XXXII. ON AN AUTOXIDISABLE CONSTITUENT OF THE CELL.

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SECTION I. INTRODUCTORY.

THE research described in this paper, though its actual point of departure had an intention quite different¹, ultimately resolved itself into an endeavour to throw light upon the chemical nature and physiological significance of a constituent of living tissues, which, though hitherto of unknown nature, has long carried a name. There can scarcely be a doubt that the substance to be described is the "Philothion" of de Rey-Pailhade.

In 1888 this author showed that yeast cells and aqueous extracts of yeast have the property of reducing sulphur to hydrogen sulphide [de Rey-Pailhade, 1888]. Later he showed that many animal tissues possess the same property. Throughout a long series of communications upon the subject he has courageously maintained the view that the labile hydrogen thus shown to exist in living cells has important respiratory functions. His views as to the probable nature of the hypothetical substance (named as above) which he supposed to carry this labile hydrogen, have been modified from time to time. In his latest writings he speaks of it as the hydride of a protein ("hydrure d'albumine"). After the publication of Heffter, to which reference will immediately be made, he accepted the view that the labile hydrogen exists in sulphydryl groups, HS—.

In 1901 Mörner employed the delicate nitroprusside reaction for the identification of cystein, and a little later Gola found by its use that a substance, which presumably contains the HS— group, is characteristically present

¹ My attention was directed to the subject in two separate ways. Some years ago I was endeavouring to discover if vitamins were to be found among sulphur-containing compounds, and was led part of the way towards the separation of the substance now described. A little later, acting on the suggestion that acid formation in muscle is a necessary factor in contraction I wished to discover if by chance in the absence of carbohydrate aceto-acetic acid from fat might function instead of lactic acid. This led me to apply the nitroprusside test to tissues. At this time Arnold's papers had not yet appeared and I was ignorant of Heffter's publication. The above mentioned enquiries were nugatory but they led to the present one.

in proliferating plant tissues. Buffa then showed that the same colour reaction is given by certain animal tissues.

In 1908 Heffter [1908] applied the nitroprusside test to a great number of tissues and tissue extracts and obtained positive results in many cases. Later, it would seem independently, V. Arnold [1911] showed that the reaction under proper conditions is displayed by practically all organised animal tissues. He at first described it as a protein reaction, though recognising that the plasma proteins gave a negative result.

A little later Arnold found that a strong nitroprusside reaction may be given by protein-free extracts of tissues, and came to the conclusion that free cystein was the responsible substance. Although he did not isolate cystein he considered that the evidence proved it to be a primary cell constituent in Kossel's sense.

Heffter, however, first gave definite form to the view that the presence of the HS— group, whatever its associations, may, owing to its labile hydrogen, be, in part at least, responsible for the reducing properties of protoplasm, and also, perhaps, indirectly for oxidations in the cell. Basing it upon known analogies he made the suggestion that during the autoxidation of the sulphhydryl group peroxide of hydrogen may be formed and the peroxide oxygen then transferred, with or without the influence of a peroxidase, to other substances in the cell. If, further, HS— groups can be supposed to act continuously as promoters of cell oxidations, their own spontaneous oxidation to —S—S— groups must be reversible. In this connexion Heffter called attention to the fact that cystine can be reduced to cystein by sodium sulphite, and suggested that in the cell some substance might, like the sulphite, act as an "acceptor" for the oxygen of the water molecule, leaving the hydrogen of this to reduce the disulphide group once more to sulphhydryl groups. Heffter's views have become generally familiar owing to the publication of an admirable discussion of the possibilities which underlie them by Thunberg in 1911.

Though very suggestive, these views have remained wholly theoretical. No experimental proof has been given of the existence of free cystein in the cell in the sense pictured by Arnold, nor has an HS— group been located in any other substance. Not without an isolation of the cell constituent actually responsible for the nitroprusside reaction, and a study of its properties, could Heffter's theoretical views receive experimental support.

Such an isolation is now to be described, together with a preliminary study of the substance from the standpoint of its possible functions in the reductions and oxidations of tissues. It proves to be a dipeptide, containing glutamic acid and cystein. It possesses properties which suggest that it may well play an important part in chemical events within the living cell. The methods of extraction have been such that there could be no possibility whatever of splitting off the dipeptide from other polypeptide material by hydrolysis.

SECTION II. THE NITROPRUSSIDE REACTION.

The nitroprusside test as applied by Heffter and Arnold consists in adding to tissue extracts, or to the fluid in which a piece of tissue is suspended, first a few drops of a moderately strong (5 %) solution of potassium nitroprusside and then excess of ammonium hydroxide. There can be no doubt that the use of ammonia is preferable to that of caustic alkali. But I have found that the colour reaction, as given by the tissue constituent to be described, is very greatly intensified when developed in the presence of a high concentration of certain ammonium salts, just as Rothera found to be the case when acetone or aceto-acetic acid is concerned. Doubtless the mechanism of the reaction is closely the same in each case, involving a reduction of the nitroprusside molecule. The colour produced resembles that of permanganate, and is less purple than when, under similar circumstances, ordinary sulphides act upon nitroprusside. If a small piece of fresh tissue, such as liver or kidney, be placed in, say, 5 cc. of a saturated solution of ammonium sulphate, two or three drops of a 5 % solution of sodium nitroprusside being then added, and finally excess of ammonium hydroxide, the tissue takes on a deep magenta colour. The reagents do not easily penetrate intact muscle fibres which should be disintegrated before applying the test. If the tissue be first heated with a little very dilute acid, acetic or other, the solution cooled, saturated with ammonium sulphate, and the test then applied, the colour will be found intensified. If the organ be ground up with sand it becomes maximal. From practically all the tissues examined the substance responsible for the colour reaction can be extracted by hot water and from such extracts it has been isolated.

SECTION III. ISOLATION OF A DIPEPTIDE.

Yeast has been chiefly used as a source of the substance to be described, but the same product has been obtained from mammalian muscle, and mammalian liver.

There was every reason in advance to expect that it would be found to have a very low concentration in the tissues. As already stated, the nitroprusside reaction is extremely delicate, and tissue extracts when completely freed from proteins contain only very small amounts of organic sulphur. The actual yield of H_2S when yeast cells or animal tissues are treated with sulphur (supposing this to measure sulphydryl groups with any accuracy) is minute.

The discovery of a method of isolation cost much labour; but could the nature and properties of the substance have been known in advance, greater difficulties might well have been expected.

Unfortunately the methods available for the separation of most types of native cell constituents are very limited. Fractional precipitation by salts of the heavy metals is at best clumsy, and as such precipitations are usually only in a very limited sense selective, it is difficult to make this method quantitative. Nevertheless after many efforts to reject it in favour of others,

it was finally adopted in the present endeavour. Needless to say, the basis of procedure was throughout largely empirical. The substance had, so to speak, to be teased out from its complex associations in the tissue. Fortunately the procedure, though tedious to describe and occupying much time in practice, is easy to repeat, and, given attention to details, invariably successful even in little practised hands.

Method of separation.

The separation from yeast may be first described. Minor modifications were made in the case of animal tissues which will be mentioned later.

Moist baker's yeast (45 kilos.) is boiled up with three successive quantities of tap water, large enamelled iron basins serving well for the purpose. For the above mentioned quantity of yeast about 10 litres of water may be used for each extraction. The extracts are filtered hot through Buchner funnels and the residue of the yeast well washed with hot water. The combined clear extracts are partially neutralised with dilute sodium hydroxide but left definitely acid to litmus. Neutral acetate of lead is then added until it ceases to precipitate. The precipitate is allowed to settle, the supernatant fluid syphoned off, and the precipitate then filtered off by use of several large Buchner funnels. It is very thoroughly washed with cold water.

The lead precipitate is next ground up in mortars and extracted with cold half-normal sulphuric acid until fresh extracts cease to give the nitroprusside reaction. With care this operation can be so conducted as to leave the lead sulphate free from the reacting substance, while the extracts contain relatively little free sulphuric acid. To the sulphuric acid extract, amounting usually to about 12 litres, uranium acetate is added until a little of the filtered fluid gives a strong brown colour with potassium ferrocyanide. Hot saturated barium hydroxide solution is next added to alkalinity and then in further excess until it ceases to produce a precipitate. The heavy precipitate is then filtered off at the pump and washed with cold water. At this stage a large proportion of certain polypeptide material originally precipitated by the lead, and all the phosphoric acid in the yeast extracts, are removed.

From the filtrate, which will measure perhaps 20 litres, barium is removed as sulphate, a small excess of sulphuric acid being left in the solution. The filtrate from the barium sulphate is now precipitated with acid mercuric sulphate solution¹, any large excess being avoided. The bulky mercury precipitate is well washed, suspended in water, and decomposed with H_2S . The filtrate and washings from the mercuric sulphide, freed from H_2S by a current of air, are measured (1000–1500 cc.) and enough sulphuric acid added to make the mixture roughly half-normal in acidity. Phosphotungstic acid dissolved in half-normal sulphuric acid is then added in rather greater excess than is necessary to produce the maximum precipitation. At this stage there is greater danger than at any other of removing part of the product sought. In

¹ Prepared as originally used by Hopkins and Cole [1901] for the isolation of tryptophan.

higher concentrations it is precipitated by phosphotungstic acid, but at the dilution indicated the loss is small. Its phosphotungstic compound is more soluble in excess of the reagent than those of the substances from which it has to be freed. If a little of the solution is tested by boiling with lead acetate and sodium hydroxide before and after the precipitation with phosphotungstic acid the reduction in the blackening given by the loosely combined sulphur should be slight. I have so far found it impossible to dispense with the use of phosphotungstic acid at this stage. The precipitate is filtered off, phosphotungstic acid removed from the filtrate by means of barium hydroxide, and the latter afterwards removed as sulphate. At this stage the solution, which need not measure more than about 4 litres, is again precipitated with the mercuric sulphate reagent, a perfectly white precipitate being obtained. This should be decomposed, and the mercury sulphide washed at the pump, with the use of as little water as safety allows, in order to keep the concentration of the product as high as possible. If a little of the solution be thoroughly boiled to remove all traces of H_2S it will be found to give an intense nitroprusside reaction, and a rich yield of sulphide when boiled with lead acetate and caustic alkali. The final treatment may be varied.

(a) In one method the copper compound may be used. The reduced dipeptide gives, like free cystein, a highly characteristic blue-grey precipitate with copper sulphate; but a more convenient method of separating it as a copper compound is to add to the solution from the above described second mercury separation, which contains a little free sulphuric acid, moist, freshly precipitated, copper hydroxide. When the vessel containing the solution is shaken the change from the appearance of the hydroxide added to that of the copper compound of the dipeptide is exceedingly characteristic. After the hydroxide has been added in excess, complete precipitation is secured by adding sodium hydroxide to the supernatant blue liquid until it is nearly but not quite neutral to litmus. The copper compound is decomposed with H_2S . The filtrate from the sulphide after removal of H_2S is made just alkaline with baryta and a stream of air is passed through it until complete oxidation of the dipeptide has occurred as shown by the disappearance of the nitroprusside colour reaction. The solution is then carefully adjusted so as to be entirely free from both barium and sulphuric acid, concentrated under reduced pressure to a bulk of about 10 cc. and then poured into 100 cc. of absolute alcohol. It should stand under the alcohol until it becomes wholly anhydrous and friable, and is then filtered off at the pump.

(b) Otherwise the dipeptide may be separated at the final stage as the lead compound. Neutral lead acetate precipitates the substance from neutral solution only when the latter is in the reduced form, and then, in the case of the pure substance, not completely. If, however, lead acetate be added to the solution obtained on decomposing the above described second mercury precipitate, the greater part of the product is thrown out. The lead salt is decomposed with H_2S and the solution treated exactly in the manner described

in the case of the copper compound. I have more than once thrown out in this way a first fraction with lead, and after removing lead from the filtrate have precipitated the remainder with copper. In such cases analyses of the two final products have closely agreed.

The substance is freely precipitated by silver salts, and completely by means of silver sulphate and baryta. I have not, however, found precipitation by silver of any advantage in purification. On a small scale one can dissolve the silver compound in hot water from which it separates on cooling. This seemed a characteristic property of which advantage might be taken; but on the large scale it is difficult to make use of it without danger of decomposition.

In making a preparation from animal organs the finely minced tissue should be allowed to extract for two or three hours with its own weight of water at 40°. The first extract is squeezed out through linen and the residue re-extracted. The extracts are finally mixed, filtered at the pump, the filtrates precipitated with neutral lead acetate and the procedure followed as with yeast. An alternative is to allow the tissue to stand with *N*/10 sulphuric acid (1 litre per kilo.) over night. The acid is nearly neutralised by the addition of 20 % caustic soda solution in small quantities at a time. The mixture is then heated and carefully neutralised as it reaches the boiling point. It is filtered at the pump and the residue extracted twice again by boiling with water. This latter method has generally been used for muscle. When it is employed it is, for some reason, advantageous to precipitate with uranium acetate at the first stage of treatment, instead of later. The reagent is added in saturated solution to the mixed extracts until it just ceases to produce a precipitate. The precipitate is filtered off and lead acetate applied to the filtrate. The sulphuric acid extract from the lead precipitate is subsequently precipitated with barium hydroxide alone.

The yield of the product from yeast has varied from 0.1 to 0.15 gram per kilo. From muscle it was about the same. The liver is, I think, undoubtedly richer in the substance, but in the one preparation made from it the weight of material taken was not recorded. Although the method of separation described is of course not quantitative, the figures given certainly represent the order of the amount actually present.

SECTION IV. PROPERTIES OF THE SUBSTANCE.

Evidence is given in later sections to show that the substance is present in the tissues in its reduced form, that is, as a dipeptide containing glutamic acid and cystein. When dissolved in this reduced form it is readily oxidised by atmospheric oxygen; most readily, as in the case of free cystein [Matthews and Walker, 1909], when its solutions are neutral or faintly alkaline. In the acid condition the HS— group of its cystein moiety is much more stable toward molecular oxygen. When in the oxidised form its solution can be easily reduced by zinc and sulphuric acid or by sodium sulphide. It will be

shown later that it is specifically reduced by tissue agencies. Needless to say the processes of reduction and oxidation can be easily followed by the use of the nitroprusside reaction, of which the oxidised form gives no trace.

As each molecule of the dipeptide contains only one HS— group oxidation, in producing the disulphide (—S—S—) grouping, must involve a linkage of two molecules. Preliminary molecular weight determinations have borne out this assumption.

Nearly all the final preparations of the substance have been made as described in the last section in the oxidised form, and this because in the final manipulation it is difficult to avoid partial oxidation of the reduced substance. It has proved impossible in the case of the oxidised dipeptide, which is a substance of high molecular weight (498), to obtain it crystalline, or to prepare a convenient crystalline derivative. I have recently found however that if all precautions are taken to avoid oxidation and the substance is separated while reduced, a crystalline preparation can be obtained. The dipeptide in this form is somewhat freely soluble in alcohol so that a mixture of alcohol and ether was used for its final precipitation (cf. last section). After it had stood under the mixture for three days its solubility in water was considerably reduced. When dissolved in hot water it separated on cooling as thorn-apple crystals which upon recrystallisation were replaced by acicular aggregates. The facts suggest that the dipeptide while standing under alcohol-ether is probably converted into the diketopiperazine anhydride. Advantage will be taken of this observation in future work.

The oxidised substance as employed for the analyses given in the next section is a snow-white non-hygroscopic powder. If heated quickly it softens at 165°–167°. At 182°–185° it melts with evolution of CO₂ and the melt runs up the tube. All its preparations have behaved in the same way either when separate or mixed.

It is exceedingly soluble in water, but apparently in no organic solvent. Its behaviour towards precipitants will have been sufficiently indicated in the previous section.

It is easily hydrolysed by mineral acids yielding glutamic acid and cystein, but it appears to be wholly resistant to the proteolytic ferments of the tissues. The effect of trypsin has not been studied.

Solutions of the reduced dipeptide when shaken with sulphur give off H₂S.

SECTION V. EVIDENCE OF CONSTITUTION.

Before it was actually obtained in substance, I was led to expect that the compound to be isolated might have the constitution of a dipeptide. By the use of nitrogen determinations made in solutions obtained at various stages of fractionation it was observed that constancy was arrived at when the amino-nitrogen estimated by van Slyke's method was about half the total nitrogen, and when the former was doubled after boiling the solution with mineral acids.

At such a stage the ratio of sulphur to nitrogen also proved constant. It should be noted that for a reason already mentioned most of the analytical studies have been made upon the oxidised product. As was pointed out in the last section, oxidation (as in a current of air) should involve the production of a disulphide grouping which must link two dipeptide molecules together and so nearly double the molecular weight of the product. This, of course, will not affect the results of hydrolysis, except that cystine should be obtained instead of cystein.

Hydrolysis.

(a) *Separation of glutamic acid.* Two grams of the oxidised product from yeast were boiled for 8 hours with 100 cc. of 25 % aqueous hydrochloric acid. The solution when concentrated under reduced pressure to about 10 cc. and cooled, deposited crystals which resembled those of the hydrochloride of glutamic acid. They were filtered off through a small linen filter, and washed at the pump with strong HCl. The filtrate was saturated ice-cold with HCl gas, and stood in ice over night. Another crop of crystals was filtered off, and the filtrate again concentrated and again saturated with HCl. A third crop was similarly obtained, all three crops being then united, dissolved in a minimal quantity of water, and again saturated with HCl at 0°. The crystals were again filtered off and the filtrate mixed with the original filtrates. The mixed filtrates on standing for a week deposited a further fraction of a hydrochloride. By careful treatment a final combined product was obtained which had all the characters of pure glutamic acid hydrochloride. It weighed 1.06 g. or 53 % of the product hydrolysed. A dipeptide of cystein and glutamic acid would have a molecular weight of 250, and the corresponding disulphide compound one of 498. The latter would yield 59.03 % of glutamic hydrochloride. The above yield from 2 g. of product is therefore equal to 90 % of theory.

The melting point of glutamic hydrochloride is often given as 198°. I have on previous occasions found it to be higher than this. A very pure carefully analysed product made from wheat proteins by Dr H. Raistrick showed softening at 200° and melted at 206° (uncorr.) with decomposition. Heated side by side with this, the product prepared as above behaved in an identical manner, and a mixture of the two gave precisely the same melting point.

Two other preparations, from different supplies of yeast, similarly treated, have given similar results. I have found that glutamic hydrochloride, being so highly dissociated in aqueous solution, will, when dissolved in a minimum of water and its solution mixed with six to eight times its bulk of alcohol, slowly yield crystals of free glutamic acid. This is a convenient method of preparation when one is working on a small scale. Small quantities of the hydrochloride prepared from the hydrolysis of yeast products were treated in this way side by side with a pure preparation from wheat gliadin. The crystals

in each case were identical, having the characteristic rhombic octahedral form. When filtered off, washed and dried, they melted in every case at 192° (uncorr.).

The nitrogen of two preparations of the hydrochlorides was determined by Kjeldahl.

Found (a) 9.47 %, (b) 9.48 %. Calculated 9.52 %.

(b) *Separation of cystine.* Filtrates from the glutamic acid preparations after evaporation on the water bath to remove HCl (the residues being subsequently dissolved in dilute sulphuric acid and precipitated with mercuric sulphate) have in all cases yielded cystine. For quantitative work, however, the original products were hydrolysed with sulphuric instead of hydrochloric acid.

Two grams of a yeast product were boiled for eight hours with 25 % sulphuric acid. The solution was then diluted with four times its volume of distilled water and precipitated with the same mercuric sulphate solution as that employed for the separation of the original product. A heavy white precipitate was produced which was filtered off and washed at the pump. The filtrate gave no sulphide when boiled with lead acetate and caustic soda. The precipitate was decomposed with H_2S , the filtrate from the mercury sulphide was freed from H_2S and sulphuric acid then removed by a slight excess of barium hydroxide. While still just alkaline it was aerated for some hours until the nitroprusside reaction had disappeared. Excess of baryta was then exactly removed. On standing without evaporation the solution (*circa* 250 cc.) deposited a small amount of cystine in typical hexagonal plates. On concentration to small bulk a white product separated which under the microscope appeared to consist of acicular aggregates. From the mother liquors of this fraction a second yield of colourless product was obtained on further evaporation. The two together weighed 0.59 g. or 60 % of theory. Although mercuric sulphate precipitates cystine from acid solutions with great completeness, it is, for some reason, difficult to recover the product quantitatively. My colleague Dr T. S. Hele, who, some years ago, studied the methods for separating this amino-acid very carefully, tells me that when he precipitated pure cystine solutions by mercuric sulphate and the cystein which resulted on decomposing the precipitate with H_2S was allowed to oxidise in the air, the amount of cystine recovered by him was of the order of 60 % only. As all who have experience of the matter are aware, the quantitative separation of cystine from other amino-bodies is always difficult¹.

Cystine was obtained in a similar manner after hydrolysing other preparations from yeast. Three such products were combined, dissolved in hydrochloric acid, and the perfectly clear solution neutralised with ammonia. The cystine separated in uniform typical hexagons.

0.3596 g. was dissolved in 25 cc. of 10 % HCl.

¹ For an experience with regard to the destruction of cystine during acid hydrolysis see van Slyke [1911].

In an 18.9 cm. tube it gave with the sodium flame a laevo-rotation of 5.45° .

$$[\alpha]_D = -200^\circ.$$

The specific rotation of *l*-cystine from proteins and keratin is usually given as -205° . The high rotation distinguishes cystine from other amino-acids, and the above figure indicates that the substance dealt with is normal *l*-cystine.

The nitrogen was determined by a micro-Kjeldahl method, the apparatus and reagents being controlled by blanks and by estimations made upon a specimen of pure cystine from hair.

(a) 11.39 %, (b) 11.56 %. Calculated 11.66 %.

Sulphur was estimated by means of a micro-Carius method which had previously given good results with similar quantities of pure cystine from hair.

0.067 g. gave 0.1169 g. $\text{BaSO}_4 = 26.45\%$ S (calculated 26.45 %).

After the separation of cystine from the products of hydrolysis and removal of the sulphuric acid the glutamic acid was either separated as the hydrochloride or precipitated by mercuric acetate and sodium carbonate and then liberated as the free acid. In all cases the properties of the product were those of normal *d*-glutamic acid. The final residues from several preparations of hydrolysed material (the glutamic acid and cystine having been in each case removed on the lines described) were combined, after removal of HCl when necessary. No trace of any other amino-acid could be obtained from these residues by crystallisation or otherwise. A minute crystalline fraction proved to be glutamic acid, and the mother liquor from this gave no more than a very small gummy residue with a slight mercaptan-like odour.

Distribution of nitrogen.

In the case of a compound suspected of being a simple peptide good evidence of constitution should be obtained by comparing with the total nitrogen the amino-N before and after hydrolysis. When, however, a substance contains cystine it must be remembered that this amino-acid gives abnormally high values in determination by van Slyke's method. Allowing for this fact the substance under description in this paper gives on these lines satisfactory evidence of being a dipeptide.

In the case of cystine van Slyke obtained the values 12.52 and 12.68 for the percentage of amino-nitrogen; theory requiring 11.67. With the reagents employed in my laboratory, however, which gave normal values for other amino-acids, considerably higher values than these were got for pure cystine and also for cystein. In a consistent series of determinations the value 14.5 was obtained for both substances. As the precise reason for the abnormal behaviour of these thio-acids in van Slyke's process is unknown it is not quite clear how a simple peptide containing one of them would behave, and a correction based on the above values would not necessarily be satisfactory.

Somewhat high values for the amino-N should at any rate be expected. A product from yeast gave the following figures:

Total N 11.9 %; amino-N 6.8 %; amino-N after hydrolysis 13.3 %.

If a calculated addition to the theoretical figures can be based on the data just given for cystine, and if it be applied to the cystine half of the molecule only, the amino-N for the oxidised product would, on the assumption that it is derived from a dipeptide, be 6.3 %, and, after hydrolysis, 12.6 %.

It is unfortunate that the properties of cystine intrude in the estimations, but in the case of a substance which on hydrolysis yields two amino-acids, and two only, and yields an amount of amino-nitrogen agreeing so closely with what special considerations would lead one to expect—this amino-nitrogen being doubled on hydrolysis—there can be no doubt that a dipeptide is in question. In the case of even a tripeptide, if it contain only monamino-acids, the amino-nitrogen must be trebled after hydrolysis and the increase grows, of course, proportionally greater with increase in the number of constituent amino-acids. Other products from yeast besides the above have given quite similar figures for amino-nitrogen and the doubling on hydrolysis has proved to be a constant happening.

Elementary analyses.

The analyses have, for a reason already discussed, been made nearly always on the oxidised product, but except in the case of hydrogen, the effect of the oxidation upon percentage composition is of course extremely small. The dipeptide $C_8H_{14}O_5N_2S$ with a molecular weight of 250 would yield with loss of two H atoms a disulphide derivative of molecular weight 498. In the case of such a substance the evidence for constitution to be got from elementary analyses is of less importance than that based upon facts such as have been already discussed.

As therefore the material costs so much labour to prepare there was a temptation to make the analyses on quantities as small as possible, though the presence of sulphur and the consequent necessity of using lead chromate made it undesirable to rely upon micro-combustions.

The results agree satisfactorily with the composition of a dipeptide having the constitution suggested by the other evidence. The nitrogen figures have, it is true, been persistently somewhat high. This is probably due, in part at least, to the presence of small amounts of ammonia in the final product. It will be remembered from the description of the preparations that immediately before evaporating the final solutions they were oxidised by aeration while only barely alkaline from barium hydrate. The process involves the passage of many litres of laboratory air, and from lack of realisation of the danger no attempt was at first made to trap off ammonia. Once the slight excess of baryta had been removed the acid nature of the free substance would have prevented removal of ammonia during evaporation. On an occasion when the above process was omitted and the product prepared for analysis in a reduced

form the theoretical figure was obtained (11.25 %) for nitrogen. The following complete analysis was made with the use of normal quantities of material. To other preparations micro-methods have been applied.

The sulphur was estimated by fusion with sodium peroxide and the nitrogen by Kjeldahl.

Product from yeast finally purified through the lead compound:

Found C, 38.21; H, 5.12; S, 12.31; N, 11.70.

Calculated C, 38.55; H, 5.22; S, 12.85; N, 11.24.

Five other determinations of nitrogen were made upon different products, all by a micro-Kjeldahl method. Of these the first two high results were obtained early in the research; the others after more experience had been gained.

Nitrogen % (12.37, 12.10); 11.68, 11.55, 11.37 (calculated 11.24).

The following results for sulphur were obtained from diverse products. All determinations had to be made upon small quantities of material—from 0.060 g. to 0.100 g.

S = 12.30, 13.22, 11.68, 12.70 %.

Mean = 12.40 %; calculated 12.80 %.

All the data so far given apply to products from yeast. A preparation from ox-muscle had exactly similar properties and gave 11.75 % N by Kjeldahl. After hydrolysis the glutamic acid, as obtained pure, amounted to 88 % of the theoretical yield. Cystine was identified but not estimated. A small preparation from liver (0.4 g.) had again identical properties. When it was mixed with a preparation from yeast the behaviour on heating was unaffected. Glutamic acid and cystine were fully identified in the products of hydrolysis but they could not be estimated.

There can be no doubt from the evidence that the compound isolated is, in its reduced form, a dipeptide of which the constituents are glutamic acid and cysteine. I have not yet determined the relation of one amino-acid to the other. An endeavour to do this is now in progress.

Until the constitution is finally established it may be premature to suggest a name for the substance. But, provisionally, for easy reference, the name *Glutathione* will perhaps be admissible. It leaves a link with the historic *Philothion*, has the same termination as *Peptone*, which has long served as a name for the simpler peptides, and is a sufficient reminder that the dipeptide contains glutamic acid linked to a sulphur compound.

SECTION VI. DISTRIBUTION.

The statement of Gola that the nitroprusside reaction is given by proliferating plant tissues I have confirmed in many instances, but its intensity is usually far less than in animal tissues. The few species of bacteria examined have all displayed it. In the animal it is given by all the tissues tested except in the case of connective tissues. Blood plasma and serum give, as Arnold found, no trace of the reaction. It is characteristic indeed of cellular tissues

with active metabolism. The constituents of the fowl's egg in native condition give no trace, but a thirty hours' embryo if observed under a dissecting microscope will, especially if first flooded with a little weak acetic acid, be seen to give a vivid reaction throughout its mass.

I cannot pretend to have explored the zoological field at all thoroughly but have obtained the reaction from tissues and tissue extracts of the following as types: muscle and other tissues of the earthworm; adductor muscle of the oyster (in most tissues of this mollusc the reaction is very slight); muscles of the blow-fly; muscles and other organs of the lobster; muscles of the skate and of the cod; other organs of fish not examined; all organs of the frog; all organs of every mammal examined. Except in the case of the muscles the reaction is much less intense in cold blooded animals than in warm. It is always possible, however, that this is a matter of tissue equilibrium; more of the substance may be in the oxidised form.

It would of course be going much too far at present to claim that the display of the nitroprusside reaction proves the presence in all these localities of the substance described in this paper. It may in different groups of animals be represented by different if analogous substances; but it is noteworthy that the cause of the reaction is the same in structures so diverse as the yeast cell and mammalian organs such as muscle and liver.

With regard to pathological relations it may be stated that actively growing cancer cells seem to show a nitroprusside reaction of surprisingly low intensity; but I have seldom had the opportunity of examining growths soon after their removal from the body.

SECTION VII. PHYSIOLOGICAL RELATIONS.

Observations will now be briefly described which seem to show that the dipeptide exercises real functions in the chemical dynamics of the cell. An attempt to relate its properties more completely with other known facts and to current theories concerning tissue respiration will be made in a later communication.

As already stated the dipeptide, when in neutral or slightly alkaline solution, spontaneously oxidises in air at ordinary temperatures though more slowly than does pure cystein. As Bach [1912] has pointed out the uptake of molecular oxygen by a substance undergoing autoxidation must by Ostwald's law of successive reactions necessarily involve preliminary peroxide formation.

The less stable state, say $R \begin{smallmatrix} \diagup O \\ | \\ \diagdown O \end{smallmatrix} + R$, however brief its duration, must precede

the more stable state $R : O + R : O$. It is therefore not without significance in any case to know that a definite cell constituent can be isolated which belongs to the class of autoxidisable substances. The existence of such, in some form or other, is assumed in accepted theories concerning oxidase systems.

But such oxidase systems, able to induce limited oxidations in constituents

which do not themselves react with molecular oxygen, are not the only type of oxidative mechanisms represented in the cell. Indirect oxidations induced by the action of catalysts in the presence of suitable hydrogen acceptors have recently received much attention, and would seem to play a very real part in biological phenomena. Bach has very fully studied what he calls "hydrolytic oxidation-reduction" reactions in which, under the influence of such catalysts as metals of the platinum group, the reduction of one substance by the hydrogen ion of water is associated with the simultaneous oxidation of another substance by the hydroxyl ion. Such a case, with an organic catalyst taking the place of the metal, is the well-known Schardinger reaction of fresh milk. Milk neither reduces methylene blue nor oxidises an aldehyde, but if both are present the former substance acts as a hydrogen acceptor and the latter as an oxygen acceptor and simultaneous reduction and oxidation are the result. Bach has shown that the same or a similar catalyst is present in the tissues.

A catalytic mechanism different from, though closely related to that just mentioned, may also exist in the cell, unless indeed as in the view of Wieland [1914] it is a more general mechanism covering the supposed activities of those already mentioned.

Wieland sees the essential cause of certain typical biological oxidations not in the activation of oxygen but in the activation of hydrogen. The proof that this activation occurs renders unnecessary he thinks any attempt to give a general application to the Engler-Bach peroxide theory. On the other hand Wieland's contention somewhat modifies Bach's conception of hydrolytic oxidation-reduction.

Starting with the conception of a catalyst, which acts as a "dehydrase" and confers lability upon hydrogen atoms, the assumption that oxydases, reductases, and "mutases" separately exist becomes from Wieland's standpoint superfluous.

His conceptions are mainly supported by a careful quantitative study of the action of Schardinger's ferment on salicylaldehyde. The aldehyde is assumed to act in the form of its hydrate (an assumption which would seem after all to negative any wide divergence between the views of Wieland and Bach). The enzyme activates two of the hydrogen atoms which are then made available for an acceptor. This acceptor may be (1) molecular oxygen, (2) such reducible substances as are represented by methylene blue, or (3) in a particular case the acceptor may be a molecule of the aldehyde itself in the unhydrated form. As an end result we have, in case (1) a frank oxidation of the aldehyde; in case (2) an observed reduction associated however with indirect oxidation of the aldehyde; in case (3) on the other hand the Cannizzaro reaction—the so-called "mutase" effect:

Whether any one of these end results is attained, or a mixture of all three, depends (as Wieland's quantitative results seem to show) upon the concentra-

tion and speed of reaction of the various acceptors. The most important hydrogen acceptor for energy production in the cell is clearly oxygen, but the reduction of other acceptors may be equally important for other aspects of cell activity.

Thunberg [1920] has accepted Wieland's conception of the events and in a remarkable paper recently published he has used the methylene blue technique to supply evidence as to the probable nature of intermediary metabolites. Tissue (muscle) deprived of its power of reducing methylene blue by thorough washing with water but still containing the catalyst is suspended in a weak solution of the pigment. Small quantities of the substances under investigation are then added. The methylene blue acts as hydrogen acceptor. If the second substance can act as a hydrogen "donator" then the dye is reduced. The ferment acts as a hydrogen "transportase." Only a limited selection out of a large number of substances tried could induce by their presence the reduction in question, and that they should do so is taken by Thunberg for presumptive evidence that they are—or are closely related to—products which appear in normal intermediary metabolism.

I have said this much to indicate the importance which is now attached to the presence of acceptors, and especially of hydrogen acceptors, co-existing with substances to be oxidised. The same importance is attached to hydrogen acceptors in fermentations.

The dipeptide when in the disulphide (oxidised) form can act of course under conditions as a hydrogen acceptor, and the labile hydrogen of the resulting sulphhydryl group, under other conditions, as an oxygen acceptor.

As already remarked, however, dynamic functions in the cell cannot be claimed for the substance unless it can be shown that its oxidation and reduction are brought about in a reversible manner by factors present in the cell itself.

That fresh tissues possess a reduction potential such as to induce rapid reduction of the substance can be at once shown by the simplest technique. In one test tube are placed a few cc. of distilled water and in another an equal amount of a weak solution of the dipeptide in its oxidised form which gives of course no nitroprusside reaction. A small weighed piece of fresh tissue (liver, kidney, muscle, etc.) is then dropped into each. If the tubes be then placed in a bath at 35° it will be found that the fluid of that containing the added dipeptide will, after an hour or two, give the nitroprusside reaction with an intensity out of all proportion to the fluid of the control tube. The latter may give a slight reaction due to diffusion from the tissue of preformed reduced dipeptide. Under anaerobic conditions the change goes faster. Antiseptics may be used. The reduction may be followed in its stages by placing crystals of ammonium sulphate mixed with nitroprusside on a tile. A couple of drops of the fluid followed by one of ammonia will show the increasing intensity of the colour reaction. It is difficult to obtain the reducing system in solution; but the dry powder obtained by grinding a tissue with sand under alcohol,

washing the residue with fresh alcohol on a filter, and allowing it to dry in the air, reduces efficiently. Such a powdered preparation from liver, deprived so far as possible of connective tissue, has served well for quantitative observations.

It is next easy to show that conditions exist on the other hand in which factors present in tissues can promote oxidation of the reduced dipeptide. It is a circumstance familiar to all who have studied the reducing power of excised tissues, by the use for instance of methylene blue as an indicator, that the reduction potential rapidly falls off as survival processes progress. It must of course be remembered that conditions for reduction and oxidation vary with the substance concerned. In the case of the dipeptide survival changes involve a complete reversal of the existing relations. A tissue which has stood under aseptic conditions for a sufficient time fails to exhibit the nitroprusside reaction and it can be shown without difficulty that conditions then develop under which the reduced dipeptide is oxidised. As this oxidation goes on anaerobically, some other substance must now act as a hydrogen acceptor. Especially upon actual autolysis does this change in the equilibrium of reducing and oxidising agencies occur. If disintegrated tissues be suspended in four to five times their weight of chloroform water and allowed to autolyse in corked flasks at 37° the fluid during the first few hours will show a strong nitroprusside reaction. This gradually fails in the fluid, and, ultimately, after a period varying with the particular tissue, also in the incompletely autolysed tissue fragments. The end-point occurs soonest with the kidney; more slowly with liver, because of its original high content of the reduced dipeptide; and still more slowly in the case of muscle. If, when the colour reaction has ceased, a further quantity of reduced dipeptide be introduced so as to restore a strong reaction to the fluid, this will be found also to disappear on standing. The process goes on with undiminished velocity, and indeed seemingly faster, under anaerobic conditions. That oxidation has occurred is shown by the fact that the process is easily reversible by reduction. If some of the fluid which has ceased to give the colour reaction, and especially if its content of dipeptide had been increased on the lines mentioned, be boiled, filtered, and placed when cool over a piece of perfectly fresh tissue an intense nitroprusside reaction once more develops.

The significance of this change of oxidation potential during survival and post-mortem changes need not be here discussed. For the moment I am concerned only to show that there exist in tissues mechanisms for both reduction and oxidation of the dipeptide. If *in vitro* the one mechanism has to disappear before the other can be displayed, it is yet perfectly probable that in the organisation of the cell both mechanisms are employed.

Considerations may now be put forward which indicate that the dipeptide does as a matter of fact play a real part in cell dynamics.

Fresh tissues of course reduce methylene blue, and so, when itself in the reduced condition, does the dipeptide. But as has just been shown the former

also reduce the latter. It would seem then that a mechanism in the tissues has a greater reduction (or lower oxidation) potential than the HS— group of the dipeptide. As a matter of fact, however, the exact relations depend upon the hydrogen ion concentration of the medium. It should be remarked that the reduced form of the dipeptide is more stable on the acid side of neutrality. The following facts are of interest.

If the substance in the oxidised (disulphide) form be added to a solution of methylene blue in contact with fresh tissue, then, if the reaction of the system is even slightly on the acid side of neutrality, say at $P_H = 6.8$, the reduction of the dye is greatly slowed, as comparison with a control preparation will at once show. The dipeptide acts here simply as a hydrogen acceptor and competes with the methylene blue in this respect, delaying or (according to its concentration) even preventing the reduction of the latter. If, however, the reaction of the medium be adjusted to $P_H = 7.4$, or made very slightly more alkaline than this, the relations change. The normal rate of reduction of methylene blue by the tissue used is then markedly *accelerated* by the addition of the *oxidised* dipeptide. The phenomena are best observed under anaerobic conditions and the observations should be made in test tubes which can be evacuated, or which are corked and so fitted with glass tubing that the air can be replaced by pure nitrogen. As the tissues contain their own supply of the substance the contrast between the behaviour of a preparation to which dipeptide has been added and a control preparation is greater if the tissue used is first washed several times with sterilised distilled water. This removes a considerable part of the pre-existing dipeptide especially from the surface of the tissue. The tissue powder from alcohol, as described above, acts well and may also be washed before use¹. Although I think the fact is not commented upon by authors who have used washed tissues in connexion with methylene blue reductions the concentration of the enzyme is reduced by extraction with water. In the experiments under discussion washing increases the contrast, but may increase the actual time required for reduction. The following data will illustrate what has been said. In the experiments given the tissue preparation was in each case the liver of the rat, ground under alcohol, air dried, and afterwards washed with distilled water. Similar results have been obtained, however, with other tissues, washed and unwashed. The first comparison shows the reversal in the effect of added dipeptide when the reaction of the system changes. The tubes were filled with nitrogen.

Tissue preparation	Oxidised dipeptide added	Methylene blue 1 : 5000	Water	P_H at beginning	Reduction time
0.5 g.	0	0.5 cc.	5 cc.	7.5	3 hrs. 35 mins.
"	4 mg.	"	"	"	1 hr. 30 mins.
"	4 "	"	"	6.2	5 hrs. 20 mins.
"	8 "	"	"	"	15 hrs. +

¹ It is noteworthy that the tissues of small animals such as the rat show under all circumstances a greater reducing power than those of larger animals.

The following shows the acceleration of reduction due to the addition of oxidised dipeptide to the system in neutral or slightly alkaline conditions. The solution in each case was a phosphate buffer solution, the P_H being adjusted after the addition of the dipeptide. Chloroform was added to all the tubes and there was a layer of toluene on the surface. The tubes were filled with nitrogen.

Tissue preparation	Buffer solution	Methylene blue	Oxidised dipeptide added	P_H	Reduction time
0.2 g.	5 cc.	0.5 cc.	0 mg.	7.4	5 hrs. 45 mins.
"	"	"	4	"	2 " 40 "
"	"	"	0	7.8	3 " 45 "
"	"	"	4	"	1 hr. 40 "
0.5	"	"	0	"	2 hrs. 30 "
"	"	"	4	"	0 hr. 55 "

The conditions can be adjusted to give more rapid reduction and sharper contrasts than the above.

Tissue	Buffer solution	Methylene blue	Oxidised dipeptide	P_H	Reduction time
Liver .15 g.	5 cc.	0.3 cc.	4 mg.	7.4	0 hr. 25 mins.
"	"	"	0	"	6 hrs. +
Muscle (washed) 0.2 g.	"	"	8	"	0 hr. 10 "
"	"	"	0	"	3 hrs. +

It seems clear that in the reactions described the —S—S— group of the oxidised dipeptide acts first as a hydrogen acceptor and under conditions of even slight acidity the resulting HS— groups are too stable to transfer the hydrogen to another acceptor. In neutral or slightly alkaline solution on the other hand the hydrogen is transferred to the methylene blue. If this view be the right one we have to recognise the important fact that the two reactions involved in the transference of hydrogen to the disulphide group under the influence of a tissue enzyme, and its subsequent transference from sulphydryl groups to the methylene blue acceptor, together run faster than the single reaction in which the dye is directly reduced by the tissue enzyme. The dipeptide then possesses what are essentially catalytic properties and would be fairly spoken of as a co-enzyme. Indeed, if tissues are very thoroughly washed, as in the observations of Thunberg referred to earlier, so that their power to reduce methylene blue is practically removed, the restoration of this power when the oxidised dipeptide is added at once gives the impression that it has the function of a co-ferment exerted on the lines suggested.

Thunberg's work, however, suggests of course another possibility. He found as already stated that a number of substances such as succinic acid, malic acid, and the like, including, as is noteworthy, glutamic acid, can, under defined conditions, restore reducing power to tissues deprived of it by washing. It is possible therefore that the dipeptide in the experiments just described was when in neutral or alkaline solution merely acting as a substance of this class. I am convinced however that this is not the case. The

compounds which act as hydrogen "donators" in Thunberg's experiments undergo an irreversible oxidation due to the removal of hydrogen atoms attached to carbon. I have evidence to show that this does not occur in the case of the dipeptide. Oxidation and reduction of this substance by tissue systems involve the thio-group alone and the strictly reversible change

It is for this reason that the substance may be justifiably supposed to have definite and permanent functions in the cell.

The question arises whether the dipeptide has relations with the respiratory substance of Meyerhof [1918]. Preliminary observations have shown that it definitely increases the uptake of oxygen by washed yeast and washed muscle, but quantitatively the effect is small compared with the results described by Meyerhof himself. I have not yet tested its effect upon the reducing power of washed yeast on the lines of Harden and Norris's [1914] experiments.

A more complete study of its behaviour in such connexions is in progress.

There can be little doubt that the chief significance of the occurrence of cystein in the dipeptide, rather than free, lies in the fact that it is thereby protected from metabolic breakdown. Why, since both the glutamic acid and the cystein contained in it are the physiological forms of these amino-acids, the dipeptide should be resistant is not fully clear; but there is no doubt of the fact.

SUMMARY.

A substance responsible for the nitroprusside reaction which is given by nearly all animal tissues, and was applied by Heffter and by Arnold in proof of the presence of sulphhydryl groups in the cell, has been isolated from yeast, from muscle, and from mammalian liver. It has the properties of the philothion of de Rey Pailhade.

Evidence is given to show that the substance is a dipeptide containing glutamic acid and cystein. The relation of the two amino-acids in the molecule has not yet been determined. Though present in low concentration (0.01 to 0.02 % of the fresh tissue) the dipeptide contains practically the whole of the non-protein organically bound sulphur of the cell.

The substance is autoxidisable, and, owing to the changes in the sulphur group of its cystein moiety from the sulphhydryl to the disulphide condition and *vice versa*, it acts readily under varying conditions either as a hydrogen acceptor or an oxygen acceptor (hydrogen "donator"). It can be both reduced and oxidised under the influence of factors shown to be present in the tissues themselves.

Evidence is discussed which suggests that the substance has actual functions in the chemical dynamics of the cell.

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REFERENCES.

- Arnold (1911). *Zeitsch. physiol. Chem.* **70**, 300 and 314.
 Bach (1912). *Biochem. Zeitsch.* **38**, 154.
 Harden and Norris (1914). *Biochem. J.* **8**, 100.
 Heffter (1908). *Med. Naturwiss. Arch.* **1**, 81; *Maly's Jahresb.* 1908.
 Hopkins and Cole (1901). *J. Physiol.* **27**, 418.
 Matthews and Walker (1909). *J. Biol. Chem.* **6**, 289.
 Meyerhof (1918). *Zeitsch. physiol. Chem.* **101**, 165.
 de Rey Pailhade (1888). *Compt. Rend.* **106**, 1683; **107**, 43.
 van Slyke (1911). *J. Biol. Chem.* **10**, 15.
 Thunberg (1920). *Skand. Arch. Physiol.* **40**, 1.
 Wieland (1914). *Ber.* **47**, 2085.