

## ON GLUTATHIONE: A REINVESTIGATION.

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### *I. Isolation of a Crystalline Substance.*

In 1927 Hunter and Eagles (1) published an account of investigations which threw doubt upon the constitution of glutathione as apparently established by myself.

At the time I was far from sharing these doubts, but I felt after the appearance of Hunter and Eagles' paper that it was desirable I should reinvestigate the matter, and, if possible, arrive at a method by which the pure thiol compound could be separated with certainty. Meanwhile a paper published by Johnson and Voegtlin (2) seemed to offer evidence in favor of the original formula, and to judge from current publications various observers have prepared the substance for use without acquiring doubts as to its nature. Nevertheless an observation of my own, to be mentioned presently, concerning products obtained essentially on the original lines made it urgently necessary to reinvestigate the constitution of the substance as well as the method of preparation.

The result of the study now to be described has been to show that Hunter and Eagles were right in doubting that the substance is a simple dipeptide of glutamic acid and cysteine though its constitution differs from their provisional suggestions concerning it.

The grave discomfort involved in making an admission of previous error is mitigated by the circumstance that I am now able to describe a method, not without special interest in itself, which with ease and rapidity separates from yeast and from red blood corpuscles a pure crystalline thiol compound with a peptide or quasipeptide structure. In the case of yeast the yield is relatively large, sufficient to show that the compound is by far the most

prominent thiol compound present in the cell and to give it quantitative importance as a general cell constituent. It has all the superficial characters previously ascribed to "glutathione."

The substance however is a compound in which cysteine is associated with glycine as well as with glutamic acid. The results of its analysis, its content of amino nitrogen as determined by titration, and other data agree exactly with the assumption that it is a tripeptide containing the three amino acids mentioned. As a tripeptide however it exhibits exceptional behavior. It would seem indeed to possess special interest in its chemical as well as in its biological aspects. While, so far, it has been prepared mainly from yeast, conclusive evidence has already been obtained for its presence in red blood corpuscles.

In beginning this fresh investigation, still on the assumption that the substance to be isolated was glutaminyl-cysteine, I realized that one desideratum was to improve the yield, since the original method yielded an amount of material which was very far indeed from accounting for the whole thiol content of yeast or tissues as determined by the iodine titration method of Tunnicliffe.

This end was partially attained by a modification of the original method which will receive brief reference in the experimental section of this paper. Some 20 gm. of material, having all the superficial properties of preparations made by the original method, could be thus obtained from 50 kilos of yeast, this amount being usually employed for each isolation. Twelve such preparations from different samples of yeast were made, slight modifications of method being occasionally employed. Some of these products were isolated in the SH form; others were first oxidized and prepared in the SS form. It became clear for various reasons that no one of them was pure glutaminyl-cysteine; but the only evidence for this which here calls for mention was first derived from the following critical observation. In the course of a study of the degree of stability displayed under various conditions by the sulfur in the preparations mentioned above, some were boiled with pure distilled water. The circumstance that hydrogen sulfide was then freely evolved is later discussed; but the significant circumstance was that when a preparation in the disulfide form was boiled with water for a sufficient length of time (8 hours or upwards) a crystalline precipitate separated on cooling which undoubtedly contained

glycine. When a reduced (SH) preparation was similarly boiled and the solution after boiling aerated at pH 7.4 (in the presence of a trace of iron or copper), the same crystalline product gradually separated. The crystalline substance proved to be the diketopiperazine, diglycyl-cystine dianhydride. The corresponding thiol compound (glycyl-cysteine anhydride) which is formed on boiling the reduced preparations is a much more soluble substance (see p. 312). The yield of each of these anhydrides was over 20 per cent of the weight of the material boiled with water.

It thus became necessary to recognize that the preparations in question contained cysteine associated not with glutamic acid alone but also with glycine. A product made on the exact lines of the original method also yielded the same diketopiperazine, and, subsequently, glycine in addition to glutamic acid and cysteine (or cystine) was isolated from every preparation which was submitted to acid hydrolysis. No other amino acid however was ever found.

Owing to misleading evidence derived from Van Slyke amino nitrogen estimations, which, as was subsequently found, are in the case of the materials in question far too high, it was still believed that the preparations must contain a mixture of the two dipeptides, glutaminy-cysteine and glycyl-cysteine (or cysteinyl-glycine). The Van Slyke nitrogen yielded by the preparations was always somewhat more than half the total nitrogen and this seemed to make unlikely the presence of higher peptides. As a result of this assumption (with which, it must be admitted, the percentage composition of the products isolated did not well agree) much labor was spent in an endeavor to separate, by many and various means, the component containing glycine from that containing glutamic acid. This endeavor wholly failed. Fractionation by heavy metal precipitation, by adsorption methods, by extraction with alcohols, by the attempted preparation of distinctive salts, etc., these and other efforts wholly failed to separate a product which would yield the crystalline glycine compound from one which would not. Such attempts at fractionation moreover seldom altered the percentage composition of the products.

The possibility of a tripeptide being present was early considered, but apart from the suggestions of the Van Slyke nitrogen

figures it was difficult to reconcile any such assumption with the percentage composition of products which up to this time had been handled.

The difficulties of the position at this stage made it essential that a new method of isolation should, if possible, be worked out.

I met success in this endeavor as a result of the following observation. During a study of the copper compounds yielded by the impure preparations described above, it was noticed that when cupric sulfate was added to a solution in dilute sulfuric acid of any one of these (in the reduced form), a precipitate exhibiting a silky sheen when the fluid was shaken slowly separated. This when filtered or centrifuged off proved to be a cuprous salt perfectly white in color, which under the microscope appeared crystalline. The metallic content and the percentage composition of this at once suggested that it might be the copper salt of a tripeptide of cysteine, glycine, and glutamic acid.

The amount of this substance obtainable under the conditions mentioned was however necessarily small. Since it is a cuprous salt, it is precipitated on addition of cupric sulfate, only because some copper is reduced by the SH group. The oxidized material simultaneously formed is not precipitated by copper, so the process cannot come to completion. A method for precipitating by cuprous copper was therefore sought.

No soluble cuprous salt suitable for use as a precipitant is available. On the other hand cuprous oxide was found not to dissolve in the peptide materials when they are in cold solution, while heating such a solution with the oxide results in decomposition.

Now the copper compound which it was sought to produce in quantity is characteristically insoluble in sulfuric acid, even when of normal, or greater, strength. It is however a familiar circumstance that when cuprous oxide is added to sulfuric acid there is immediate decomposition into cupric sulfate and metallic copper.

I found on the other hand that when the thiol peptide material is dissolved in the sulfuric acid (0.5 N to N) it entirely inhibits the above decomposition. When under these circumstances the cuprous oxide is added, no metallic copper separates unless the oxide is added in considerable excess. Instead, the microcrystalline cuprous compound rapidly separates from the solution.

It was next found that if the precipitate obtained by adding mercuric sulfate dissolved in sulfuric acid<sup>1</sup> directly to an aqueous yeast extract be decomposed by  $\text{H}_2\text{S}$ , and if to the filtrate from the mercury sulfide, freed from excess of hydrogen sulfide, enough sulfuric acid is added to make it 0.5 N, addition of cuprous oxide then throws down a bulky precipitate of the characteristic cuprous salt just described. Finally, it was found that the copper salt, even at this stage, is essentially a pure compound, that the copper-free substance obtained from it crystallizes under suitable conditions with extreme ease, successive fractions yielding identical analytical figures, and, lastly, that the yield of the substance is of the order of 1 gm. per kilo of moist pressed yeast. Red blood corpuscles, as I have already mentioned, have been shown with certainty to contain the same substance, though no statement of the amount can at present be made. It has not yet been sought elsewhere. For details concerning its separation the experimental section should be consulted.

The impure preparations previously made have been proved to contain a large proportion of the tripeptide now described, and it is extremely unlikely that any appreciable quantity of a dipeptide having the constitution previously ascribed to glutathione exists in cell extracts. In correcting this serious error I feel it fortunate that the extreme ease with which a product, unquestionably pure, can now be obtained will enable all who are interested in the subject to test with little effort the statements contained in the present paper. By the methods now described a satisfactory yield of the crystalline thiol peptide can be obtained with certainty in the course of a few days. A strictly quantitative separation is doubtless more difficult, but this is a circumstance common to most cases of the kind. The details concerning yields are discussed in the experimental section.

<sup>1</sup>The acid mercuric sulfate solution is frequently referred to as Hopkins and Cole's reagent. When Cole and I used it for the isolation of tryptophane we were wholly unaware of the use previously made of it by Denigès as an agent in the preparation of many organic mercury compounds. As his paper appeared 2 years before ours (*Ann. chim. et physique*, **18**, series 7, 382 (1899)) it is clear that if any name is to be attached to the reagent it should be that of the French author.

*II. General Nature and Some Properties of the Crystalline Substance.*

1. The pure compound melts sharply at  $190^{\circ}$  (uncorrected), with decomposition, but without browning until heated above its melting point. As bearing however upon the value of melting points as a test of purity in compounds of the kind, it may be remarked that the original preparation of glutathione, now known to have been impure, melted though with less sharpness at  $187^{\circ}$ .<sup>2</sup>

Its optical rotation in 2 per cent solution is for the mercury green line  $[\alpha]_{\text{H}_{81}}^{15^{\circ}} = -18.5^{\circ} \pm 0.3$ .

The crystallography of the substance will be fully dealt with in a later communication.

2. *Evidence for the Tripeptide Constitution.*—The consistent data from numerous analyses made on preparations from diverse samples of yeast, from blood, and on successive crystalline fractions from individual preparations (p. 292) correspond exactly with the composition of a tripeptide containing glutamic acid, glycine, and cysteine.

	C	H	N	S
Average of analyses.....	38.89	5.53	13.42	10.51
Calculated for $\text{C}_{10}\text{H}_{17}\text{N}_3\text{SO}_6$ , ....	39.09	5.54	13.68	10.42

The metallic content of its pure cuprous compound, on the assumption that this contains 1 atom of copper in its molecule, indicates an equivalent weight for the substance (307) which is that of such a tripeptide.

	<i>per cent</i>
Average copper found.....	17.21
Calculated for $\text{C}_{10}\text{H}_{16}\text{N}_3\text{SO}_6\text{Cu}$ .....	17.26

Determinations by titrations, moreover, confirm this equivalent weight with great exactness (p. 292). From the products of acid hydrolysis glutamic acid, glycine, and cysteine (as cystine) have all been separated pure, while no other amino acid has been found, and no evidence for the liberation of any other constituent on hydrolysis.

On the other hand, the nitrogen evolved when the method of Van Slyke is applied to it (6.89 to 7.00 per cent) is far too high

<sup>2</sup> Compare also statements by Stewart and Tunnicliffe (6), p. 214.

for correspondence with the single free amino group of the tripeptide (4.56 per cent). Experience, however, with the cruder products obtained by earlier methods of separation had given warning that Van Slyke determinations may be entirely erroneous in the case of peptides containing the amino acids now in question. It is important, indeed, to recall that Van Slyke in one of his first papers on the subject points out that in the case of glycylglycine no less than 40 per cent of the nitrogen concerned in the peptide linkage is liberated by nitrous acid. I personally observed long ago that on applying titration methods to the earlier non-crystalline preparations of glutathione much lower figures were obtained for amino N than those yielded by the Van Slyke method. My colleague, Dr. L. J. Harris, whose publications on the titration of ampholytes are well known, is possessed of a technical experience in this branch of experiment much greater than my own, and I therefore asked him to undertake an independent investigation of the behavior of the crystalline peptide when submitted to various methods of differential titration. The results of his observations are reported under his name in a separate section of this paper. They justify very completely the claim that the substance is a tripeptide of the nature claimed. Further confirmation, on slightly different lines, is yielded by the results of the electrometric titrations described by Pirie and Pinhey in a separate paper in the present number of this *Journal*.

3. *Instability of the Substance as a Tripeptide.*—If the crystalline substance be a tripeptide as claimed (and it seems impossible on the evidence above to conceive of any other constitution), its molecule displays in certain circumstances a degree of instability which, if not peculiar to itself, has not been described as characteristic of any other peptide.

(a) The circumstance that its cysteine sulfur is much less stable towards alkalis than the sulfur of free cysteine may indeed prove to be true of all cysteine and cystine peptides. Bergmann (3) called attention to the extreme ease with which the sulfur of the dianhydride of dialanyl-*L*-cystine is removed by dilute NaOH in the presence of lead acetate and attributed this instability to the oxypiperazine configuration. Brand and Sandberg (4) showed however that the sulfur of the corresponding dipeptide itself (dialanyl-cystine) is much more readily removed than is that of free

cystine. That this was true of the original preparations of glutathione was also shown by the latter authors and has long been known in this laboratory. In these, the rate of desulfuration has been often estimated and compared with that of cystine. In the case of the crystalline substance the same relative instability is sufficiently illustrated by the observation that when dissolved in a 0.3 N barium hydroxide solution, with excess of lead acetate, 55 per cent of its sulfur was removed as lead sulfide in 5 hours at 37°. Under similar conditions after the same period cystine and cysteine solutions showed no trace even of blackening (p. 310).

(b) The pure substance undergoes decomposition during such gentle treatment as that involved in aeration (in the presence of a trace of iron) at room temperature and at pH 7.6. When this treatment is adopted for the conversion of the thiol into the disulfide form and continued until the solution no longer gives a nitroprusside reaction (5 to 6 hours approximately), some 80 per cent is found to undergo reversible oxidation alone, but the remainder loses both nitrogen and sulfur.

The evidence for this statement is the following: Two different preparations were treated as above and the solution was in each case freed from the barium used to adjust the pH, evaporated *in vacuo* at 40°, and, finally, taken to dryness in a desiccator and dehydrated with absolute alcohol. The friable white products were then dried *in vacuo*. Their analysis will be found in the experimental section.

In the oxidation from the SH to the SS form the small loss of hydrogen creates of course little difference in the percentage analyses. It will be seen however that the N of the above products fell from 13.43 per cent (that of the original preparation) to 12.33 in one case, and from 13.39 to 11.99 per cent in the other. The S fell from 10.5 and 10.65 to 9.61 and 9.60 per cent, respectively. When the products were redissolved, the solutions reduced by saturation with H<sub>2</sub>S in the presence of a few globules of mercury (after about 24 hours the whole of any SS form present is thus fully reduced to SH), the application of the cuprous salt method showed that about 80 per cent of the product could be again obtained in the crystalline thiol form. The whole of the loss in N and S must therefore have fallen upon the other 20 per cent. The nitrogen must have been removed as ammonia and the

sulfur either as sulfuric acid (precipitated by the barium present during the aeration) or in volatile form, since, as stated, the oxidized solutions before analysis were not fractionated but taken to dryness as a whole and then showed the loss of N and S in question. It is noteworthy that this secondary irreversible oxidation of part of the material only continues during the existence of the SH group in solution. An observation following those just described showed that even very prolonged aeration after the disappearance of the nitroprusside reaction brings about no further fall in nitrogen or sulfur. This suggests that the secondary oxidation might be due to peroxide formed during the oxidation of the SH group. Attempts to prevent it by adding manganese dioxide and catalase, respectively, to the solutions when aerated failed however, though this possibility has not been fully explored and there is good reason for believing that there are protective conditions in the cell.

In the case of the earlier impure preparations this loss of S and N was never observed. The SS products were repeatedly prepared from SH products by aeration, and on the average analyses showed no more than a minimal variation in the percentage composition of such preparations. It would seem as though some associated substance in these acted in a protective sense. The facts are obscure and call for further study, but the removal of N and S under the circumstances described illustrates the instability of the tripeptide when pure.

A practical result of the occurrence of this secondary oxidation is that the disulfide form of the pure tripeptide has never yet been obtained. It does not crystallize, at any rate not from the mixture which results from its production on the above lines.

(c) The most noteworthy illustration of the instability of the tripeptide is seen in its decomposition when boiled in pure aqueous solution. As already stated, the prominent result of such treatment is the formation of the diketopiperazine of glycine and cysteine; or when the oxidized form of the dipeptide is concerned, of the disulfide form of this (diglycyl-cystine dianhydride). At the same time, glutamic acid is liberated, being ultimately found chiefly in the form of its derivative, pyrrolidonecarboxylic acid. While decomposition goes only very slowly to completion, the formation of the diketopiperazine can be early detected and may even be demonstrated after an aqueous solution of the peptide

has been taken to dryness a few times on the water bath. After about 12 hours boiling—a period required for the complete decomposition of some peptides even under the influence of strong acids—the greater part of the glutamic acid is already free. It would seem that, while the linkage which binds the glutamic acid is resolved with exceptional ease, on its occurrence the cysteine and glycine residues are free to complete the double linkage which is involved in piperazine formation. On these direct lines, however, the decomposition proceeds by no means quantitatively. Of the yield of diketopiperazine which would be expected if the whole of the cysteine and glycine were concerned in its formation, only about half can be isolated (22 to 25 per cent of the weight of original tripeptide decomposed, instead of 52 per cent). To a small extent this deficiency is due to the circumstance that the diketopiperazine undergoes further decomposition on boiling, though this is so slow a process that it is nearly negligible in the result. But decomposition of the peptide on other and secondary lines is always involved. On boiling with water desulfuration for instance occurs from the first, and the evolution of  $\text{H}_2\text{S}$  is continuous throughout long periods of such treatment.

Quantitative studies of the phenomena were in the case of impure products carried out long ago, though the results have never been published. Since these products (as is now known) contained some 80 per cent of the tripeptide, the data are not without significance and the results of one experiment, typical of many, are given in Table I. It will there be seen that the sulfur is liberated partly as  $\text{H}_2\text{S}$  and partly as free sulfur which collects in the condenser tube. The latter is not evident till about the 20th hour of boiling, when for a time its amount exceeds that of the sulfur eliminated as  $\text{H}_2\text{S}$ . After about 40 hours boiling one-fourth of the sulfur was found to have been eliminated as above and about one-third appeared in the diketopiperazine produced. The material in the experiment chosen for illustration was in the disulfide form, but results scarcely differing from these were obtained from the crude preparations when reduced. The purest crystalline preparations of the tripeptide desulfurize continuously when boiled with water, as may be demonstrated by lead papers exposed to the vapors leaving the vertical condenser. The loss of sulfur is slower however, though the amount of diketo-

piperazine formed in a given time is not increased as a result. Thus, in one experiment with a pure preparation in the thiol form the substance yielded in 14 hours boiling only 3.6 per cent of its sulfur content as  $\text{H}_2\text{S}$ , and in 42 hours only 12.3 per cent. The yield of diketopiperazine at the latter stage was just 20 per cent of the tripeptide weight. In a second experiment the disulfide form of the tripeptide (containing some 8 to 10 per cent of the products of more complete oxidation; see above) was employed. Of its original S content 11 per cent was eliminated, as  $\text{H}_2\text{S}$  and free S, during 42 hours boiling. The yield of diketopiperazine was at this stage 17.5 per cent.

In each of the above experiments the evolution of  $\text{H}_2\text{S}$  was shown by means of lead papers to proceed continuously for many hours after the quantitative observations were stopped.

In the experimental section the details of an experiment are given in which an aqueous solution of the pure tripeptide (4 gm. in 150 cc. of water) was boiled for a long period (50 hours). From the products 0.88 gm. of the pure diketopiperazine was obtained (22 per cent). Just over 1 gm. of free glutamic acid, partly as such but chiefly as pyrrolidonecarboxylic acid, was obtained. While the amounts of these latter together represent little more than half of the glutamic acid present in the tripeptide, this quantity was obtained analytically pure and certainly represents, under the circumstances, only a minimal recovery. Owing to the presence of the products of secondary decomposition (the products, for instance, from the desulfurized cysteine, which have not been identified) a quantitative separation is difficult. It is easy to show (*cf.* p. 315) however that no glutamic acid remains attached to cysteine. This is the case indeed (to judge from experience with the less pure products) after periods of boiling much shorter than in the experiment under reference. It is more difficult to prove that none remains attached to glycine, but no fraction giving definite evidence of this association has ever been obtained from the products of boiling with water. On the other hand it has always proved impossible (as in this experiment) to isolate free glycine from these products, and to judge from the results of subjecting non-crystallizing mother liquors from the water-boiled materials to subsequent acid hydrolysis it would seem that much of the glycine which has not shared in piperazine formation remains attached

to cysteine. In the experiment under notice a very small amount of free cysteine was identified (as cystine).

It is not without interest to note the contrast between the results of direct acid hydrolysis and those of boiling with water. Under the former treatment the tripeptide splits cleanly into its three constituent amino acids. The more rapid hydrolysis, or the fact that the peptide linkages are split with velocities more nearly equal, prevents changes which depend upon the influence of the whole original structure of the tripeptide molecule on the course of its slow resolution. I am not aware that any analogous facts have been yet described in the literature of peptide chemistry.

The decomposition in water just described clearly involves relatively slow processes; but they proceed, it must be remembered, without an added catalyst of any sort. The elimination of sulfur is out of all proportion faster than any similar elimination in the case of pure cysteine or cystine. That it is faster in the case of impure preparations would seem to be due, not to the circumstance that the impurities contribute any appreciable amount to the eliminated sulfur, but to some catalytic effect of an unknown constituent. The formation of the diketopiperazine is as rapid in the case of the pure tripeptide as in that of the crude preparations and so probably is the liberation of free glutamic acid.

It should be here stated that decomposition on lines similar to those just described proceeds when the tripeptide is boiled with alcohol instead of water. In the case of the less pure products this has been repeatedly shown. In one experiment, carried out on a scale larger than usual (14 gm.), one of these preparations in the reduced condition (containing N 12.10 per cent, and S 11.56 per cent) was boiled for 30 hours with pure absolute alcohol. Evolution of  $\text{H}_2\text{S}$  was observed to be continuous throughout the process. The alcohol was distilled off and the residue dissolved in water and aerated at room temperature. By the time when the nitroprusside reaction was no longer given the solution had yielded a crystalline precipitate of the diglycyl-cystine dianhydride, and, on evaporation, a further quantity. In all 3.53 gm. of the anhydride were obtained, just 25 per cent of the weight of the material employed. Evidence from this and other experiments indicates that the anhydride formation is somewhat more rapid in alcohol than in water, while desulfuration is slower.

The pure tripeptide behaves similarly. While however the impure preparations decomposed freely when boiled with absolute alcohol, the pure substance is more resistant unless the alcohol is slightly diluted. This appears to be mainly a question of solubility; the impurities present in the preparations made by the earlier method appreciably increase the solubility of the tripeptide in alcohol. When the pure substance is first dissolved in a minimal quantity of water and alcohol added till the mixture contains 96 per cent, boiling the solution then induces desulfuration and anhydride formation at rates not appreciably different from those observed on boiling with water. In the disulfide form neither the pure nor the impure products decompose in boiling alcohol in which they are insoluble.

(d) Instability is further illustrated by the circumstance, for which there is some evidence, that on boiling with water decomposition extends to the carbon chains. In the case, at least, of more than one of the earlier preparations which from present evidence must have consisted mainly of the tripeptide, experiments indicate that after 50 hours boiling some 14 per cent of the carbon was removed in volatile compounds. This has not yet been proved in the case of the pure substance.

4. *Naphthalene-Sulfo Derivatives*.—An aspect of the behavior of the tripeptide which is puzzling should here receive brief mention. During the study of the impure products endeavors were made to prepare from them crystalline substitution products; in particular the classical type of naphthalene-sulfo derivative. Repeated endeavors to obtain this consistently failed, although the conditions (concentration of alkali, etc.) were widely varied and though typical derivatives from various amino acids and dipeptides were easily prepared by the use of identical reagents. It has since been found that the pure crystalline product fails equally to yield a naphthalene-sulfo derivative, a circumstance at present without explanation. It is possible of course that strong alkalis may induce some structural change involving the amino group.

### *III. Nature of Impure Preparations Previously Obtained.*

Some of the data obtained from the earlier preparations (made not by the method of 1921 (5) but by the modification already

mentioned and to be described) are published in this paper. The justification for this is the recent proof that they contained a large proportion of the tripeptide itself. This has been shown by the easy isolation of the crystalline product on applying the new cuprous salt technique to residual stocks. Incidentally it should be said that while these earlier preparations all yielded on acid hydrolysis the three amino acids of the tripeptide, they yielded no other. They gave results on titration which do not depart widely from those given by the pure substance, and all the evidence suggests that their content of this was on the average some 80 per cent. Nevertheless their analysis showed always lower figures for nitrogen (on the average just under 12 per cent) and higher for sulfur (average 11.7 per cent) so that the ratio of sulfur to nitrogen in them was considerably higher than in the pure substance. At present I have no definite knowledge as to the nature of the admixtures. Until the new copper method was worked out it had proved impossible, as already stated, to fractionate the preparations effectively, and the problem has not since received attention. Free cysteine, which might have accounted for the higher sulfur content, could never be demonstrated with certainty. It can only be said that the preparations usually gave an appreciable color reaction with  $\alpha$ -naphthol and sulfuric acid. This reaction however is of course very delicate, and the presence of carbohydrate could not be otherwise demonstrated.

This question would under present circumstances scarcely call for discussion except for the result of some observations which are now being extended. Certain of my colleagues who are concerning themselves with the behavior of the purified tripeptide have found it to differ in some respects from the older products in its relations with tissue preparations. Briefly put, it would appear that the original products contained something (not a metal) which in a sense "activates" the thiol group in the tripeptide, and it is at least possible that the observations in question may throw fresh light on a chemical system in the cell. For this reason a summary of the method by which the earlier preparations were made is given below.

I may choose this place to make certain remarks which must necessarily appear in this paper. It is sure, as I have already

stated, that the original preparations of glutathione as described in 1921 must certainly have contained glycine, and indeed almost certainly contained, as did the intermediate preparations just discussed, a large proportion of the tripeptide. I now find it hard to understand how I originally missed the presence of glycine, so easy has been its isolation from later products. Two circumstances may be advanced, not in excuse for the error, but as some explanation for its commission. The first is the small quantities of material then employed for hydrolysis, and the second the bias in one's mind induced by the high amino nitrogen figures as given by the Van Slyke method.<sup>3</sup> These seemed to make impossible the presence of any peptide more complex than a dipeptide, and having identified two amino acids I feel (on looking back to that period) that the search for a third may have been perfunctory.

I am more concerned however to make reference to the syntheses (6) which seemed to confirm the constitution of glutathione as a glutaminyl-cysteine dipeptide. My junior colleagues who were responsible for these syntheses devoted, I know, much labor to their supposed accomplishment, and I can testify that the results obtained were discussed with myself, and afterwards published, in the most perfect good faith. I may say further that the material they obtained by their second method<sup>4</sup> so exactly resembled in all its more obvious characters the preparations from yeast then in my hands that, when I compared them, all doubt concerning the constitution of glutathione was banished from my mind. This agreement could only have been a remarkable coincidence; but it was a coincidence which actually occurred. Fortunately any who may now devote their efforts towards the synthesis of the crystalline tripeptide will, I imagine, find no difficulty in deciding with certainty when they have reached their goal.

It is sure that but for Hunter and Eagles' researches the present correction of the former error would never have come from myself. It is right that I should freely acknowledge this circumstance.

<sup>3</sup> This classical and admirable method gives very accurate results with some peptides; for a recent instance of this see Bergmann and Miekeley.

<sup>4</sup> *Cf.* (6) p. 214.

## EXPERIMENTAL.

1. *The New Method of Separation.*

*Separation from Yeast.*—It is quite easy to prepare the crystalline substance in a pure state from as little as 1 or 2 kilos of pressed bakers' yeast, while the method is equally convenient for preparations on the large scale. The yeast is extracted by boiling it for about 5 minutes with water containing 0.1 per cent acetic acid. In the first extraction it is convenient to use 1 liter of fluid for each kilo of yeast; a second extraction may be made with half that quantity. The extract filters well at the pump if the papers on the Buchner funnels be first covered with a thin layer of kieselguhr. To the clear pigmented extract a saturated solution of neutral lead acetate is added (about 20 cc. per liter of the extract) and then, without filtering, the 10 per cent solution of mercuric sulfate in 5 per cent sulfuric acid. The latter is gradually added until no more precipitate is produced, any considerable excess of the reagent being avoided.<sup>5</sup> About 150 cc. per liter are usually required. After a few hours the precipitate settles sufficiently to allow much of the supernatant fluid to be syphoned off. It is then filtered off and washed, once with dilute (2 to 3 per cent) sulfuric acid, and twice with distilled water. After removal from the filter it is ground in a mortar to a thin paste, washed into a flask of suitable size, and gassed with hydrogen sulfide. In practice it has been found satisfactory if the suspension of the precipitant before gassing contains roughly 250 cc. of water for each kilo of yeast employed. The decomposition is slow and it should be remembered that the black sulfide may cloak a large proportion of undecomposed precipitate. When one is working on a large scale, with use of the closed vessel method, 48 hours should be allowed for complete decomposition, and the vessel should be frequently shaken. The mercury sulfide is then filtered off and well washed.

From the filtrate the  $H_2S$  is removed, at first by a current of air, and finally, to avoid any possible oxidation of the product, by a

<sup>5</sup> In the case of some samples of yeast, probably those containing more glycogen than usual, the precipitate obtained on addition of the mercury reagent alone tends to be somewhat colloidal, and rather difficult to filter. The preliminary partial precipitation with lead seems always to obviate this.

stream of hydrogen. Sufficient sulfuric acid is then added to make the solution approximately 0.5 N; say, 12 cc. of strong acid (first diluted somewhat) per liter.<sup>6</sup> The solution is then ready for precipitation of the product by cuprous oxide. The oxide is conveniently prepared by boiling ordinary Fehling's solution with excess of glucose cautiously added. It should be of bright red color, and not brown or purple, and is washed first by decantation and then on a filter. It can be safely dried and kept as a powder. A known amount should be rubbed up in a little water at the time of using.

The copper compound of the peptide may be precipitated from the yeast extract at room temperature, but it is of advantage first to heat the extract to about 50° before the precipitation is begun, though this temperature need not be further maintained. Local overheating should of course be avoided. The aqueous suspension of cuprous oxide should be added gradually in quite small quantities at a time and the fluid should be well stirred on each addition. Precipitation is not quite immediate, but after a certain minimum of the oxide has been added it is seen to begin and to increase with each addition. As an excess of copper must be avoided (see below) some care is necessary towards the end of the process. A fairly good indication of the end-point is given when the red color of suspended, undissolved, copper oxide is seen for a brief period to survive vigorous stirring. It will usually be found that about 0.3 gm. of the oxide per kilo of yeast is required. Familiarity makes recognition of the end of precipitation quite easy, but when one is first carrying out the process it may be well to let the precipitate settle from time to time and note the effect of further very small additions of copper to 20 cc. or so of the supernatant fluid.

Two circumstances involved in the precipitation call for remark: (1) For a reason not yet investigated the cuprous compound is soluble in excess of copper. It contains 1 atom of copper to a molecule of the tripeptide and it is possible that a more soluble salt containing more of the metal exists. A relatively small excess

<sup>6</sup> When lead is used in the precipitation as described above, a certain amount of sulfuric acid is of course already present in the filtrate from the sulfides. The copper compound remains however but slightly soluble throughout a wide range in acid concentration. The solution need be only approximately 0.5 N.

of the added oxide may at any rate when a separation from a yeast extract is in progress, cause resolution in dramatic fashion of a quite large precipitate. This result however is easily avoided on the lines suggested above. If it has occurred, all the copper present must be removed as sulfide, and the process of precipitation repeated, a procedure which need not involve loss. (2) As was pointed out in an earlier section of this paper, presence of the peptide in the solution prevents the decomposition into cupric sulfate and metallic copper which otherwise occurs instantaneously when cuprous oxide is dissolved in sulfuric acid. When the peptide is in pure, or moderately pure, solution no trace of metallic copper is formed until a marked excess of the oxide has been added. The cuprous compound comes out in pure white microcrystalline form. In the crude yeast extract however the contact between the peptide and the added cuprous oxide is not always immediate enough to prevent small amounts of metallic copper being formed. The precipitate which first forms is apt therefore to be gray in color, though later additions of the oxide produce a white precipitate. Unless it is desired to prepare the copper salt in pure form, this circumstance is of no moment. It does not of course affect the purity of the copper-free product which is obtained when the metal is removed as sulfide. (If a preparation of the pure copper compound is desired at this stage, the precipitate when centrifuged off may be digested at 30–40° with its own bulk of 0.5 N sulfuric acid with occasional stirring. The compound is then obtained perfectly white and gives accurate copper values on analysis. Otherwise of course the copper compound may be prepared by precipitating as above a solution of the pure peptide previously prepared. It then separates in micro crystals which give a silky sheen to the fluid, and is a pure white substance.)

The precipitate should not be filtered off but centrifuged; washing on a filter is slow, and the compound tends to become blue from oxidation. It should be washed by use of the centrifuge, once with 0.5 N sulfuric acid, and then with successive quantities of distilled water (previously well boiled and allowed to cool in a corked flask) until absolutely free from sulfate. It centrifuges extremely well and the first appearance of a slight non-settling cloud nearly always coincides with complete freedom from sulfate.

If it is wished to estimate the yield of copper compound, the

washed precipitate should be stirred up with alcohol, again centrifuged, the centrifuge tubes placed in a vacuum desiccator overnight, and the product weighed. The dry precipitate is then ground up with water for decomposition. Otherwise the original moist precipitate is suspended in 4 or 5 times its bulk of distilled water and decomposed with hydrogen sulfide. The sulfide must be well washed. The filtrate from the copper sulfide, which should be water-clear and completely colorless, is freed from  $H_2S$  by a stream of hydrogen, and then evaporated in a good vacuum at  $40^\circ$ . Evaporation should stop before the material is syrupy, the solution being removed from the vacuum flask when it measures, say, 6 to 8 cc., or thereabouts, for each kilo of yeast extracted. It is placed in a large crystallizing dish, mixed with half its volume of alcohol, a little more alcohol being poured upon the surface of the solution, and the dish then allowed to stand in a thoroughly evacuated desiccator containing fresh sulfuric acid. Crystallization may be somewhat slow to begin, but in from 24 to 36 hours foci of crystals will be seen and thereafter separation rapidly proceeds. If, as very rarely happens, no crystals have appeared by the time the solution has become syrupy, slight disturbance, such for instance as is involved in transference to a smaller crystallizing dish, is invariably followed by rapid crystallization. The solution of the peptide is sometimes apt to become supersaturated, but then if the familiar method of scratching the surface of the basin is adopted, it is always followed by a rapid separation of thick magma consisting of microscopic prisms.

In general it is found that the product thus obtained is so pure that if the solution be taken quite to dryness the residue gives good figures on analysis, or if successive crystalline fractions have been separated this remains true of the contents of the final mother liquors. More rarely a small syrupy uncrystallizable residue is obtained, from which however cuprous oxide precipitates a large proportion of the normal copper salt. Experience suggests that this is not necessarily because the original copper preparation was impure, but may be because in such cases the tripeptide has undergone slight decomposition owing, possibly, to the process of evaporation at  $40^\circ$  (*supra*) being, as the result of a poor vacuum, overprolonged.

The crystalline fractions should not require recrystallization

for purity. If however it be desired to recrystallize them, they must be dissolved in a minimum of water and, after the addition of alcohol, left in the desiccator as before. No solvent has been found from which more direct recrystallization is possible.

Owing to the nature of the substance, I have described its crystallization in (perhaps unnecessary) detail. As a matter of fact the product, although a tripeptide, crystallizes so readily that in dealing with it one need only remember that it is not very insoluble in the only medium at present available, namely, dilute alcohol.

However carefully the end-point is adjusted, precipitation by cuprous oxide as described above does not remove the whole of the tripeptide which is contained in the original mercury precipitate. It may happen indeed that no more than from 70 to 75 per cent is so precipitated. If the clear solution from which the first copper precipitate has been removed be again treated with the mercuric sulfate reagent (without the trouble to remove any excess of copper) and if the resultant precipitate be decomposed by  $\text{H}_2\text{S}$  and the solution as before be made 0.5 N with sulfuric acid, it yields on addition of cuprous oxide a further precipitate of the characteristic copper salt. This is not due solely to an increase in concentration, but largely to a readjustment of somewhat obscure equilibria in the solution. Precipitation may be yet incomplete however carefully the end-point is adjusted. A further (now small) quantity of the pure copper salt may be obtained after a second precipitation with mercury. On these lines upwards of 1 gm. of the crystalline product per kilo of yeast has been obtained.

At the risk of some repetition I feel it may be helpful to describe a separation, as actually carried out, in a case where a record was kept of the stages involved.

12 kilos of French pressed yeast were boiled up in a large enamelled iron basin with 12 liters of water containing 0.1 per cent of acetic acid. The extract was kept boiling for 3 or 4 minutes and the thick brew then transferred while still hot to six large Buchner funnels in which the filter papers were covered with a thin layer of kieselguhr. A moderate vacuum being maintained in the filter flasks, the residue became completely dry overnight. It was again boiled up with 5 liters of acidulated water and returned to the filters. The final residue was sucked dry but not further washed.

To the mixed filtrates (16.5 liters) 250 cc. of saturated neutral lead acetate solution were added, and then, more gradually and with constant stirring, 2400 cc. of the mercuric sulfate reagent. When the mixture had stood overnight the mercury precipitate settled well, the supernatant fluid, though not quite clear, containing only a negligible quantity of suspended material. The fluid was syphoned off and the precipitate transferred to a Buchner funnel. It filtered well with the pump, though somewhat slowly. It was washed thrice with distilled water and then transferred to a mortar and rubbed into a thin uniform paste. This was transferred to a 5 liter flask and the suspension of the precipitate finally made to measure about 2 liters. The flask being provided with cork and tubes, the decomposition by  $\text{H}_2\text{S}$  delivered from a Kipp was carried out with the exit tube closed, the flask being frequently shaken. After about 40 hours decomposition was complete, as evidenced by the absence of bubbles in a wash bottle when the flask was vigorously shaken. The mercury precipitate was filtered off and washed thrice at the pump.  $\text{H}_2\text{S}$  was removed from the filtrate, the greater part by aeration, and the last traces by a stream of hydrogen. The solution now measured 2300 cc. and was acidified by the addition of 25 cc. of strong sulfuric acid (diluted before addition). The flask was heated on the water bath, the fluid being kept in motion till its temperature reached  $45^\circ$ . 3 gm. of  $\text{Cu}_2\text{O}$  in fine aqueous suspension were gradually added, the flask being well shaken between each addition. The precipitate showed a silky sheen and soon became bulky. The contents of the flask were transferred to a tall beaker and in the course of a few hours the precipitate had completely settled, when the clear fluid was syphoned off. The precipitate and associated fluid were then centrifuged thoroughly and the fluid returned to the main bulk of solution. To the latter 0.1 gm. of  $\text{Cu}_2\text{O}$  was added and a small, but appreciable, further quantity of precipitate formed with the usual crystalline appearance. On standing however instead of settling, this small fraction became completely redissolved.

The first copper precipitate was washed at the centrifuge with oxygen-free distilled water until free from any trace of sulfuric acid. Contained in four tubes, each holding 45 cc. of wash water, it had to be centrifuged ten times before the acid was wholly

removed. It was finally stirred up with 97 per cent alcohol, again centrifuged till as dry as possible, and then, still in the centrifuge tubes, transferred to a vacuum desiccator. When dry the copper salt, still completely colorless, weighed 7.9 gm.

To the solution from which this first fraction of the copper salt had been removed mercuric sulfate was added and the resultant precipitate washed and decomposed. To the filtrate, again made about 0.5 N with  $\text{H}_2\text{SO}_4$ , 0.4 gm. of cuprous oxide was added. The typical silky precipitate when separated, washed, and dried weighed 1.6 gm. On similar lines another 0.48 gm. was obtained. The total yield of practically 10 gm. of the copper compound (= 0.69 gm. of tripeptide per kilo of moist yeast) was in this case below the average. It should be understood however that the substance is strongly adsorbed on the successive sulfide precipitates. Complete washing takes much time and when a supply of materials is alone required may not in the end prove economical. A more nearly accurate estimate of the amount of tripeptide actually present is got by using small quantities of yeast. Thus from 2 kilos of fully extracted yeast, with thorough washing of successive precipitates, 2.1 to 2.4 gm. of tripeptide have been obtained.

*Separation from Blood Corpuscles.*—Before the cuprous compound method had been discovered a preparation made from ox blood by the modification of the original method had been found to yield the characteristic diketopiperazine of glycine and cystine on being boiled with water, and from the products of its acid hydrolysis glutamic acid, glycine, and cystine were isolated. Later the new copper method was applied to a remainder of this preparation and the presence of the tripeptide demonstrated. The new technique was then applied to blood itself and the tripeptide easily isolated pure. Only one such preparation has so far been made however, and as the optimal conditions for the isolation from blood have not been fully studied, the yield obtained must not be taken as evidence for the amount of the tripeptide actually contained in the corpuscles.

20 liters of whole ox blood were mixed with 10 liters of water and then with 7 liters of 0.1 N sulfuric acid and the mixture heated to 80°. The coagulum was filtered off and to the cooled filtrate the mercuric sulfate reagent was added. Whether or not because of a difference in the buffers present the blood preparation at this

stage does not yield the mercury precipitate so readily as does a yeast extract. Sodium hydroxide must be added till the acidity of the solution is considerably reduced. By adding the mercuric sulfate gradually, alternating the additions with small additions of alkali, the point of maximal precipitation can be determined without much difficulty. When, in the present case, this point was attained, the precipitate was filtered off, washed, and decomposed. The solution was for a second time precipitated with mercury and the precipitate treated as before. To the solution thus finally obtained (900 cc.), when freed from  $H_2S$  and warmed to  $45^\circ$ , cuprous oxide was cautiously added. The characteristic silky precipitate of the copper compound quickly formed. It was allowed to settle and then centrifuged off and thoroughly washed on the centrifuge with oxygen-free water. Dehydrated with alcohol and dried in the vacuum desiccator, it weighed 0.92 gm. After the copper salt was decomposed the colorless filtrate from the copper sulfide was evaporated *in vacuo* and, finally, in the desiccator. It readily yielded crops of crystals and the product so obtained agreed in every respect with the product from yeast. It yielded consistently accurate analytic figures for the tripeptide, as will be seen below.

*Note on a Modification of the Original (1921) Method of Separation.*

The following brief account of this method is given because certain experiments described in this paper were carried out on products obtained by its use, but also because these less pure products, though they certainly contained a large proportion of the tripeptide, show differences of behavior which may have significance (p. 282).

The yeast or tissue extract was first precipitated by the acid mercuric sulfate solution. The mercury was removed as sulfide and sulfuric acid removed from the filtrate, the solution being next precipitated by neutral lead acetate with careful avoidance of excess. The lead precipitate was ground up with 0.5 N sulfuric acid and successively extracted with this until the extracts no longer gave a nitroprusside reaction. The filtrate from the lead sulfate was precipitated by phosphotungstic acid added in quantity somewhat larger than that required to produce a maximal precipitate. The filtrate was cooled with ice and the excess of phosphotungstic acid removed with ice-cold barium hydroxide solution. The filtrate from the barium phosphotungstate was precipitated by mercuric sulfate; this precipitate was decomposed, and another lead precipitate obtained. The latter, decomposed as usual, yielded the solution of the final product. This was concentrated *in vacuo* at  $40^\circ$  and the product (with or without previous oxidation to the disulfide form) thrown out of solution with alcohol.

2. Analytical Data.<sup>7</sup>*Metallic Content of the Pure Copper Compound.*

The following data were from preparations which had been washed at the centrifuge with oxygen-free water, then with alcohol, and, after standing in a well evacuated desiccator, dried to constant weight *in vacuo* at 50°. Only with such precautions is oxidation of the cuprous copper completely avoided. The dried products should show no blue color. All the figures given were obtained by igniting the compound in a stream of oxygen and weighing the CuO.

Product	A.	4.931 mg.:	1.102 mg. CuO.	Cu = 17.45 per cent.
"	B.	6.130 " :	1.290 " "	16.80 " "
"	C.	5.341 " :	1.181 " "	17.41 " "
"	D.	8.110 " :	1.746 " "	17.20 " "
"	E.	100.00 " :	21.540 " "	17.21 " "
Calculated for $C_{10}H_{16}N_3O_6Cu$ .....				17.26 " "

*Crystalline Tripeptide.*

*Preparation A (Yeast).*—In this 5 gm. of the copper were decomposed and two successive fractions of the crystalline product were analyzed. The mother liquor from these was taken right to dryness and the crystalline residue analyzed. The products were first dried in a vacuum desiccator, and then, till of constant weight, at 50° in an evacuated tube connected with a bulb containing  $P_2O_5$ .

*Fraction 1 (2.2 Gm.).*

	C	H	N	S
4.773 mg.:				
6.840 mg. $CO_2$ and				
2.30 mg. $H_2O$ .....	39.11	5.45		
3.180 mg.:				
0.366 cc. N at 21° and				
761 mm.....			13.38	
5.570 mg.:				
4.260 mg. $BaSO_4$ .....				10.50

<sup>7</sup> The nitrogen and sulfur determinations were made by A. Colwell of this laboratory; the carbon and hydrogen by Schoeller of Berlin-Schmargendorf.

*Fraction 2 (1.19 Gm.).*

4.951 mg.: 7.010 mg. CO <sub>2</sub> and			
2.42 mg. H <sub>2</sub> O.....	38.80	5.45	
3.288 mg.: 0.380 cc. N at 20° and			
761 mm.....		13.43	
5.715 mg.: 4.435 mg. BaSO <sub>4</sub> .....			10.65

*Residues from Mother Liquor.*

5.181 mg.: 7.34 mg. CO <sub>2</sub> and			
2.62 mg. H <sub>2</sub> O.....	38.64	5.61	
6.477 mg.: 4.957 mg. BaSO <sub>4</sub> .....			10.51
Micro-Kjeldahl.....		13.41	

The following preparations from yeast were made by colleagues.

*Preparation M.*

	C	H	N	S
4.303 mg. required 4.11 cc. 0.01 N				
acid.....			13.37	
5.602 mg.: 4.249 mg. BaSO <sub>4</sub> .....				10.42

*Preparation P.*

4.485 mg. required 4.39 cc. 0.01 N				
acid.....			13.40	
5.556 mg.: 4.237 mg. BaSO <sub>4</sub> .....				10.47

*Preparation C (from Yeast of Different Provenance).*

Fraction 1.....	38.91	5.41	13.56	10.41
“ 2.....			13.41	10.45

*Preparation from Blood.*

	C	H	N	S
4.610 mg.: 6.590 mg. CO <sub>2</sub> and				
2.38 mg. H <sub>2</sub> O.....	39.00	5.71		
6.286 mg.: 4.882 mg. BaSO <sub>4</sub> .....				10.67
4.427 “ required 4.24 cc. 0.01				
N acid.....			13.41	
Mean of all analyses.....	38.89	5.53	13.42	10.51
Calculated for C <sub>10</sub> H <sub>17</sub> N <sub>3</sub> SO <sub>6</sub> .....	39.09	5.54	13.68	10.42

*3. Results of Acid Hydrolysis.*

Before the crystalline product had been prepared a somewhat long apprenticeship had been served in isolating the amino acids derived from the earlier less pure preparations. Only cysteine (or cystine), glycine, and glutamic acid were found, and a satisfactory technique for isolating these, with at least approximately quantitative results, became familiar. In the case of the particular amino acids in question preliminary esterification served no good purpose from a quantitative point of view; and for the method used (removal of cysteine by precipitation with mercury, and reliance upon fractional crystallization for the separation of the others) the employment of relatively small amounts came to be preferred.

The pure tripeptide has been hydrolyzed both with hydrochloric and with sulfuric acids. The former has the disadvantage that in removing the acid remaining after evaporation with silver sulfate or silver oxide it is impossible to avoid simultaneous precipitation of part of the cysteine; the use of sulfuric acid of course needs care in the avoidance of loss when one is dealing with the large barium sulfate precipitate.

In respect to the isolation of glutamic acid and glycine the results of hydrochloric acid and sulfuric acid hydrolysis have been so similar that the latter alone need be described in detail.

2 gm. of a crystalline product were boiled for 16 hours with 30 cc. of 25 per cent sulfuric acid. The greater part of the acid was removed with baryta and the barium sulfate precipitate very thoroughly washed. The solution was then precipitated with the acid  $\text{HgSO}_4$  reagent and practically every trace of cysteine thus removed. From the filtrate the mercury was removed as sulfide and the sulfuric acid (exactly) as barium sulfate. The solution was then evaporated and the successive crystalline fractions which separated were filtered off quantitatively through very small filters. The first three fractions, weighing together 0.726 gm., proved to be glutamic acid analytically pure. Late fractions weighing 0.250 gm. consisted of pure glycine. Intermediate fractions however with an average nitrogen content of 17.5 per cent (glycine 18.66, glutamic acid 9.52) were resistant to further separation. Each yielded however, when treated with picric acid, a

copious precipitate of glycine picrate. In making the yield of this approximately quantitative advantage was taken of the detailed study of Levene and Van Slyke (7). The combined fractions were dissolved in distilled water (1.5 cc. for each decigram) and picric acid added in the proportion of 1.8 parts to 1 part of the material. When the mixture was boiled the picric acid went completely into solution. When cooled to room temperature, the solution was sown with a crystal of pure glycine picrate and immediately became nearly solid with a crystalline precipitate. After standing on ice for an hour, the mother liquor was drained off, the crystals washed with a little 50 per cent alcohol, and then with absolute alcohol and ether. Without further treatment the picrate so obtained was completely pure. When dried it melted sharply at  $202^{\circ}$ , after softening at  $200^{\circ}$ . The melting point was not altered by admixture with pure glycine picrate. The amino N was determined in a Van Slyke apparatus. When multiplied by the factor 0.94 the result obtained was 7.51 per cent. Calculated for  $(C_2H_5NO_2)_2C_6H_3N_3O_7$ , 7.39 per cent.

On concentration of the filtrate another small yield of glycine picrate was obtained and finally, after acidification and removal of the picric acid with ether, a crystalline fraction consisting of glutamic acid.

The non-crystalline mother liquors from the crystalline fractions as first obtained (see above) gave a residue weighing 0.14 gm. This largely consisted of glycine, as shown by the yield of pure picrate.

The mercury precipitate obtained as above was decomposed by  $H_2S$  and the sulfuric acid removed with baryta. The solution when quite free from  $H_2S$  was made alkaline with ammonia, a trace of iron being added to secure oxidation of the cysteine to cystine, then evaporated slowly on the water bath, and allowed to stand. The cystine which was finally separated weighed 0.710 gm. The uncrystallizable mother liquor from this gave a residue weighing 70 mg. only.

By the procedure described 0.710 gm. of cystine (90.0 per cent of theory for tripeptide) was obtained analytically pure; of pure glutamic acid 0.810 gm. (84.5 per cent of theory); and of glycine, either weighed pure or calculated from the picrate 0.3900 gm. (92 per cent of theory). The small deficiencies were due to manipula-

tive losses. If the data be scrutinized, it will be seen that no other amino acid could have been present.

*Analyses.*

		N	S	C
Cystine fraction.	Found.....	11.48	26.20	
	Calculated.....	11.57	26.44	
Glutamic fractions (combined).	Found.....	9.44	6.20	40.71
	Calculated.....	9.52	6.12	40.82
Glycine fraction.	Found.....	18.54		
	Calculated.....	18.66		

The evidence for the purity of the glycine picrate is given above.

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*IV. Determination of Amino and Carboxyl Groups, and of the Minimal Molecular Weight, by the Acidimetric Titration Technique.*

BY LESLIE J. HARRIS.

An extension of the theory of titrations was elaborated some years ago (Harris, 1923, etc.<sup>1-5</sup>) as a result of which it became possible to determine acids or bases which had previously been considered too weak to fall within the scope of acidimetric methods. It was shown, in particular, that the amino as well as the carboxyl groups in amino acids, polypeptides, and the like, could be determined by acidimetric titrations, and several alternative procedures for this purpose were described. The applications of the new technique were also shown to include methods for (1) determining combining weights, (2) estimating certain individual amino acids (or homologous series of amino acids) when present in complex mixtures of amino acids, (3) determining polypeptides or proteins volumetrically, and (4) measuring dissociation con-

<sup>1</sup> Harris, L. J., *Proc. Roy. Soc. London, Series B*, **95**, 440 (1923).

<sup>2</sup> Harris, L. J., *Proc. Roy. Soc. London, Series B*, **95**, 500 (1924).

<sup>3</sup> Harris, L. J., *J. Chem. Soc.*, **123**, 3294 (1923).

<sup>4</sup> Harris, L. J., *Biochem. J.*, **17**, 693 (1923); *Nature*, **115**, 119 (1925); *J. Soc. Chem. Ind.*, **44**, *Chem. and Ind. Rev.*, **3**, 1016 (1925); *Proc. Roy. Soc. London, Series B*, **97**, 364 (1925).

<sup>5</sup> Harris, L. J., *Proc. Roy. Soc. London, Series B*, **104**, 412 (1929).

stants. As of further direct interest in connection with the present note, it should be added that among other deductions it was shown also, for the first time, that the protein molecule is constituted as a "zwitterion,"<sup>6</sup> and that the peptide linkages, contrary to former supposition, are incapable of combining with acids or alkalies under any of the conditions examined.

As far as the general principle of these methods is concerned, it is sufficient here to say that any single amino acid (or any mixture of ampholytes) is regarded as equivalent to a mixture of single simple mono-acids and bases having the same separate dissociation constants as the several titration dissociation constants of the body in question, and therefore that when one is dealing with weak acids or bases, or when one is titrating to a sufficiently highly acid or alkaline end-point, the "strength" of the *water*, present as solvent, is comparable with the "strength" of these other weak acids or bases, and hence cannot be neglected; *i.e.*, the *water* present must be regarded as one of the *constituents* of the mixture.

Below is described the application of these methods to glutathione. Since the Van Slyke gasometric method<sup>7</sup> for amino groups gives irregular results with many peptides, often also with sulfur compounds, no less than with glycine derivatives, it would appear that the titration methods offer the only alternative at present available for gaining that knowledge of the number of amino and carboxyl groups, pre-necessitated in arriving at the structure of the body.

The several methods described below all comprise simple titrations to some sharp color change with a pH indicator. (When applied to a large number of amino acids—and also to a few peptides—these same titration methods have invariably been found to give satisfactory results, generally to within 1 or 2 per cent of the theoretical.<sup>8</sup>) To obtain fullest information, such colorimetric estimations were studied, it will be remembered, in conjunction with complete titration curves, in water, formol, etc. The latter aspect in the present tests has been undertaken by Pirie and Pinhey and is described by them separately.<sup>9</sup>

<sup>6</sup> Similarly, isoelectric oxidized glutathione ( $G_2S_2$ ) appears to be a complex zwitterion, with four ionized groups.

<sup>7</sup> Van Slyke, D. D., *J. Biol. Chem.*, **9**, 185 (1911).

<sup>8</sup> Except that arginine sometimes behaves as a mono-base rather than a di-base mono-acid.

<sup>9</sup> Pirie, N. W., and Pinhey, K. G., *J. Biol. Chem.*, **84**, 321 (1929).

All the findings to be recorded were obtained upon crystalline specimens of (reduced) glutathione which had been isolated by the newer method described by Hopkins in the present paper.

1. *Estimation of  $\text{NH}_2$  and  $\text{COOH}$  by the Alcohol Titration Method,*  
(A)  $\text{NH}_2$ .

This method while having a slightly lower percentage accuracy than other methods to be described below, is marked by extreme simplicity and rapidity. Martens,<sup>10</sup> who has recently submitted it to a critical study, using not only amino acids but also peptides, concludes: "La méthode procure des résultats très précis pour tous les acides aminés. . . . frappé par la précision des résultats, séduit la simplicité du mode opératoire. . . . Elle a sur la méthode de Sørensen [et de Foreman] des avantages marqués."

*Method.*—A suitable weight of the glutathione (about 0.5 or 0.1 mg. equivalent) is taken and dissolved in a small quantity of water (*e.g.*, enough to make a 0.1 M solution); next sufficient alcohol may be added to make a final concentration of about 80 per cent; and, then from a micro burette that amount of soda (best, N) is run in which is required to titrate all the carboxyl groups present, as determined by a separate duplicate titration (Section 2, below). Finally methyl red indicator is added, and the amino groups are then determined by titrating with HCl (best, N) to a sharp change from yellow to orange (to match aqueous pH virage of about 5.4). (Formaldehyde must be absent in this titration.) (The titrations with N acid or alkali are conveniently carried out in a new type of micro burette which is graduated in 0.01 cc. intervals, holds a total of 2 or 5 cc., and is conveniently filled from the top like an ordinary macro burette.)

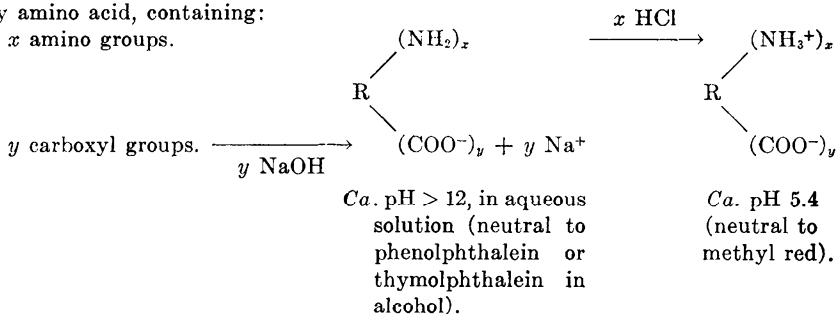
*Note (a). On an Alternative Method.*—When one is dealing only with known amino acids, and in the absence of tyrosine, it was shown in the early paper<sup>2</sup> that the above procedure could for convenience be somewhat shortened by performing the two titrations upon the same specimen. The carboxyl titration was first made with NaOH against thymolphthalein, and then in continuation the amino determination was made by a back titration with HCl against methyl red. In the presence of the weakly basic phenolic OH of tyrosine, however, the preliminary

<sup>10</sup> Martens, R., *Bull. Soc. chim. biol.*, **9**, 454 (1927).

titration with NaOH naturally gives a high reading (since part of the OH titrates) so that the back titration with HCl was found to be increased by the same amount. The shortened procedure is, then, clearly *less* advisable when one is investigating the behavior of unknowns, more definitely as in the present instance when the weakly basic —SH of glutathione is closely comparable in weak basicity with the phenolic OH of tyrosine.<sup>11</sup> The preliminary titration of carboxyl groups with soda should therefore be made by the alternative method described in my early paper; *i.e.*, (1) to phenolphthalein in 80 per cent alcohol and with formalin, instead of to thymolphthalein and without formalin; and (2) on a duplicate, since the subsequent amino titration is best performed in absence of formalin.

*Note (b). On the Possibility of Omitting Alcohol in the Back Titration.*—A small blank correction is made for the amount of soda required to take the solvents and indicator alone over the same pH range. This blank correction is naturally the smaller (and the titration correspondingly the more accurate) when the HCl titration is carried out in the absence of alcohol instead of with an excess of alcohol. Such an omission is generally permissible, indeed sometimes slightly advantageous in intensifying the sharpness of the end-point, when the NaOH titration is performed on a separate duplicate in advance, according to the slightly lengthened technique here adopted. The NaOH and HCl titrations give rise to the following type reactions, respectively, when stated in the zwitterion mode.

Any amino acid, containing:  
 $x$  amino groups.



A detailed setting out of the different possible types of amino acid—acidic, basic, and neutral—will be found in the earlier paper,

<sup>11</sup> For glutathione  $\text{pK}_{\text{SH}} = 9.8$ ; for tyrosine  $\text{pK}_{\text{OH}} = 9.7$  or greater.

where the alternative classical mode of representation is employed in place of the present zwitterion style. The method may be said to depend on the fact that the following bodies in solution have a reaction near pH 5.4 (pK of methyl red): the monoamino-monocarboxylic acids, the monosodium salts of the dicarboxylic monamino acids, the monohydrochlorides of the diaminomono-amino acids.<sup>12</sup> The results of the analyses are given in Table A.

*Results.*—It will be seen that the titration values *found* are in very close agreement with those *calculated* (*i.e.*, for a tripeptide consisting of cysteine, glycine, and glutamic acid). With the

<sup>12</sup> The criticism might possibly be raised that there may exist, for all we know, among ampholytes as yet unisolated, some having pK values so widely divergent from the normal pK range of all the known ampholytes as to give inaccurate titration readings in this method. It is therefore worth recording that the technique in question has already been applied experimentally, so far with remarkably accurate results, to practically all the known natural amino acids, as well as to a number of polypeptides. Thus, giving our fullest available list of forty-three instances here appended, we see that the calculated error is always so small as to lie well under 1 per cent in all except the five instances where the slightly higher figures are cited. (We are here referring to titrations to pH 5.4 *without alcohol*,—the similar, or in some cases even better, experimental accuracy obtained *in the presence of alcohol* has already been recorded for the first seventeen of these bodies.<sup>2,10</sup>

Glycine, alanine, valine, leucine, phenylalanine, tryptophane, cystine, cysteine, proline.

Aspartic acid (error = + 2 per cent, but much less in alcohol), glutamic acid (error = + 4 per cent, but much less in alcohol), tyrosine (see text).

Lysine, arginine (see foot-note above), histidine (100 per cent of amino plus 90 per cent of imino in aqueous solution, but 100 per cent of amino only in alcohol).

Glycyl-glycine, leucyl-glycine.

Serine, isoleucine, norleucine, oxyproline,  $\beta$ -hydroxyglutamic acid (error = + 6 per cent in aqueous solution, ? less in alcohol),  $\alpha$ -,  $\gamma$ -, and  $\delta$ -amino-*n*-valeric acids, sarcosine.

Alanyl-alanine, alanyl-glycine, aminobutyl- $\alpha$ -aminobutyric acid, glycyl-alanine, glycyl-asparagine, glycyl-leucine, glycyl-valine, glycyl-sarcosine, leucyl-asparagine, leucyl-isoserine, sarcosyl-sarcosine.

Aspartyl-glycine (error = + 12 per cent in aqueous solution, ? less in alcohol), glycyl-aspartic acid (error = + 10 per cent in aqueous solution, ? less in alcohol).

Glycyl-glycyl-glycine, alanyl-glycyl-glycine.

Glycyl-alanyl-alanyl-glycine.

Leucyl-octaglycyl-glycine.

TABLE A.  
*Analytical Details.*

	Specimen 1, F.G.H.*	Specimen 2, F.G.H.	Duplicate of Specimen 2.	Specimen 3, N.U.M.
Weight of glutathione taken.....	<i>gm.</i> 0.0614	<i>gm.</i> 0.0500	<i>gm.</i> 0.0500	<i>gm.</i> 0.0601
Water added (CO <sub>2</sub> -free).....	<i>cc.</i> 1	<i>cc.</i> 0	<i>cc.</i> 0	<i>cc.</i> 1
EtOH added.....	20	0	20	8
Standard NaOH run in ( <i>i.e.</i> theoretical amount as determined in Section 2).....	4.1† × 0.1 N (Macro burette.)	3.258‡ × 0.1 N (Micro burette.)	3.258‡ × 0.1 N (Micro burette.)	0.397† × N (Micro burette.)
Phenolphthalein (0.5 per cent in 50 per cent alcohol).....	1	0	0	1
Methyl red (0.02 per cent in 60 per cent alcohol).....	1	0.1	0.5	1
HCl blank.....	0.15	0		0.005
Found.				
Standard HCl required (corrected for blank).....	1.95, 2.00 × 0.1 N (Macro burette.)	1.605 × 0.1 N (Micro burette.)	1.605 × 0.1 N (Micro burette.)	0.205 × N (Micro burette.)
Sensitiveness of end-point color change, in cc. HCl added.....	<0.05	<0.02	<0.03	<0.01
Calculated.				
For tripeptide of cysteine, glycine, glutamic acid.....	2.00 × 0.1 N	1.629 × 0.1 N	1.629 × 0.1 N	0.196 × N
Maximum divergence. Between "found" and "calculated" readings.....	<i>per cent</i> < -2.5	<i>per cent</i> -1.5	<i>per cent</i> < -1.8	<i>per cent</i> +4.6

\* Specimens 1 and 2 were both prepared by F. G. H.; Specimen 3 by N. M. Meldrum. Hopkins' method was used for all three specimens.

† NaOH blank was included here, and hence increased by the same amount as the HCl blank (*i.e.* in the back titration).

‡ NaOH blank was omitted.

most sensitive conditions, the divergence falls within 1.5 per cent of the reading.

2. *Estimation of NH<sub>2</sub> and COOH by the Alcohol Titration Method, (B) COOH.*

Although according to Willstätter and Waldschmidt-Leitz<sup>13</sup> the COOH of polypeptides may be titrated quantitatively in 40 per cent alcohol (against phenolphthalein), nevertheless preliminary trials with glutathione showed that the method gave extremely *low* readings. Even when the alcohol was increased up to 95.5 per cent,<sup>14</sup> 86 per cent only of the theoretical was reached (titrating to the first visible color change); or only 93.5 per cent of the theoretical, when titrating in 97 per cent alcohol (and to a fuller pink). On the other hand titration to thymolphthalein in alcohol gave, as anticipated, *high* results, owing to the ionization of the weakly basic —SH group. Accordingly the method previously described by the writer, which is a convenient, very slight modification of Foreman's COOH titration method, was used. It has previously given accurate results when applied to all the better known amino acids and to several peptides.

*Method.*—A 0.1 mg. equivalent of glutathione, more or less, is dissolved in water to make say 1 cc. of 0.1 M solution, to which are added about 8 cc. of alcohol, 1 cc. of formalin, and 1 cc. of phenolphthalein indicator. The mixture is then titrated to a sharp end-point (change from colorless to red) with 0.1 N, or preferably N, NaOH, with use of the micro burette already alluded to. The final alcohol concentration should be about 80 per cent. A small blank correction is made for the solvents.

*Analytical Details.*

1. First specimen (F. G. H.); macro titration, 0.1 N NaOH.  
0.0614 gm. glutathione (F. G. H.)  
1.0 cc. water (CO<sub>2</sub>-free)  
20.0 " alcohol (97 per cent)

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<sup>13</sup> Willstätter, R., and Waldschmidt-Leitz, E., *Ber. chem. Ges.*, **54 B**, 2988 (1921).

<sup>14</sup> 97 per cent alcohol is required in the Willstätter method when free amino acids rather than peptides are to be titrated.

- 1.0 cc. phenolphthalein (0.5 per cent in 50 per cent alcohol)  
 Required 3.8 cc. 0.1 N NaOH  
 Added 1.0 cc. of formalin just neutralized to phenolphthalein  
 Required 4.1 cc. 0.1 N NaOH  
 Less 0.1 " blank correction =  
 4.0 " (corrected)
2. Duplicate. 4.0 " "  
 Theoretical for tripeptide, cysteine, glycine, glutamic acid... 4.0 cc.  
 Found..... 4.0 "  
 End-point is sensitive to <0.1 cc., hence "found" = 100 per cent  $\pm$   
 <2.5 per cent of "theoretical."
3. Second specimen (N. U. M.); micro titration, N NaOH.  
 0.0578 gm. glutathione (N. U. M.)  
 1.0 cc. water (CO<sub>2</sub>-free)  
 8.0 " alcohol (absolute)  
 1.0 " phenolphthalein (0.5 per cent in 50 per cent alcohol)  
 Titrated from micro burette  
 Required 0.35 cc. N NaOH  
 Added 1.0 cc. of formalin just neutralized to phenolphthalein  
 Required 0.38 cc. N NaOH  
 Less 0.005 " blank correction  
 0.375 " (corrected)  
 Theoretical for tripeptide, cysteine, glycine, glutamic acid. 0.375 cc.  
 Found..... 0.375 "  
 End-point is sensitive to <0.01 cc., hence "found" = 100 per cent  $\pm$   
 <2.7 per cent of "theoretical."

*Results.*—The readings agree with the theoretical values calculated for the tripeptide within the experimental error of the method (*viz.* about 2.5 per cent).

### 3. Estimation of the Second COOH, by Titration in Water.

It was shown <sup>1,3</sup> that the second (*i.e.*, unbalanced) carboxyl group in dicarboxylicmonoamino acids, or the second (*i.e.*, unbalanced) amino group in diaminomono-carboxylic acids, can be estimated with great accuracy by titrating to a sharp color change to a pH slightly more acid than 7. This principle was made use of to estimate both the diamino and the dicarboxylic acids when present simultaneously in mixtures with other amino acids. It provides, in general, a method of determining unbalanced carboxyl or amino groups (*i.e.*, carboxyl in excess over amino, or *vice versa*).

It will now be understood that a titration to phenolphthalein

(pH 8.3), which is so commonly used for estimating single organic mono-acids, is liable to give erroneous results when applied to di-acid mono-bases or di-base mono-acids, or polyampholytes in general, since it estimates a fraction of a second group as well. Thus with glutathione, titration to a faint pink with phenolphthalein (? about pH 8.2) was found to give 115 per cent of the theory, or, to a more distinct color, a still higher figure.

*Analytical Details.*

First specimen (F. G. H.).

0.0614 gm. glutathione (F. G. H.)

1.0 cc. water (CO<sub>2</sub>-free)

0.5 " brom-thymol blue indicator

Titrated from micro burette

Required..... 0.205 cc. N NaOH

Theoretical (for the tripeptide)..... 0.200 " " "

Method sensitive to..... 0.005 " " "

Another preparation (N. U. M.).

0.0304 gm. glutathione (N. U. M.)

1.0 cc. water (CO<sub>2</sub>-free)

0.5 " brom-thymol blue

Titrated from micro burette

Required..... 0.10 cc. N NaOH

Theoretical (for the tripeptide)..... 0.10 " " "

Method sensitive to..... 0.005 " " "

*Results.*—These agree with theoretical (tripeptide) within the experimental error of the method (0.25 per cent) and are in confirmation of conclusions reached above in Sections 1 and 2.

*4. Estimation of Total COOH, "Neutral COOH," and Hence NH<sub>2</sub> by Means of Formaldehyde Titrations.*

As has been pointed out by Brown,<sup>15</sup> and by the present writer, the method of performing a formol titration (*cf.* Schiff, 1900;<sup>16</sup> Sørensen 1908<sup>17</sup>) as described in certain text-books gives fallacious results. One is directed, according to these, first to adjust the unknown solution to phenolphthalein (Stage 1), next to add neutralized formalin, and then to retitrate to phenolphthalein (Stage 2). The error arises in the first adjustment of

<sup>15</sup> Brown, J. H., *J. Bact.*, **8**, 245 (1923).

<sup>16</sup> Schiff, H., *Ann. Chem.*, **319**, 59 (1901).

<sup>17</sup> Sørensen, S. P. L., *Biochem. Z.*, **7**, 45 (1908).

the unknown (Stage 1) since quite an appreciable fraction of the carboxyl to be estimated becomes neutralized during this stage, so that the titer found after addition of formalin (Stage 2) is short by the same amount.<sup>18</sup> From a recent detailed study<sup>5</sup> it is clear that an accurate estimation of total carboxyl can be obtained if one adds the specimen, not previously neutralized, to already neutralized formalin and titrates direct with NaOH. (If sufficient formalin be added, it is no longer necessary to titrate to so alkaline an end-point as phenolphthalein, and indeed results of somewhat greater precision are then possible, the relative blank corrections being less.)

Titration to pH 6 *ca.*, before addition of formalin gives the excess carboxyl over amino; or *vice versa*, as the case may be as shown in the last section. A continuation of the titration to a final end-point in the presence of formol then shows total carboxyl; hence by difference one estimates amino or carboxyl groups not in excess (*i.e.* carboxyl or amino groups equally balanced in number). Results of analysis are given in Table B.

*Results.*—The values found agree with the theoretical calculation, within the small experimental error and confirm the preceding section.

*5. NH<sub>2</sub> Determined by Titrating to a Highly Acid End-Point in Absence of Water.*

In my first paper on amino acid titrations, a method for the volumetric estimation of amino groups was devised, according to which they were titrated with standard acid to a highly acid end-point; *e.g.*, less than pH 0.4. It was then shown that the sharpness of the end-point, in other words the accuracy of the method, was limited by the quantity of water present as solute in the titrated solution. Therefore to obtain optimal conditions it was directed that the test substance should be made up with as little water as possible and that the standard acid should also be as concentrated as convenient. Recently I have been experimenting along the lines of reducing the effective water concentration by such means as titrating in the presence of large amounts of glucose,

<sup>18</sup> *E.g.* 20 per cent in the case of phenylalanine, 50 per cent in the case of glycyl-glycine, only 5 per cent in the case of glycine.

TABLE B.  
*Analytical Details.*

	Specimen a, N.U.M.	Specimen b, N.U.M.	Specimen c, F.G.H.	Specimen c, F.G.H.
	<i>gm.</i> 0.0304 <i>cc.</i>	<i>gm.</i> 0.0280 <i>cc.</i>	<i>gm.</i> 0.0307 <i>cc.</i>	<i>gm.</i> 0.0614 <i>cc.</i>
Weight of glutathione taken.....	1.0 1.0*	1.0 1.0†	1.0 1.0*	1.0 1.0*
Water added (CO <sub>2</sub> -free).....	10.0	2, 3, 5, 7.5	10	10
Indicator added.....				
Formalin (previously neutralized to the same indicator).....				
Total carboxyl found. ( <i>I.e.</i> standard NaOH required at final stage of titration).....	$0.20 \times N$ (Micro burette.)	$1.75 \times 0.1 N$ (Macro burette.)	$0.21 \times N$ (Micro burette.)	$0.40 \times N$ (Micro burette.)
Total carboxyl calculated. ( <i>I.e.</i> theoretical for tripeptide of cysteine, glycine, glutamic acid).....	0.20 0.01	" "	0.20 0.01	" "
Method sensitive to.....				
Standard NaOH required in same titration in absence of formalin.....	0.10	"	0.11	"
By difference.				
Total amino groups found, standard NaOH.	0.10	"	0.10	"
" " " calculated.				
( <i>I.e.</i> theoretical for tripeptide).....	0.10	"	0.10	"
Method sensitive to.....	0.01	"	0.01	"

\* Brom-thymol blue.

† Phenolphthalein.

$\text{CaCl}_2$ , etc. Only partial success has attended these devices. But, by titrating in the entire absence of water I have succeeded in obtaining extremely accurate readings with remarkably sensitive end-points. A sharp indicator color change occurs under the conditions described below, with the addition of less than

TABLE C.  
*Analytical Details.*

	Specimen A. Twice separated as Cu salt, Fraction I (F.G.H.).	Duplicate of Specimen A.	Specimen B. Once separated as Cu salt.	Duplicate of Specimen B.	Specimen C. (N.U.M.).	Duplicate of Specimen C.
	gm.	gm.	gm.	gm.	gm.	gm.
Glutathione taken.....	0.0307	0.0307	0.0307	0.0307	0.0345	0.0280
	cc.	cc.	cc.	cc.	cc.	cc.
Volume of 0.1 N perchloric acid added.....	1.10	1.10	1.10	1.20	1.31	1.10
Volume of brilliant cresyl green (0.01 per cent in glacial acetic acid) added.....	1.0	1.0	1.0	1.0	1.0	1.0
0.1 M glycine (in glacial acetic acid) required in back titration.	0.13	0.10	0.08	0.18	0.20	0.19
Blank correction.....	0.02	0.02	0.02	0.02	0.01	0.01
NH <sub>2</sub> found.....	0.95	0.98	1.00	1.00	1.10	0.90
“ calculated for the tripeptide formula.....	1.00	1.00	1.00	1.00	1.12	0.91

0.01 cc. of titrant. Glacial acetic acid is used as solvent. Its property as a general solvent for bases has been known for a great many years past, and it has recently been employed by Hall and his coworkers in their important work on E.M.F. measurements.<sup>19</sup>

As a titrant perchloric acid in place of hydrochloric acid has been recommended by a number of recent writers (*e.g.*, Smith,<sup>20</sup>

<sup>19</sup> Hall, N. F., and Conant, J. B., *J. Am. Chem. Soc.*, **49**, 3047 (1927). Conant, J. B., and Hall, N. F., *J. Am. Chem. Soc.*, **49**, 3062 (1927). Hall, N. F., and Conant, J. B., *J. Am. Chem. Soc.*, **50**, 2367 (1928).

<sup>20</sup> Smith, G. F., *Chem.-Analyst*, **17**, 20 (1928).

Hall, *et al.*) and may also be used dissolved in glacial acetic acid solvent. It is prepared for me at N dilution by Messrs. British Drug Houses, London.

Since I have found that the amino acids themselves may be much less readily soluble than are their acid salts in the glacial acetic acid, I have adopted as a routine in estimating the basic groups in amino acids, peptides, and proteins, the practice of adding a slight measured excess of the standard perchloric acid and then back titrating the excess with base.

It is intended to publish elsewhere the detailed results of the method as applied to other peptides, and to proteins and amino acids.

*Method.*—To about 0.1 mg. equivalent of the substance, an accurately measured slight excess of 0.1 N perchloric acid (in glacial acetic acid) is added from a micro burette. When dissolved, a suitable indicator is added. (The indicator should have a pK value of about 0.) A search of the current catalogues shows that brilliant cresyl blue (Messrs. British Drug Houses) is the only suitable indicator which is at all readily available, and I have therefore adopted it for this work. Water must be rigorously excluded and I have found it easy to use a stock solution of the indicator dissolved in glacial acetic acid.

For the back titrations I have used as a base, a 0.1 M solution of glycine ( $pK = 2.4$ ) dissolved in glacial acetic acid.<sup>21</sup> This keeps reasonably well. The titration is carried on until there occurs an extremely sharp discharge of the brilliant blue tint. The perchloric acid titer less the glycine back titer gives the amino groups present. (A small blank correction may be made for the amount of perchloric acid which is required to titrate the same volume of glacial acetic acid solvent plus the same quantity of indicator, in the absence only of the solute.) Data are given in Table C.

*Results.*— $NH_2$  "found" agrees with  $NH_2$  "calculated" within 2 per cent (5 per cent in one case).

<sup>21</sup> The back titration should be carried out fairly soon after the indicator has been added, without its being left too long in contact with the highly reducing glutathione.

### 6. Determination of Equivalent Weight of Glutathione.

The results in Table C all agree in showing that glutathione has an equivalent weight of 307 with a possible error of about 2 per cent (*i.e.*, it lies between 301 and 313). (The figure 307 is arrived at as being the weight in gm. of glutathione which we determined to combine with 1 unit equivalent of acid, or with 2 unit equivalents of soda, the latter of course in two separate stages.) In order to obtain a more exact value for the equivalent weight, the glutathione was lastly titrated to as sensitive an end-point as could be found. The most sharp change was found to occur at about pH 6.2 (*e.g.* with brom-cresol purple); a reading which is in concordance with the relation

$$x = \frac{pK_3 - pK_2}{2}$$

where the observed  $pK$  values in question are roughly 8.8 and 3.6 respectively ( $x$  being the pH for maximal value of  $d.pH/dA$ ).

#### *Experimental Details.*

0.056233 gm. glutathione (prepared by N. U. M.), plus 0.1 cc. brom-cresol purple indicator; no water, titrated from micro burette:

Required.....	1.83	cc. 0.1 N NaOH.
Reading was sensitive to.....	0.005	" 0.1 " "
Calculated for the tripeptide.....	1.83	" 0.1 " "

*Results.*—The value here found leads to a minimal molecular weight for glutathione of  $307 \pm 0.8$ . That calculated from the formula, for a tripeptide comprising cysteine, glycine, and glutamic acid is 307. (This may be taken of course as the actual molecular weight in water, since there is no evidence of polymerization.)

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#### *Desulfuration of the Tripeptide by Alkali in the Presence of Lead Acetate.*

A number of experiments were earlier carried out on impure products, involving estimations of the percentage desulfuration at successive periods. It does not seem worth while to occupy space in this paper with these data. Their chief result was to demonstrate that their sulfur was much more unstable to alkali than that of free cysteine or cystine.

In the case of the pure tripeptide it has been thought sufficient to estimate the percentage removal of its sulfur at the end of one definite period.

The alkali chosen was, as in the earlier experiments, 0.33  $N$  barium hydroxide. In 30 cc. of this 0.2 gm. of the substance were dissolved and 2 cc. of 20 per cent neutral lead acetate added. A stream of hydrogen was passed for a short time through the fluid and the flasks then corked air-tight. The solutions stood for 5 hours in a hot room at 37°. They were then strongly acidified with acetic acid, the lead sulfide filtered off, and thoroughly washed. The residue was then converted into lead sulfate in a crucible and weighed.

It was found that in these circumstances no less than 55 per cent of the sulfur of the tripeptide is removed in 5 hours, while similar solutions of cystine and cysteine showed in the same time no trace even of blackening. In one experiment cysteine treated as above lost only 4.5 per cent of its sulfur in 24 hours.

*Results of Boiling with Water. Rate of Desulfuration and  
Diketopiperazine Formation.*

With the less pure products a number of experiments were carried out. It appears desirable to publish the results of one of these as given in Table I. The rates of desulfuration and diketopiperazine formation during boiling with distilled water are there illustrated. In this and in most experiments of the kind the product was boiled in 2 per cent solution. The pH of such a solution is about 3.00. The product used for the experiment was in the oxidized form and happened to be one in which the original percentage of sulfur was somewhat higher than the average among preparations separated by the intermediate method. A current of air was passed through the boiling solution and led into a cylinder packed with glass beads and containing an ammoniacal solution of hydrogen peroxide. Beyond this the air passed through lead acetate solution but no blackening of this was observed. Oxidation with the peroxide was complete and the sulfur was estimated as barium sulfate. The free S, which after about the 20th hour began to accumulate in the condenser, was (after the condenser tube was dried) dissolved in  $CS_2$  and weighed. At the end of each

period given in Table I the process was interrupted, the solution evaporated to a small bulk, the crystalline piperazine filtered off, washed with cold water, dried, and weighed. The filtrate was then returned to the flask, made up to the original volume, and the boiling continued. That the method secures considerable accuracy is suggested by a striking similarity in the figures and time

TABLE I.

*Product (SS) Containing 12.00 Per Cent Sulfur (1.975 Gm. Containing 237 Mg. of S Boiled in 100 Cc. of Water).*

Period No.	Time boiled.	S evolved as SH <sub>2</sub> .	S eliminated as free S.	Total S eliminated.	S eliminated during each period in per cent of original.	Total S eliminated in per cent of original.	Amount of diketopiperazine formed.	Containing S.
		hrs.	gm.	gm.	gm.		gm.	gm.
1	24	0.028	0.0044	0.0324	13.5		0.2100	0.0420
2	17	0.0087	0.0155	0.0242	10.2	23.7	0.1270	0.0254
3	9	0.0048	0.0051	0.0091	4.2	27.9	0.0290	0.0058
	50	0.0415	0.0250	0.0657			0.3660 = 18.5% of original product.	0.0732 = 31.0% of total S.

Total S accounted for:

As SH<sub>2</sub> and free S..... 27.9 per cent.

In diketopiperazine..... 31.0 " "

58.9 " "

of S in original product.

relations displayed in experiments on the same lines in other cases of the less pure preparations.

When the same technique was applied to the pure crystalline substance, it was found, as stated earlier in the paper, that the rate of desulfuration is much slower than that displayed by the less pure materials, though that of diketopiperazine formation is almost the same. Thus in an experiment similar to that just discussed the sulfur eliminated from the pure substance in 42 hours boiling was only 12.3 per cent of the whole, instead of 23 per

cent, and no free S appeared, while the diketopiperazine formed was 20 per cent of the weight of the original material, as against 17.0 per cent (43 hours, end of second period, see Table I). This experiment was with the thiol form of the pure substance. In another experiment the disulfide form (not wholly free from desulfurized products yielded 10.8 per cent of its sulfur (of which a small part was in the form of free S) in 42 hours, and 17.2 per cent of diketopiperazine.

*Anhydrides Formed during Boiling with Water.*—The diglycylcystine dianhydride produced during the boiling of the disulfide form of the tripeptide with water is, like most diketopiperazines, readily crystallizable. It is soluble in boiling water, from which it separates on cooling as a magma of acicular crystals. Its solubility in cold water is 0.06 per cent. When obtained pure it melts sharply at 262° (uncorrected) with decomposition. It gives the color reactions described for diketopiperazines, but no nitroprusside reaction. It yields no trace of nitrogen when treated with nitrous acid in Van Slyke's apparatus, and no ninhydrin reaction. The reduced form of the above (glycyl-cysteine anhydride) formed on boiling the tripeptide in its thiol form, is a much more soluble substance. It is most simply prepared by reducing the disulfide form described above, and very easily by dissolving the latter in a large quantity of hot water, and precipitating the solution while still hot with  $\text{HgSO}_4$ . The white precipitate is suspended in a minimum of water and decomposed as usual with  $\text{H}_2\text{S}$ . The solution now contains the anhydride completely reduced, and on being evaporated *in vacuo* to a small bulk it yields well formed crystals of the pure substance. This melts sharply at 203° (uncorrected) without decomposition. It gives the diketopiperazine reactions and a strong nitroprusside reaction, yields no Van Slyke nitrogen, and gives no ninhydrin reaction. It is easily oxidized by aeration in the presence of iron, giving rise to the dithiodianhydride described above. The sulfur of each of these piperazine derivatives is removed with exceptional ease under the influence of even weak alkalis.

A number of experiments were performed with the less pure products to determine the yield of these anhydrides on boiling with water. The maximum yield was obtained at about the 60th to 70th hour and was somewhat greater when, in the case of SS

preparations, the crystalline product was separated in successive fractions; the contents of the flask were at each stage evaporated to small bulk, the crystals filtered off, washed with cold water, and the filtrate boiled for a further period. The yield only varied between 22 and 25 per cent. In two similar experiments with the pure tripeptide the yields were 20.15 and 22 per cent.

The oxidized form (the dithiodianhydride) has been frequently analyzed, always with consistent results. The following figures were given for example by a sample consisting of mixed products obtained in various experiments on the less pure preparations. It was once recrystallized before analysis.

- A. 4.076 mg. required 5.15 cc. 0.01 N acid.  
 5.934 " : 8.785 mg.  $\text{BaSO}_4$ .

The following data refer to a product obtained from the crystalline tripeptide.

- B. 5.200 mg.: 7.170 mg.  $\text{CO}_2$  and 2.13 mg.  $\text{H}_2\text{O}$ .  
 6.201 " : 9.138 "  $\text{BaSO}_4$ .

Nitrogen by micro-Kjeldahl, 17.58 per cent.

Found. A. N 17.70, S 20.33.

B. C 37.42, H 4.55, N 17.58, S 20.24.

Calculated for  $\text{C}_{10}\text{H}_{14}\text{N}_4\text{O}_4\text{S}_2$ . C 37.73, H 4.40, N 17.61, S 20.12.

The reduced form (glycine-cysteine anhydride) prepared from the pure substance has been once analyzed.

	C	H	S	N
5.163 mg.: 7.140 mg. $\text{CO}_2$ and				
2.54 mg. $\text{H}_2\text{O}$ .....	37.70	4.92		
5.100 mg. required 6.39 cc. 0.01				
N acid.....				17.53
12.552 mg.: 18.000 mg. $\text{BaSO}_4$ ...			19.77	
Calculated for $\text{C}_5\text{H}_8\text{N}_2\text{O}_2\text{S}$ .....	37.50	5.00	20.00	17.50

From the products of acid hydrolysis of the anhydrides pure cystine and glycine have been readily obtained; the circumstance that the cysteine or cystine is completely precipitated by the acid  $\text{HgSO}_4$  reagent, and the glycine not at all, makes the separation easy.

*Other Results of Boiling with Water.*—Identification of the residual products presents difficulties because the decomposition is complex. Useful experience in dealing with such residual products was obtained from numerous observations on the earlier prepara-

tions and certain points of interest have been established. One experiment with the pure crystalline product need alone be reported however, as the general indications have been the same in all cases.

4 gm. of this, in thiol form, were dissolved in 150 cc. of water, and the solution boiled for 50 hours. The evolution of  $H_2S$  was continuous throughout. The solution when cool was precipitated with mercuric sulfate, which removes the whole of the diketopiperazine and any material still associated with cysteine. From the filtrate excess of mercury was removed as sulfide and the sulfuric acid as barium sulfate. The solution was then evaporated to a thin syrup and allowed to stand. A crystalline fraction separated (0.141 gm.) which when once recrystallized analyzed as pure glutamic acid. Another small fraction was next removed (22 mg.) which contained 9.55 per cent N and was also pure glutamic acid. From this stage onwards the solution began to deposit long silky prismatic needles which separated very slowly. Three successive fractions were removed after long standing at each stage. All of these had exactly similar characters, and gave a nitrogen content of 10.80, 10.54, and 10.75 per cent, respectively (pyrrolidonecarboxylic acid, 10.85 per cent). The residue was next extracted with absolute alcohol and from the extract further separation of the silky needles occurred, the product containing nitrogen 10.75 per cent. Successive fractions were found to have a melting point of  $160^\circ$  which remained unchanged on being mixed with pure pyrrolidonecarboxylic acid, and all the fractions in question undoubtedly consisted of that substance.<sup>8</sup> They gave no ninhydrin reaction until after they had been boiled with HCl. The concordant data for carbon and hydrogen appear below.

In all 0.78 gm. of the pyrrolidone acid was isolated, equal to 0.90 gm. of glutamic acid; amounting, with that isolated as such, to 1.06 gm. of the latter.

Analysis of pyrrolidone fraction.

	C	H	N
5.243 mg.: 8.890 mg. $CO_2$ and 2.68 mg. $H_2O$ .....	46.24	5.68	
Mean of micro-Kjeldahl estimation.....			10.71
Calculated for $C_5H_7O_3N$ .....	46.51	5.42	10.85

<sup>8</sup> That free glutamic acid when boiled with water is readily converted into pyrrolidonecarboxylic acid has been shown by Foreman (Foreman, F. W., *Biochem. J.*, 8, 463 (1914)).

The mercury precipitate mentioned above was decomposed in the usual manner and the solution obtained, after being freed from  $\text{H}_2\text{S}$  and  $\text{H}_2\text{SO}_4$ , was aerated at pH 7.5 in the presence of a trace of iron. Crystals of the piperazine compound separated which were filtered off (weight 0.43 gm.; N 17.61, S 20.44 per cent). The barium used to adjust pH was then exactly removed and the solution evaporated to dryness. The residue was extracted with cold hydrochloric acid in which the piperazine derivative is but little soluble. The acid extract contained a very small quantity of free cystine, identified by its hexagonal crystals. The anhydride left behind was not quite free from admixture, but after one recrystallization it was pure, and weighed 0.32 gm. The syrupy mother liquor from the pyrrolidonecarboxylic acid (mercury filtrate) and those from the diketopiperazine (mercury precipitate) were separately hydrolyzed with HCl. From the former 0.34 gm. of pure glutamic acid was separated. It is uncertain whether this was still in combination before the acid hydrolysis or in the form of the pyrrolidone acid which had failed to crystallize. A very small amount of glycine was identified as picrate. From the latter 0.12 gm. of pure glycine and 0.19 gm. of pure cystine were obtained, but no trace of glutamic acid. Final mother liquors contained products derived from decomposition during the boiling with water. These could not be identified.

As the mercury precipitates all products still containing sulfur, it is certain that after the tripeptide had been boiled with water no glutamic acid remained attached to cysteine. While the greater part of this amino acid was undoubtedly free, a small amount may have remained in association with desulfurized cysteine, though this was not proved. The greater part of the glycine which had escaped diketopiperazine formation would seem to remain in association with cysteine.

*Evidence Suggesting That There Is Some Destruction of Carbon Chains during Boiling with Water.*

In the case of two of the earlier preparations (one SS and one SH) an inlet tube was passed through the condenser into the boiling flask and  $\text{CO}_2$ -free air passed with positive pressure through an outlet tube into standard barium hydroxide solution. (The apparatus was made completely air-tight.) In each case after a few

hours the baryta became cloudy and in the course of 6 hours a considerable precipitate formed. Titration indicated that in both cases after this relatively short period some 3 per cent of the total carbon of the product had appeared as  $\text{CO}_2$ .

To obtain more definite evidence a product (SS) was boiled for 50 hours and the carbon estimated in the original and in the residue obtained after boiling. The absence of any error due to manipulation or incomplete drying, etc., was proved by the figures obtained for nitrogen. It was found that the results could be made most accurate by working on a relatively small scale with compact apparatus.

0.5 gm. of the product of which the percentage composition was known was boiled, as stated, for 50 hours.

The residue after boiling, when dried to constant weight, weighed 0.4068 gm., showing therefore a total loss of 0.0932 gm., or 18.5 per cent. Of this loss part of course was due to the removal of sulfur and to anhydride formation, but that there was also loss of carbon is shown by the following data:

	Original.	Residue.
C.....	37.65	39.58
H.....	5.80	4.90
N.....	11.55	13.53

$0.5 \times 37.65 = 0.188$  carbon originally present;  $0.4068 \times 39.58 = 0.161$  carbon in the residue. There was therefore a loss of carbon  $= 0.188 - 0.161 = 0.027$  gm. This is equal to 14.3 per cent of the carbon in the original product.

On the other hand the nitrogen content of the original product and that of the residue were, within the limits of experimental error, identical.  $0.5 \times 11.55 = 0.0575$  N;  $0.4068 \times 13.53 = 0.0550$  N. There is thus an apparent loss of nitrogen of 2.5 mg. only, or 0.5 per cent of the original, showing therefore that there was no appreciable loss due to the manipulation.

These results were obtained with care and were reproduced in the case of other preparations. As, however, similar experiments have not been made upon the pure product, it is not proposed to insist upon them. It is extremely probably however that the decomposition induced by boiling with water extends to the carbon structures of the tripeptide.

*Oxidation of the Thiol Product by Aeration.*

1 gm. of a crystalline preparation (A) was dissolved in 30 cc. of water and barium hydroxide added to make the pH 7.6. After the addition of a trace of ferrous sulfate air was passed through the solution until it no longer gave a nitroprusside reaction (5 hours). The barium was then exactly removed and the solution evaporated *in vacuo* at 40°. The residue was taken two or three times to dryness with alcohol until completely dehydrated. It was a white friable powder and was dried to constant weight *in vacuo* over P<sub>2</sub>O<sub>5</sub> at 50°.

A second preparation (B) was treated in a similar manner.

The oxidized products yielded the following figures on analysis.

A. C 39.51, H 6.36, N 12.07, S 9.52.

B. " 39.89, " 6.98, " 11.99, " 9.60.

The nitrogen and sulfur values are thus considerably below those proper to the disulfide form of the tripeptide (N 13.68, S 10.42).

The product (A) was dissolved, and the solution precipitated with mercuric sulfate. The precipitate on decomposition with H<sub>2</sub>S (which reduces the product) yielded a solution which in the presence of 0.5 N sulfuric acid gave the typical cuprous salt of the thiol compound, and from this the latter (0.7 gm.) was obtained crystalline and pure. While the greater part of the oxidized product was therefore in the disulfide form, a portion had suffered loss of N and S. For a discussion of these results see p. 276.

*Concluding Remarks.*

The evidence that the crystalline substance, of which the isolation has been described, has the constitution of a tripeptide seems to be so conclusive that I have not hesitated to speak of it as such throughout this paper. The analytical data, the evidence for its equivalent weight, the results of titrating its amino and carboxyl groups, and the yield of the three amino acids upon complete hydrolysis, all unite in support of this constitution.

Nevertheless aspects of its behavior have been described which may raise at least some measure of doubt concerning the linkages involved in its structure; a question as to whether they are normal amide peptide linkages. So difficult is it however to picture likely

alternatives for these linkages that an explanation of the exceptional instability displayed by the substance may rather be sought in the nature of its constituent amino acids themselves. The glutamic acid determines the presence of two free carboxyl groups and the consequent high acidity of aqueous solutions. The inherent reactivity of the thiol group may well contribute to instability, and the simultaneous presence of the extra carboxyl and thiol groups, especially characteristic of the substance, may be sufficient to determine its behavior when boiled with water.<sup>9</sup>

The present paper, with full intention, has followed lines that are merely descriptive. It is certain that conclusive views concerning the constitution of the newly isolated compound must await the results of synthetic studies, and these, owing to the apparent difficulties experienced in the preparation of cysteinyl derivatives, may prove troublesome. An explanation of the behavior of the compound moreover may call for comparison with related synthetic cysteine peptides of a kind not yet available. In the following paper by Pirie and Pinhey certain conclusions as to its constitution are based upon the results of electrometric titrations. These, though of real interest, must I feel be regarded as strictly provisional.

The relatively large amount of the tripeptide found in the yeast cell indicates that it is a normal cell constituent of some prominence, and is certainly no accidental fraction of ordinary intracellular protein breakdown. That it is also present in red blood corpuscles, so remote metabolically from the yeast cell, is a further suggestion for this, and also perhaps for the general significance of

<sup>9</sup> M. Bergmann and his coworkers have shown that in the case of dipeptides in which serine is one member, acidity in solution tends to shift the acyl group of the second member from an amide linkage derived from the  $\text{NH}_2$  group of the serine to an ester linkage derived from the OH group. Alkalinity in solution induces the opposite change. An intermediate stage involves the formation of an oxazolone ring with the acyl carbon attached to both nitrogen and oxygen (Bergmann, M., and Miekeley, A., *Z. physiol. Chem.*, **140**, 128 (1924). Bergmann, *Naturwissenschaften*, **12**, 1155 (1924)). If it may be supposed that the SH group of cysteine functions in a manner analogous with the OH group of serine, a thioxy ester linkage may, under conditions, be established in the tripeptide, and its presence may determine aspects of the behavior of this substance.

its presence. An attempt to identify it in other tissues is already in progress.

Any who may undertake its isolation will find that the method described is extremely easy to carry out. They may feel I think that the unusually high selectivity displayed by cuprous copper as an agent for separation has an interest of its own; and they will certainly realize that the tripeptide is to be obtained crystalline with an ease seldom found in dealing with compounds of its class.

The question perhaps arises as to whether the name glutathione should be continued as a label for the tripeptide. Whatever propriety it may have had when attached (as supposed) to a compound containing only glutamic acid and cysteine is lessened under the circumstance. Like other names of the kind however it was and must remain a mere label, and some convenience may be served in retaining it. There are chapters in the literature of glutathione which are not falsified by the circumstance that its constitution is other than was thought, and on the assumption that the literature may increase it may be well to avoid a breach of continuity.

I have to acknowledge the skilful assistance of E. J. Morgan in all manipulative operations. To the Medical Research Council my thanks are due for generous financial support.

#### SUMMARY.

A rapid and easy method is described for separating from yeast a pure crystalline substance with the characters of a tripeptide. It yields on hydrolysis glycine, glutamic acid, and cysteine, and its percentage analysis corresponds exactly with the composition of a tripeptide containing these three amino acids.

The yield from yeast is 1 gm. per kilo and upwards. The substance is present in red blood corpuscles.

Acidimetric titration methods have been applied (by L. J. Harris to the determination (1) of the  $\text{NH}_2$  and  $\text{COOH}$  groups, and (2) of the minimal molecular weight. The values so found agree with those calculated for a tripeptide of the above constitution within the experimental error of 2 per cent when the routine methods generally applicable to amino acids, polypeptides,

etc., were used; or less (*e.g.* within 0.3 per cent) when special refinements were adopted.

The substance is unstable. On boiling with pure distilled water for instance it yields the diketopiperazine of cysteine and glycine, together with free glutamic acid; but also slowly undergoes a more deep seated decomposition.

The tripeptide has been shown to constitute a large proportion of the preparations of glutathione made by earlier methods. The description of that substance as a dipeptide was therefore erroneous.

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