XXIX. GLUTATHIONE.

SYNTHESIS.

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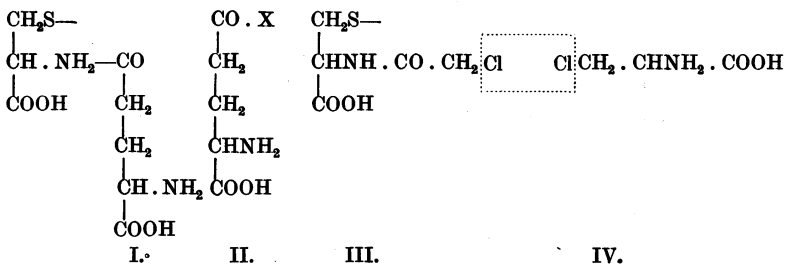
In a previous paper [1923] the authors, with Mr J. H. Quastel, put forward the view that glutathione possesses the constitution represented by Formula I.



I. II. III. IV.

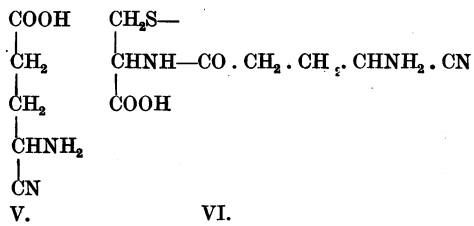
The evidence on which they based that view was derived from a study of the breakdown of the glutathione molecule in various directions. The fact that condensation of glutathione with 2 . 3 . 4-trinitrotoluene (which condenses with primary amine groups) and subsequent hydrolysis of the product yielded free cystine showed that in glutathione the free amino group was that of the glutaminic portion. Confirmation of this was given by treatment with nitrous acid followed by hydrolysis, when α-hydroxyglutaric acid was isolated. Oxidation by hydrogen peroxide gave a compound from which succinic acid was obtained after hydrolysis. This, by analogy with the work of Dakin [1905] on the oxidation of amino acids, was held to indicate that that carboxyl group of glutaminic acid remote from the amino group was involved in the peptide linkage. It was pointed out, however, that the constitution of glutathione could only be regarded as definitely established when a substance of the proposed formula had been synthesised and shown to be identical with the natural product.

The problem presented in the synthesis—that of linking the amino group of cystine with that carboxyl group of glutaminic acid which is remote from

the amino group—resolved itself into the preparation of the appropriate mono-acid halide of glutaminic acid (II) or of some substance which can subsequently be converted to glutaminic acid.

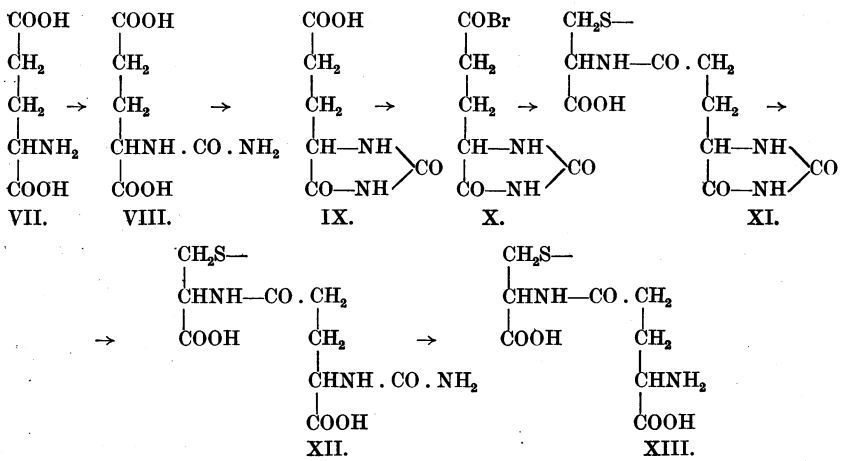
Alternatively, the following synthesis, which, though indirect, would definitely establish the constitution, was considered. Cystine readily condenses with chloroacetyl chloride [Fischer and Suzuki, 1904] giving dichloroacetyl-cystine (III). By elimination of chlorine from this and β-chloroalanine (IV) glutathione should result. This method was abandoned, however, not only because of its tedious nature—it involves the preparation of serine—but also because it was found that the condensation of even simple chloro compounds in the presence of "molecular" silver, sodium, etc., was extremely unsatisfactory.

The preparation of the compound V by Strecker's reaction on the semi-aldehyde of succinic acid presents itself as a solution until it is remembered that after halogenation and coupling of the acid halide to obtain the compound VI, prolonged hydrolysis would be necessary to convert the nitrile group to the required carboxyl group. Such hydrolysis would certainly disrupt the peptide linkage and thus defeat the end in view.



V. VI.

The method finally adopted is shown in the scheme below.



VII. VIII. IX. X. XI. XII. XIII.

Glutaminic acid (VII) was converted to the hydantoinpropionic acid (IX) by the method of Dakin [1919]. This, on treatment with phosphorus tribromide is converted to the acid bromide (X) which is then coupled with cystine

dimethyl ester hydrochloride [Fischer and Suzuki, 1905] in the ordinary way. The opening of the hydantoin ring presents a problem similar to that encountered by Dakin in his synthesis of β-hydroxyglutaminic acid. Boiling with barium hydroxide, he found, brought about complete decomposition of the bulk of his substance (hydantoin-β-bromopropionic acid). Hydantoin-acrylic acid, on prolonged treatment with the same reagent, gave off ammonia and carbon dioxide with formation of the amino acid, but considerable losses were again experienced. His alternative method of heating in a sealed tube with fuming hydrochloric acid at 130° is obviously inadmissible in the present case owing to the presence of a peptide linkage. Boiling for a short time with calcium hydroxide, however, appeared to open the ring with, probably, the formation of a uramino acid (XII), and with the destruction of only a small amount of cystine. Rohde [1918] has shown that uramino acids, treated with nitrous acid, lose half their nitrogen. She did not, however, identify the products of the reaction. Dakin (private communication) has found that from β-*p*-hydroxyphenyl-α-uraminopropionic acid, under such treatment, tyrosine can be obtained in considerable yield. It is reasonable to suppose that glutaminyl-cystine-uramino acid (XII) would behave in the same way, and accordingly the solution of the substance obtained by the action of calcium hydroxide on di-hydantoinpropionyl-cystine (XI) is treated with nitrous acid in slight excess of the amount required for the removal of one nitrogen atom. The isolation of the dipeptide (XIII) obtained in this reaction is accomplished through the mercuric sulphate compound.

Whilst the substance so obtained agrees with the natural substance in chemical properties and in certain physical properties, it has not the same optical activity. This is most probably due to racemisation during the formation and subsequent opening of the hydantoin ring [Dakin, 1910]. The agreement in all other respects, however, is so close that it is difficult to imagine any difference between the synthetic and natural substances save that of configuration. The differences between the three *possible* constitutions of glutathione are so marked, that did glutathione not possess the constitution proposed, and, consequently, not that of the synthetic product, the two substances must inevitably differ more widely. Especially is this true of the melting point, even though such a criterion is of lessened value when one is dealing with amorphous substances.

Nevertheless, it seemed desirable, since resolution of the racemic peptide appeared a well nigh hopeless task, to attempt a synthesis, which, while yet affording confirmatory evidence of the constitution, should give a substance completely identical with natural glutathione. This object was successfully attained by a direct coupling of cystine and glutaminic acid.

Glutaminic acid was found to react with phosphorus tribromide. The ease with which the product of this reaction was hydrolysed, and its ability to condense with aniline in slightly alkaline solution, showed it to be an acid bromide. Analysis of the anilide gave figures in close agreement with those required for

the hitherto unknown glutaminyl mono-anilide. The reaction of phosphorus tribromide and glutaminic acid had therefore yielded a *mono*-acid bromide. The two-fold object of the synthesis—to confirm the constitution and to obtain a product identical with natural glutathione—demanded that before proceeding further, the constitution of this acid bromide should be definitely established. For this purpose glutaminyl mono-anilide was prepared in sufficient quantity, and use was made of the oxidation method employed in determining the constitution of glutathione itself. It was there argued [Quastel, Stewart and Tunnicliffe, 1923] that the two compounds: $R.NH.CO.CH_2.CH_2.CHNH_2.COOH$ and $R.NH.CO.CHNH_2.CH_2.CH_2.COOH$ should behave differently on oxidation with hydrogen peroxide. Dakin [1905] has shown that α-amino acids, treated in this way, eventually yield fatty acids containing one carbon atom less, glutaminic acid for example, being converted to succinic acid. The first, therefore, should yield $R.NH.CO.CH_2.CH_2.COOH$ which, on hydrolysis, should be broken up to $R.NH_2$ and succinic acid. The second should yield $R.NH.CO.CO.CH_2.CH_2.COOH$ which further oxidation should split up, possibly to succinic acid. At any rate, the complete breakdown should, in this case, take place before hydrolysis. In the particular case of glutaminyl mono-anilide, the problem is simplified by the fact that succinyl mono-anilide is known (see Beilstein's *Handbuch III*, II, 413) so that hydrolysis after oxidation is unnecessary. In actual fact, oxidation of glutaminyl mono-anilide yielded a substance extractable by ether, which was identified by properties, melting point, and analysis, with succinyl mono-anilide. The acid bromide from which it was derived was thus represented by the formula:

$$BrOC.CH_2.CH_2.CHNH_2.COOH.$$

That is, it had the bromine atom attached to the carboxyl group remote from the amino group, and was therefore the one required for the synthesis of a compound having the constitution proposed for glutathione.

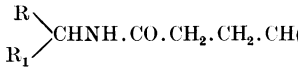
Condensation of the acid bromide with cystine yielded a dipeptide which had all the properties of natural glutathione, and which, on oxidation with hydrogen peroxide behaved in exactly the same way, yielding succinic acid only after hydrolysis of the oxidation product.

The comparison of the physiological properties of the natural and synthetic dipeptides has not yet been fully worked out. It has been observed, however, that like natural glutathione, both the synthetic products restore to a thermostable preparation of dog muscle the power of reducing methylene blue. Further, when either of them is added in "oxidised" (disulphide) form to such a preparation, and is incubated *in vacuo* at 37° for a few minutes, the —S—S— grouping is reduced to —SH.

Both the methods of synthesis involve the coupling of an acid bromide with cystine dimethyl ester. In both of these reactions considerable losses were experienced, so that the final yields of dipeptide were small. Little better, however, is to be expected in such a case where, besides the main

reaction, an acid bromide and an ester are undergoing simultaneous hydrolysis in a slightly alkaline medium.

The methods described in this paper, are, of course, generally applicable to the synthesis of dipeptides of the type:



Experimental.

Synthesis I.

Preparation of the Acid Bromide of Hydantoinpropionic Acid.

The dry, finely powdered hydantoinpropionic acid was covered with excess of phosphorus tribromide, and the mixture was heated on the water-bath for two to three hours in a wide-mouthed flask under a reflux condenser. The yellowish coloured mass was freed from as much as possible of the excess of phosphorus tribromide by decantation, and the remainder was removed by repeated trituration with dry chloroform. It was then transferred to a vacuum desiccator containing sulphuric acid and paraffin wax. The bromine content of the product was estimated by hydrolysis for 20 minutes with excess of a twice normal solution of potassium hydroxide, acidification with nitric acid, and precipitation of the silver bromide with silver nitrate.

0·512 g. of the product gave 0·369 g. of silver bromide. Hence 90 % of the hydantoinpropionic acid had been converted to the acid bromide. No further purification of the crude product was attempted.

Preparation of Di-hydantoinpropionyl-cystine.

5 g. of cystine dimethyl ester hydrochloride, prepared according to the method of Fischer and Suzuki [1905], were dissolved in 50 cc. of ice-cold water giving a clear solution. After further cooling in a freezing mixture (as short as possible since the ester begins to be hydrolysed at once) solid sodium bicarbonate was added until the solution was just alkaline to methyl red. While still in the freezing mixture, the solution was vigorously stirred by a mechanical device, and alternate small portions of the hydantoinpropionyl bromide and solid sodium bicarbonate were added, the solution being kept just alkaline to methyl red, excess being avoided, and 30 % excess over calculated quantity of the acid bromide was added—in the case described, 11 g. At the completion of the reaction the p_H was adjusted to the neutral point (to methyl red). Standing overnight completed the precipitation of unchanged cystine, which was then filtered off. The filtrate contained di-hydantoinpropionyl-cystine along with hydantoinpropionic acid, traces of cystine, and sodium salts. It was acidified with sulphuric acid, and treated

with a little dilute solution of sodium nitrite. Any uncombined cystine was thus destroyed, whereas the di-hydantoinpropionyl-cystine was unattacked owing to the absence of any free amino group from the molecule. The solution was then precipitated with the Hopkins-Cole mercuric sulphate reagent, and, after standing overnight, the heavy white precipitate was filtered at the pump and washed thoroughly with distilled water. Since hydantoinpropionic acid is not precipitated by mercuric sulphate, this precipitate consisted solely of the mercuric sulphate compound of di-hydantoinpropionyl-cystine. It was suspended in water and decomposed by hydrogen sulphide in the usual way. The filtrate was freed from hydrogen sulphide by aeration, and then made alkaline with barium hydroxide. The aeration was then continued until the vivid nitroprusside reaction had disappeared, barium was removed quantitatively with sulphuric acid, and the filtrate was concentrated *in vacuo* to a syrup. Considerable excess of a mixture of one part of absolute alcohol to two parts of dry ether was then added and the sticky product which separated was allowed to stand under the mixture until it became friable. It was filtered off rapidly and dried in a vacuum desiccator containing sulphuric acid, phosphorus pentoxide, and paraffin wax.

The product so obtained, which had a slight yellow colour, was exceedingly soluble in water giving an acid solution. It was insoluble in ether and in acetone. It was precipitated from aqueous solution by mercuric sulphate. The aqueous solution gave no nitroprusside reaction, but after reduction with magnesium and dilute hydrochloric acid an intense reaction was obtained. Heated in a capillary tube, it softened at 140° and melted at 174° (uncorr.).

Nitrogen by Micro-Kjeldahl. Found: N = 15·15 %. Calculated for $C_{18}H_{24}O_{10}N_6S_2$: N = 15·32 %.

Amino Nitrogen by the method of Van Slyke. Traces only of nitrogen were evolved. After hydrolysis, however, a qualitative test showed the presence of free primary amino groups.

Sulphur was determined by fusion with sodium peroxide. Found: S = 11·32 %. Calculated: S = 11·68 %.

1 g. of cystine was recovered from the precipitate which separated during the coupling of the cystine dimethyl ester with the acid bromide. Even allowing for this recovery, however, the yield was poor, as seems to be usual in reactions of this type. Part of the loss may no doubt be referred to the use of mercuric sulphate as a precipitant for cystine and its derivatives [Hopkins, 1921]. From 5 g. of cystine dimethyl ester hydrochloride 0·8 g. of di-hydantoinpropionyl-cystine was isolated.

Opening of the Hydantoin Ring.

The di-hydantoinpropionyl-cystine was dissolved in water and a moderate excess of finely-powdered calcium hydroxide was added. The mixture was boiled gently under a reflux condenser for three-quarters of an hour. At the end of that period the undissolved calcium hydroxide was filtered off and the

filtrate was acidified with dilute sulphuric acid so that the resulting concentration of acid was approximately 1·5 %. On acidification, a certain amount of hydrogen sulphide was evolved showing that a partial decomposition of the cystine portion of the molecule had occurred. This decomposition, however, was less than with sodium or barium hydroxide, the use of which, moreover, would probably result in a greater disruption of the peptide linkage. After standing, the precipitated calcium sulphate was filtered off. No attempt was made to isolate the uramino acid from this solution; it was, however, observed to be precipitated by the mercuric sulphate reagent.

Conversion of the Uramino Acid to the Amino Acid.

Rohde [1918] observed that a uramino acid, treated with nitrous acid, loses half its amino nitrogen. Dr H. D. Dakin, in a private communication, states that by the action of nitrous acid on β-*p*-hydroxyphenyl-α-uraminopropionic acid, he has obtained tyrosine. Presumably, an imino acid is first formed and subsequently loses carbon dioxide.

The solution obtained in the previous stage was therefore treated with a dilute solution of potassium nitrite in slight excess of the quantity required for the conversion of the uramino to the amino acid. The solution was allowed to stand several hours, and the dipeptide was then precipitated by mercuric sulphate reagent. The further procedure was as described in the isolation of di-hydantoinpropionyl-cystine. Usually this sufficed, but occasionally it was found advisable to purify the substance further by precipitation of the copper compound of its "reduced" (sulphydryl) form [Hopkins, 1921].

Properties of the Dipeptide.

The dipeptide as thus prepared was a white non-hygroscopic amorphous powder. Like natural glutathione, it was extremely soluble in water, but insoluble in alcohol, ether, and the usual organic solvents. Its aqueous solution was acid to litmus. In neutral solution it gave a violet coloration with triketohydrindene. It gave no coloration with sodium nitroprusside in ammoniacal solution except after reduction with magnesium and hydrochloric acid. On hydrolysis with sulphuric acid, cystine was isolated; with hydrochloric acid, glutaminic acid hydrochloride was isolated.

Amino Nitrogen by the method of Van Slyke. Found: 6·4 %. Calculated for $C_{16}H_{26}O_{10}N_4S_2$: 5·62 %.

It will be recalled that in the case of natural glutathione, Hopkins [1921] obtained a similar high result (6·8 %).

Amino Nitrogen after hydrolysis. After hydrolysis of the substance with 20 % sulphuric acid for six hours, the amino nitrogen was doubled, and, allowing for the somewhat high value to be expected in the presence of cystine, was equal to the total nitrogen. Found: 12·2 %. Calculated: 11·24 %.

Total Nitrogen by Micro-Kjeldahl. Found: N = 11·26 %. Calculated: N = 11·24 %.

Heated in a capillary tube, the substance softened from 165–170° and melted at 187°. A sample of natural glutathione, heated side by side with the synthetic substance, behaved in exactly the same way. Not too much reliance, perhaps, should be placed on the melting point of an amorphous substance, but not only did the two substances melt at precisely the same temperature, but the melt of the synthetic product behaved in the manner characteristic of glutathione, in that there was an evolution of gas, and the liquid ran up the sides of the tube. Further, the melting point was not lowered by mixing the natural and synthetic products.

Optical Activity.

There is thus good agreement between the properties of natural glutathione and of the synthetic dipeptide—and it may be noted that no dissimilarities were observed. The best criterion of complete identity is, of course, the optical activity. From the mode of preparation of the synthetic substance, involving as it does, the closing and opening of a ring by somewhat drastic means, it is obvious that a partial racemisation, at any rate, is to be expected. However, the specific rotation of glutathione being still unknown, it was thought of some interest, at this point, to measure the rotation both of the natural and the synthetic product.

The Specific Rotation of "Oxidised" Glutathione.

I. *In aqueous solution.* 15 cc. of solution in distilled water contained 0·5194 g. of "oxidised" glutathione. The rotation, measured in a 2·0 dm. tube, using the mercury green line, was (mean of six concordant observations) $\alpha = -6{\cdot}82°$. The above solution was diluted to twice its volume with distilled water. Mean of six concordant observations: $\alpha = -3{\cdot}40°$, whence

$$[\alpha]^{15°}_{\text{Hg. j}} = -98{\cdot}3°.$$

II. *In acid solution.* 15 cc. of solution in 10 % hydrochloric acid contained 0·2597 g. of "oxidised" glutathione. Mean of six concordant observations: $\alpha = -3{\cdot}09°$, whence

$$[\alpha]^{15°}_{\text{Hg. j}} = -89{\cdot}2°.$$

The Specific Rotation of the Synthetic Dipeptide.

10 cc. of solution in distilled water contained 0·545 g. of the synthetic dipeptide. The rotation, measured in a 1·0 dm. tube using the mercury green line, gave (mean of twelve concordant observations): $\alpha = -1{\cdot}67°$, whence

$$[\alpha]^{15°}_{\text{Hg. j}} = -30{\cdot}6°.$$

SYNTHESIS II.

Preparation of the acid bromide of glutaminic acid. 10 g. of free glutaminic acid were suspended in 50 cc. of dry toluene. To this suspension were added 3·0 cc. of phosphorus tribromide, *i.e.* a 50 % excess over the quantity theoretic-

ally required to introduce a bromine atom into one carboxyl group. The mixture was boiled gently under a reflux condenser for three to four hours. After cooling it was rapidly filtered at the pump, and the light yellow solid was repeatedly triturated with dry chloroform. It was dried over sulphuric acid and paraffin wax (*in vacuo*). The bromine content of the crude product was estimated in the manner described for the acid bromide of hydantoinpropionic acid. In a typical case, 0·4806 g. gave 0·123 g. of silver bromide, corresponding to a 30 % yield of a mono-acid bromide.

Condensation of the acid bromide with aniline. 4·0 cc. of aniline were suspended in 40 cc. of water and coupled with the acid bromide in the manner previously described for the condensation of hydantoinpropionyl bromide and cystine dimethyl ester (p. 211), except that the solution was not cooled below room temperature. 10 g. of the crude acid bromide were used. After removal of the excess aniline by repeated extraction with ether, the reaction mixture was acidified with hydrochloric acid, and evaporated to dryness *in vacuo*. The residue was extracted several times with absolute alcohol. The combined extracts were decolorised by boiling with a little animal charcoal, and were then evaporated on the water-bath. The crystalline residue was recrystallised from absolute alcohol.

The colourless spear-head crystals were readily soluble in water and alcohol, but insoluble in ether and acetone.

Nitrogen by Micro-Kjeldahl. Found: N = 10·4 %. Calculated for $C_6H_5NH.C_5H_8O_3N.HCl$: N = 10·83 %.

Chlorine by precipitation with silver nitrate. Found: Cl = 13·51 %. Calculated: Cl = 13·73 %.

Calculated for the di-anilide, $C_6H_5NH.C_5H_7O_2N.C_6H_5NH.HCl$:

N = 12·59 %; Cl = 10·64 %.

Oxidation of glutaminyl mono-anilide. A solution in distilled water of the glutaminyl mono-anilide was made faintly alkaline with ammonia, a trace of ferric chloride was added, and the liquid was warmed on the water-bath with excess of hydrogen peroxide. As the hydrogen peroxide was used up, more was added until the reaction had been in progress for one-and-a-quarter hours. At the end of this time it was no longer possible to obtain a coloration with triketohydrindene. The solution was then acidified with hydrochloric acid, and thoroughly extracted with ether, the combined ethereal extracts being dried over sodium sulphate, and evaporated to dryness. There remained a mass of long, fine, colourless needles. These were dissolved in hot water, from which they separated on cooling, a qualitative test on the crude product having shown the presence of nitrogen. As, after recrystallisation, the test for nitrogen was still strongly positive, an estimation by micro-Kjeldahl was carried out. Found: N = 7·34 %. Calculated for $C_6H_5NH.C_4H_5O_3$: N = 7·25 %. The crystals melted sharply at 144–145°. In Beilstein's *Handbuch*, 3rd edition, **2**, p. 413, the melting point of succinyl mono-anilide is given as

148·5°. Later determinations, however, are rather lower than this for it is given (Ergänz. **2**, p. 210) as 144·5–145·5°. Melting point and nitrogen determination, then, agree in identifying the oxidation product as succinyl monoanilide. There is similar agreement in the reactions. The substance is sparingly soluble in cold water, but readily so in hot water, alcohol or ether. Its aqueous solution is acid to litmus. The addition of silver nitrate to its aqueous solution precipitates the crystalline silver salt. The barium salt is soluble, and cannot be obtained by adding barium salts (or hydroxide) to a solution of the anilide. The calcium salt, on the other hand, is sparingly soluble, and separates in small needles when calcium chloride solution is added to a neutral solution.

Condensation of glutaminyl mono-bromide with cystine dimethyl ester. The coupling was carried out exactly as described in the previous synthesis, care being taken to keep the temperature as low as possible during the reaction. As before, a variable amount of cystine was recovered from the reaction mixture. In one case, from 6 g. of cystine dimethyl ester, 3 g. of cystine were recovered. The dipeptide was isolated through its mercuric sulphate compound as previously described..

Identification of the dipeptide with natural glutathione. The substance thus prepared gave all the qualitative chemical reactions described in Hopkins' original paper [1921] and in this paper for the racemic dipeptide (p. 213). The melting point was identical with that of the natural product, and was not lowered by admixture.

Total Nitrogen by Micro-Kjeldahl. Found: N = 11·35 %. Calculated for $C_{16}H_{26}O_{10}N_4S_2$: N = 11·24 %.

Amino Nitrogen by the method of Van Slyke. Found: 6·4 %. Calculated: 5·62 %. As before, the value obtained for the amino nitrogen is high as is characteristic of the natural product and of cystine compounds in general.

Amino Nitrogen after hydrolysis. The substance was hydrolysed by boiling under reflux condenser for six hours with 20 % sulphuric acid. Found: amino N = 13·26 %. Calculated: amino N = 11·24 %. Found for natural glutathione [Hopkins, 1921]: amino N = 13·3 %.

Sulphur by the method of fusion with sodium peroxide. Found: S = 12·3 %. Calculated: S = 12·80 %.

Specific Rotation. 10 cc. of a solution in distilled water contained 0·5435 g. The rotation, measured in a 1·0 dm. tube, using the mercury green line, gave (mean of twelve concordant observations): $\alpha = -5{\cdot}295°$, whence

$$[\alpha]^{15°}_{\text{Hg. j}} = -97{\cdot}4°.$$

Summary.

I. Glutathione has been synthesised by two methods.

(*a*) Glutaminic acid—hydantoinpropionic acid—hydantoinpropionyl bromide—di-hydantoinpropionyl-cystine—di-uraminoglutaryl-cystine—di-glutaminyl-cystine (glutathione).

(*b*) Glutaminic acid—glutaminyl monobromide—di-glutaminyl-cystine.

II. The product obtained by the first of these methods differed from the natural substance only in its optical activity.

III. The second method yielded a substance completely identical with natural glutathione.

IV. The first method of synthesis substantiates the constitution of glutathione previously advanced by the authors.

V. It is shown that in the acid bromide of glutaminic acid used in the second synthesis, the bromine atom has entered the carboxyl group remote from the amino group. This synthesis, therefore, is also considered to confirm the constitution already deduced.

IV. The specific rotation of glutathione has been measured.

In aqueous solution

$$[\alpha]^{15°}_{\text{Hg. j}} = -98{\cdot}3°.$$

In 10 % hydrochloric acid solution

$$[\alpha]^{15°}_{\text{Hg. j}} = -89{\cdot}2°.$$

VII. The methods described are generally applicable to the synthesis of dipeptides of the type: $\begin{matrix}\text{R}\\ \text{R}_1\end{matrix}\!\!>\!\text{CH.NH.CO.CH}_2\text{.CH}_2\text{.CH(NH}_2\text{).COOH.}$

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