. A part of the phosphoric acid in this case, except in very thin sections, diffuses and forms a slight deposit of phospho-molybdate crystals on the preparation. Preparations of renal tubules and the cat's placenta illustrate this well.

Owing to the abundance and general distribution of lecithin in animal and vegetable tissues, it is necessary to extract this compound from them in order to ascertain the distribution of the other phosphorus compounds. Bitto* has shown that the extraction can be regarded as complete only when the material, first treated with ether, has been acted on by boiling ethyl alcohol thirty times, each period of extraction lasting about ten minutes. Adopting this process, I subjected samples from all the material used to extraction in a Soxhlet apparatus for five hours, the condensed but still hot alcohol being siphoned off every 6—10 minutes. This treatment is specially necessary in the case of nerve tissues in which it makes a marked difference in the phospho-molybdate reaction.

A much more difficult problem is that of the removal of the inorganic phosphates from tissues. Jolly† used acetic acid of 20 per cent. strength for this purpose, claiming that this fluid removes all the phosphates except that of iron. It does indeed remove a large part of them, but not those which may be in the nuclear elements. In order, therefore, not to confuse the inorganic phosphorus with that of organic combinations, I have always endeavoured to determine in any given material what extent of molybdo-phosphate reaction may be obtained in the first ten minutes after the nitric-molybdate reagent is added. This reaction indicates whether the tissues are rich or poor in inorganic phosphates, and it may be compared with what may be obtained after a longer stay in the reagent, any enhancement in the reaction thus demonstrating the phosphorus of organic compounds.

* "Ueber die Bestimmung des Lecithingehaltes der Pflanzenbestandtheile," 'Zeit. für Physiol. Chemie,' vol. 19, p. 488, 1894.

† 'Comptes Rendus,' vol. 125, p. 538, 1897.

*Results of the Method.*

I. *General.*—The chromatin of all nuclei gives, after eighteen hours' treatment with the nitric-molybdate reagent a strong phospho-molybdate reaction. This is so marked that the nuclei appear under ordinary microscopic magnification as if they were stained with a dark-green dye for the express purpose of showing the chromatin structures. Even the finer fibrils constituting the so-called reticulum are prominently brought out. This is well illustrated in the nuclei of the epithelial cells of the skin, alimentary tract, renal tubules, and olfactory region, and of the muscle fibres, liver cells, testicular and ovarian cells, nerve cells (spinal cord), pancreatic cells, connective tissue cells, and leucocytes of *Menobranchus* (*Necturus*) *lateralis* and *Amblystoma punctatum*. In vegetable cells, as shown in *Erythronium americanum*, the same result was found. In brief, wherever true chromatin was found, there the reaction for phosphorus was obtained. In the chromatin of the mitotic loops in dividing animal and vegetable cells, no reaction more marked than in the chromatin of the resting nuclei was in any case obtained. This fact definitely contradicts the view of Lilienfeld* that the chromosomes in mitosis are composed of nucleic acid only, a view which Heine,† as a result of experiments in staining with mixtures of dyes, also rejected. The phosphorus in nucleic acid amounts to 9—10 per cent., but in nuclein it is 3—4 per cent. If Lilienfeld's view is correct, then the reaction for phosphorus in the chromosomes should be at least twice as marked as in resting chromatin elements, taking volume for volume. The results obtained by Lilienfeld in his staining experiments must be explained on some other hypothesis than that which he adopted.

The eosinophilous nucleoli in animal and vegetable nuclei give a strong reaction for phosphorus, but less marked than in the case of the chromatin. On the other hand, the nucleolar elements in the nucleus of the ovary of *Erythronium* which, as I have pointed out,‡ are rich in "masked" iron, give a deep reaction for phosphorus. A similar result was obtained in the nucleoli of the nuclei of the embryo-sac of the same form, in the peripheral nucleoli of the maturing ovarian ova of *Menobranchus*, in the nucleoli of *Corallorhiza multiflora* and of *Spirogyra*, all rich also in "masked" iron. The

* "Ueber die Wahlverwandtschaft der Zellelmente zu gewissen Farbstoffen," 'Verh. Berl. Physiol. Gesell.,' 'Arch. für Anat. und Phys.,' Phys. Abth. 1893, p. 391.

† "Die Mikrochemie der Mitose, zugleich eine Kritik mikrochemischer Methoden," 'Zeit. für Physiol. Chemie,' vol. 21, p. 494, 1895–96.

‡ "On the Distribution of Assimilated Compounds of Iron, other than Hæmoglobin and Hæmatins, in Animal and Vegetable Cells," 'Quart. Journ. Micr. Sci.,' vol. 38, p. 175, 1895.

nucleoli of the nerve cells in the spinal cord of *Menobranchus* and of the ox and dog, give a deep reaction, but it is not uniform throughout the nucleolus, portions of a granular form, giving a deeper colour than the surrounding material.

In the cytoplasm of various cells the organic phosphorus present is usually small in amount, and, unless inorganic phosphates are present, the lecithin being extracted, the reaction is a very faint one. In the cells of the nucellus and in the bast cells of *Erythronium* a deeper reaction is obtainable in the cytoplasm; and this appears to be due to the presence of chromatin—at least in the case of the nucellar cells. The cytoplasm of the latter is also, as I have pointed out elsewhere, iron-holding. Other exceptions are found in the pancreatic cells, liver cells, nerve cells, striated muscle fibres, in maturing and mature ovarian ova of Amphibia, and in the spermatozoids of *Ascaris*. These exceptions are referred to at greater length below.

In dividing cells the achromatic spindle gives no reaction for phosphorus. This result is quite the opposite of that which Heine obtained when he used stannous chloride as a reducing reagent after the employment of the nitric-molybdate reagent. Heine advanced the view that his result showed that the molybdic reagent could not be depended on to indicate the presence of phosphorus in tissues. It is rather to be interpreted as indicating that stannous chloride does not distinguish between the molybdate and the phospho-molybdate compounds.

In no case has the centrosome or centrosphere in animal and vegetable cells given a reaction for phosphorus.

II. *Special.*—The zymogen granules in the pancreas of *Diemyctylus*, from which the lecithin was thoroughly extracted, gave a deep reaction for phosphorus after eighteen hours' treatment with the nitric-molybdic reagent. The phosphorus apparently is less firmly bound than is the case in the nuclear chromatin in the same cells, for the reaction in the latter is slower in appearing. A very distinct but less deep reaction was obtained also in the protoplasm in which the granules lie, more especially in the part adjacent to the lumen, and a marked reaction also was produced in the antecedent substance of the zymogen, found usually in the outer or protoplasmic zone of the cells. This substance, which I have named prozymogen,* contains iron in a "masked" form, and it stains in every way like chromatin. The presence of phosphorus, as well as of "masked" iron, seems to indicate very clearly that it is a nucleo-proteid.

The demonstration that zymogen and prozymogen are phosphorus-

* *Loc. cit.*, p. 224; also "Contributions to the Morphology and Physiology of the Cell," 'Trans. Can. Inst.,' vol. 1, p. 247, 1891.

holding confirms the view which I advanced seven years ago,* that both are primarily derived from the nuclear chromatin.

The deeper reaction for phosphorus which is obtained in that part of the pancreatic cell immediately adjacent to the lumen, may be due to ferments dissolved in the cytoplasm at this point or to a phosphorus-holding substance derived from the zymogen at the same time the ferments are formed.

A diffuse reaction for phosphorus, slow in appearing, was obtained in the cytoplasm of liver cells of dog and man. These cells also frequently contain abundance of inorganic phosphates whose presence may render the demonstration of the organic compound difficult.

I have been unable to determine whether organic phosphorus compounds are present in the cytoplasm of the renal cells, for in the dog and human subject these cells are rich in inorganic phosphates which are difficult to extract, and, consequently, obscure the reaction for the other compounds if these occur here.

Mr. F. H. Scott, who is at present working on the micro-chemistry of nerve cells, has found that Nissl's granules also give a distinct reaction for phosphorus. He has also found that the substance forming the granules does not digest in artificial gastric juice. Mackenzie† had previously found that these granules contain "masked" iron. They stain like chromatin. These facts lead one to conclude that the substance of the granules is a nucleo-proteid.

A feeble reaction for phosphorus has been obtained in the axis cylinders of medullated nerves from which the lecithin was extracted.

In the muscle fibres from the chelæ of the crayfish a deep phosphorus reaction was obtained in the dim bands and in the beadlets which constitute Dobie's line, while no reaction occurred in the lateral discs of Engelmann. The phosphorus-holding substance is coterminous with the anisotropous element. The phosphorus demonstrated is not due to presence of lecithin, for this was wholly extracted from the preparation before it was treated with the nitric-molybdic reagent, and it was not due to inorganic phosphates, for the reaction did not come out, except very feebly, during the first twenty minutes.

In the striated muscle fibres of Amphibian larvæ the iron-holding substance appears to be also limited in its distribution, as it was found only in the dim bands,‡ Dobie's line giving no evidence of its presence, perhaps because this structure in Amphibia is too minute to permit a proper determination of this point. In my experiments on crayfish muscle both the dim band and Dobie's line appear to give

* 'Trans. Can. Inst.,' vol. 1, p. 247, 1891.

† "Investigation in the Micro-chemistry of Nerve Cells," 'Brit. Assoc. Report,' 1897, p. 822.

‡ 'Quart. Journ. Micr. Sci.,' vol. 38, p. 220.

a reaction for "masked" iron, and thus in muscle this element and phosphorus would seem to have the same distribution.

The matrix of cartilage in *Menobranchus* and the frog gives a marked reaction for phosphorus, which seems in large part to be due to inorganic phosphates, for it appears soon after the addition of the nitric-molybdate reagent. The reaction in some specimens appears in areas or zones about cartilage cells or groups of them, the areas being separated by narrow zones in which no reaction was observed.

In the maturing and mature ovarian ova of Amphibia the cytoplasm is very rich in organic phosphorus, though not so much so as the nucleus. As the yolk spherules form, the amount of phosphorus-holding substance seems to lessen, possibly through its being taken up by the spherules which, even when freed from traces of lecithin, give a marked phospho-molybdate reaction in about six hours. It is to be noted that these spherules are also iron-holding.

In the spermatozoids of *Ascaris* the organic phosphorus is, on the whole, distributed as I have found the "masked" iron to be in these structures.* The "nucleus" gave a deep phospho-molybdate reaction, and a less marked reaction was obtained in the surrounding cytoplasm.

A diffuse but distinct reaction for phosphorus was obtained in human chorionic villi of the seventeenth (?) day, and in the placental villi of the sixth week and third and sixth months. A part of this reaction is due to inorganic phosphates, for it is obtained to a certain extent in about ten minutes after the nitric-molybdic reagent is added. The cat's placenta is very rich in inorganic phosphates distributed throughout the tissue, but more abundant in the deeper portions of the organ.

The colloid bodies of the thyroid are phosphorus-holding according to Gourlay,† who relied in his experiments on Lilienfeld and Monti's interpretation of the action of pyrogallol on the phospho-molybdate compound. Through the kindness of Dr. J. H. Elliott, I obtained an abundance of free colloid bodies of the ox, fixed in alcohol, which, after extracting the lecithin, I fused in a platinum cup with crystals of pure potassic nitrate. The mass, treated with a quantity of the nitric-molybdate solution, became yellowish, owing to the formation of the phospho-molybdate, the characteristic crystals of which could be found under the microscope. The reaction was not due to inorganic phosphates, for when thin sections of the ox's thyroid, freed from lecithin, were placed in the reagent, the phospho-molybdate compound formed very slowly, and the maximum reaction appeared only after eight hours. The presence of organic phosphorus in these elements does not, as Gourlay

* 'Quart. Journ. Micr. Sci.,' vol. 38, p. 229.

† *Op. cit.*

believes, necessarily indicate the existence of a nucleo-proteid in them, for Dr. Elliott has found that they digest in artificial gastric juice, leaving no residue, which would not be the case were a nucleo-compound present.

The outer portions of the rods and cones in *Menobranchus* and *Diemyctylus* are rich in organic compounds of phosphorus. It is more abundant in the rods than in the cones, and it is not due to lecithin, for the retinæ used were freed from the latter, nor is it owing to the presence of inorganic compounds of phosphorus, for the reaction is not obtainable during the first twenty minutes after placing the organs in the nitric-molybdate solution, while it is a progressive one up to the sixth hour. The chromatin of the nuclei of all the layers of the organ also gives the reaction.

The chromatophore in *Spirogyra* gives a weak phospho-molybdate reaction, and it appears to be due to the presence of an organic compound of phosphorus. A more marked reaction, however, is usually found in the pyrenoids in the same genus, and also in those of *Œdogonium*, *Cladophora*, and *Conferva*. In fresh specimens of *Spirogyra*, taken during daylight and put into the nitric-molybdate reagent, the pyrenoids appeared to give a stronger reaction than those of specimens taken at ten o'clock at night. The reaction develops slowly.

A diffuse reaction for phosphorus, slow in developing, was obtained in the cytoplasm of *Saccharomyces Ludwigii*. In apparently normal cells this may be the only reaction which will be obtained, but in cells cultivated in the sap of the iron-wood tree a spherical body occurs, at first sight like a nucleus, but frequently homogeneous, which after about ten hours' treatment with the nitric-molybdate reagent gives a reaction for phosphorus which may be very marked. This body is in no sense a nucleus,* nor does the phospho-molybdate reaction reveal any structure that corresponds to the latter. The fact that the "masked" iron in these cells has a distribution parallel to that of the organic phosphorus, also points distinctly to the absence of a nucleus.

In Cyanophyceæ the "central body" always gives evidence of the presence of organic phosphorus compounds. A stronger reaction for phosphorus was obtained in the iron-holding, chromatin-like granules which are to be found in the central body, or on its periphery, in *Tolypothrix* and *Oscillaria*. The "cyanophycin" granules, on the other hand, have not given any evidence of the presence of organic phosphorus except in some few filaments of a preparation of *Oscillaria tenuis*, in which case a marked reaction was developed in about an hour.

* I have discussed the nature of this body, 'Quart. Journ. Micr. Sci.,' vol. 38, p. 246.