XXVI. ON AMINO-ACIDS.

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THE following paper contains results of investigations on the separation, preparation and properties of various amino-acids and their derivatives. Some new methods have been made use of which promise to be of considerable value and have already led to the isolation of a new amino-acid and peptide. The complete working out of the experimental details will take a considerable time, but as certain of the results already obtained are of a definite character and the methods appear to have a wide applicability, it appears desirable to give an account of the work at the present time, leaving many obvious extensions as subjects for further study. The material here presented is divided into three main portions, which will be considered separately:

I. The extraction of amino-acids by means of partially miscible solvents.

II. Hydroxyglutamic acid, a new hydroxyamino-acid of the dicarboxylic acid group.

III. A new peptide, isoleucylvaline anhydride, obtained by the acid hydrolysis of caseinogen.

I. THE EXTRACTION OF AMINO-ACIDS BY MEANS OF PARTIALLY MISCIBLE SOLVENTS.

The writer has long sought some means of extracting amino-acids from their aqueous solutions. Experiments were made with a variety of acid or basic solvents, in the hope of extracting basic or acidic amino-acids, but without success. Later experiments were made in which attempts were made to extract the strongly acid methylene amino-acid derivatives obtained by the action of formaldehyde, but the results were unsatisfactory. Recently, however, observations were made which led to a surprisingly simple method of extracting practically quantitatively many amino-acids from their aqueous solutions. For a particular purpose, the writer was anxious to obtain some

optically pure proline, and as the ester method invariably gives a largely racemised product, it was decided to attempt the direct extraction of proline from the products of hydrolysis of caseinogen by means of alcohols. The probability of success seemed small, since Fischer and Abderhalden [1904], in writing of the separation of proline from other amino-acids by means of alcohol, strongly emphasise the necessity of a previous distillation of the amino-acid esters. However, on hydrolysing gelatin with sulphuric acid and subsequently removing the latter as barium sulphate and then extracting the resulting concentrated solution of amino-acids with amyl alcohol in a continuous extractor, it was found that the proline was readily extracted. Soon after the extraction was started, the surprising observation was made that large quantities of solid amino-acids separated from the alcoholic extract and this seemed the more curious since they are practically insoluble in ordinary amyl alcohol. The phenomenon seemed sufficiently interesting to warrant further investigation and similar experiments were made with other alcohols. Propyl, butyl and isobutyl alcohols and various mixtures were used and it was found that butyl alcohol was the most satisfactory. This substance is now obtainable at nominal cost, owing to its large production in the fermentation process of acetone manufacture. Its boiling point, lower than that of iso-amyl alcohol, is a practical convenience and its rate of extraction is as good as that of any of the others.

On extracting amino-acid solutions from caseinogen and other proteins with butyl alcohol, it was found that the proline and practically the whole of the monamino-acids were extracted, together with some peptide anhydrides, which will be referred to later. The strongly ionised dibasic and dicarboxylic acids were left behind practically quantitatively. The only "neutral" monamino-acids detected in the extracted material have been minute traces of serine and in the case of amino-acids from gelatin the complete extraction of glycine seemed more difficult than that of the other amino-acids. In general, the extraction is surprisingly rapid and with the simple apparatus employed the extraction of two or three hundred grams of mixed amino-acids is complete in 36 hours. The bulk of the monamino-acids separate out in the extraction flask as a cream-coloured granular powder. It is convenient to filter off the monamino-acids occasionally during the extraction in order to avoid bumping or local overheating, using the mother liquor for continuing the extraction. The solid monamino-acids are washed first with a little butyl alcohol, then with a little ether, which removes traces of pigment and water. The butyl alcohol mother liquor contains the whole

of the proline, together with small amounts of amino-acids and peptide anhydrides, which can be largely separated through their sparing solubility in alcohol or water.

On examining the residue of non-extracted amino-acids, it was found to contain practically the whole of the diamino-acids, as judged by quantitative precipitation with phosphotungstic acid, and also the dibasic acids, aspartic acid, glutamic acid and a new dibasic acid to be described later. Larger yields of the dibasic acids were obtained from caseinogen than have previously been obtained by other methods. None of the dibasic acids could be detected among the extracted monamino-acids, so that it is clear that for practical purposes, they may be regarded as quantitatively separated from the latter.

It is thus seen, that by the use of butyl alcohol as a solvent, the products of hydrolysis of a protein may be readily separated almost completely into the following five groups:

(1) Monamino-acids, both aliphatic and aromatic, insoluble in alcohol but extracted by butyl alcohol.

(2) Proline, soluble in alcohol and extracted by butyl alcohol.

(3) Peptide anhydrides (diketopiperazines) extracted by butyl alcohol, but separated from (2) by sparing solubility in alcohol or water.

(4) Dicarboxylic acids, not extracted by butyl alcohol.

(5) Diamino-acids, not extracted by butyl alcohol, but separable from (4) by phosphotungstic acid and other means.

Those who have had much experience in the separation of amino-acid mixtures will appreciate the following points. (*a*) The groups are composed of chemically similar individuals. (*b*) Practically the whole of the products of the hydrolysis of an amino-acid may be divided into the five groups without serious loss and each can be readily obtained in solid form, aliquot parts of which may be used in the search for individual acids. (*c*) No indications of any racemisation have been observed. All the amino-acids thus far separated possessed their full optical rotation, thus limiting the number of isomers in the various mixtures and permitting greater use of the polarimeter for identification purposes. The absence of racemisation has an obvious bearing on the use of the method for purposes of preparing material for bacterial and animal metabolism experiments. (*d*) Materially higher yields of many amino-acids may be obtained from proteins than by existing methods, thus permitting a more nearly quantitative analysis of the proteins themselves.

The apparent paradox of the extraction from aqueous solutions of monamino-acids, which are essentially insoluble in all alcohols, by means of butyl alcohol or similar compounds requires some reference. It soon became clear that the presence of water was a conditioning factor and that the passage of a certain proportion of water from the fluid undergoing extraction to the butyl alcohol medium, was essential. It was found that a concentration of amino-acids rapidly extractable by butyl alcohol yielded almost no amino-acids in the extract, if the aqueous phase contained an excess of salts such as calcium chloride. The obvious inference was that the passage of water from the aqueous to the butyl alcohol phase was impeded with corresponding reduction in the rate of extraction. Butyl alcohol at room temperature is miscible with about twelve parts of water, but I know of no experiments on the relative distribution of water and butyl alcohol when shaken together in excess. It can hardly be doubted that the successful extraction of monamino-acids from aqueous solutions by butyl alcohol is dependent both on the limited passage of butyl alcohol into the aqueous layer and of water into the alcohol layer. The conditions empirically found in the present case seem excellent for the purpose in view, but it would seem well worth while to consider the possibility of a much more extensive use of the present principle with other extractable substances and solvents. So far as I am aware, no systematic investigation of the possibilities of extractions by partially miscible solvents has been undertaken, though the common method of extraction of aqueous solutions with ether is in reality an extraction with ether plus water, and various substances, *e.g.* hydantoins, can be quantitatively extracted in this way from aqueous solutions, although they are almost insoluble in pure ether, and readily soluble in water. It can hardly be doubted that the use of butyl alcohol and similar solvents, will be found of value for many other purposes than the one referred to. It is not unlikely that certain sensitive substances may be selectively extractable from tissue extracts with a minimum of alteration, especially if the extraction be carried out under reduced pressure and hence at a reduced temperature. Tryptophan, formed from caseinogen by tryptic digestion, is readily extracted practically quantitatively in an almost pure condition after a single preliminary purification with mercuric sulphate. Another obvious application of the method is in the study of the products of the hydrolysis of proteins by enzymes.

Experimental.

The earlier experiments in which solvents other than butyl alcohol were used, will not be described, since the latter solvent appears preferable. Hydrolysed gelatin was used in many preliminary experiments, but as the results with caseinogen are rather more complete, they alone will be considered.

Hydrolysis. Purified caseinogen varying in amounts from 100–400 g. was hydrolysed by boiling for 12 to 16 hours, with five to six times its weight of diluted sulphuric acid prepared by mixing the concentrated acid with three volumes of water. Hydrolysis under these conditions is still incomplete, judged by the subsequent separation of peptide anhydrides in significant amount. After the heating was ended, the solution was diluted with water and the sulphuric acid quantitatively removed with barium hydroxide in the usual way. The filtrate, which still reacts acid to litmus but not to congo-red, was concentrated moderately and about 2 per cent. of crude tyrosine allowed to crystallise out. The filtrate was again concentrated and made approximately neutral to litmus by the further addition of barium hydroxide. The optimum reaction has still to be determined and probably varies with different proteins. In the case of gelatin feebly acid and alkaline reactions obtained by adding acetic acid or ammonia were successively tried, but this is not necessary with caseinogen and it is doubtful if it is ever desirable.

Extraction. The approximately neutral solution of amino-acids prepared as above described was concentrated on the water-bath until crude leucine began to separate and then the whole mixture was transferred whilst still warm to the extraction apparatus. Usually an apparatus of the simple Kutscher-Steudel type was employed, although others proved equally satisfactory. The amino-acids from 100 g. of caseinogen may be conveniently extracted in an apparatus holding about 350 cc. It is preferable to arrange conditions so that the aqueous layer occupies three-quarters or five-sixths of the available space and the volume of supernatant butyl alcohol is relatively small. It has been found convenient to substitute a rather larger extraction flask made of tough glass of the Jena type for the smaller flask usually employed. Rubber connections can be employed, although they are attacked by the hot alcohol to a considerable extent and their exposed surface should be reduced to a minimum. The extraction flask is heated over a sand-bath, preferably with rather high sides, so as to reduce condensation in the flask, and the flame is adjusted so that a reasonably rapid flow of alcohol returns

into the flask. When using a 300–400 cc. or smaller apparatus, the aqueous solution soon attains a temperature of from 60°–80° and neither cooling nor heating is required, but when larger volumes of fluid are being extracted, it is a good plan to warm it to 75 or 80° by means of a water-bath.

Soon after the extraction has begun, a separation of cream-coloured granular particles of amino-acids is observed in the extraction flask and the amount steadily increases. After extraction has continued during a working day, it will be found convenient to allow the extract to stand overnight and to filter off the separated amino-acids on a Buchner funnel, washing them with a little butyl alcohol and finally with a little ether. The combined alcoholic filtrates which are light brown in colour are used for continuing the extraction on the second day. Risk of breakage due to the accumulation of solid amino-acids on the bottom of the flask is thus avoided and it is possible to judge the completeness of the extraction by observing the increment of solid amino-acids on continued heating. When after several hours' extraction no significant additional amount of solid matter separates out of the butyl alcohol solution, the operation is regarded as finished. With the apparatus employed and extracting 1 to 400 g. of mixed amino-acids, the operation usually took about 36 hours; the time varying naturally with the rate at which the butyl alcohol is boiled.

When the extraction is over, the aqueous solution is reserved for examination for bases and dicarboxylic acids, as will be described later. The butyl alcohol extract, containing the proline and some peptide anhydrides, is filtered from the separated amino-acids, after standing at room temperature for some hours, while the monamino-acids, filtered and washed as previously described, are dried and weighed.

Butyl alcohol extract. Separation of proline, etc. The filtered extract is evaporated to dryness in a distillation flask under reduced pressure and the sticky brownish residue boiled with about ten parts of absolute alcohol. In the case of caseinogen at least, the whole of the residue is soluble in ethyl alcohol, indicating the absence of any considerable quantity of monamino-acids other than proline. On allowing the alcoholic solution to stand overnight, crystallisation occurs with separation of a small quantity, averaging 2 per cent. of the caseinogen, of an impure mixture chiefly composed of peptide anhydrides with traces of monamino-acids. This product was filtered off, washed with alcohol and examined later. The alcoholic filtrate was then evaporated under reduced pressure, dissolved in hot water, boiled with charcoal, filtered and concentrated to syrup.

A further crystallisation of peptide anhydrides occurs on standing, averaging 2 to 3 per cent. of the caseinogen taken, and this is filtered off and examined together with the similar product obtained above. The total yield of peptide anhydrides in different experiments was as follows: 2·7, 2·2, 3·2, 5·0 and 5·7. The larger yields were obtained from experiments with the larger amounts of caseinogen.

The clear aqueous solution after separation of the two fractions just described contains the whole of the proline. It is already much purer than might be anticipated from its method of preparation and compares favourably with the alcohol-extracted product obtained by the ester method.

It appears that the actual proline content can be closely estimated by means of van Slyke's methods for differentiating between amino and imino nitrogen, for a characteristic derivative of proline was isolated in amount corresponding closely to that indicated by analysis. It was found that no purification was effected by converting the proline into its copper salt for the whole of the product was soluble in methyl alcohol. It will be convenient to give the yields of proline as indicated by van Slyke's differential analysis and then to refer to a new derivative of proline which seems useful for purposes of characterisation.

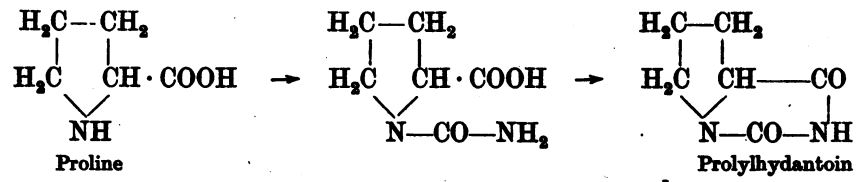
Caseinogen analysed	Nitrogen of Proline Fraction		Percentage Proline calculated
	Total	Non-Amino	
150 g.	1·70 g.	1·34	7·3
64	0·683	0·557	7·1
50	0·611	0·521	8·5
18	0·224	0·184	8·2
232	2·94	2·45	8·7

The average of the above determinations is close to 8·0 per cent. proline and evidence other than analytical will be presented to show that no considerable amount of other substance than proline is contained in the above estimate. It is of interest to compare the above figures for the proline content of caseinogen with that obtained by other workers. Van Slyke [1911], using his analytical methods on amino-acids separated by the ester method, obtained 6·70 per cent., while his analysis of caseinogen by direct hydrolysis indicated that 7·13 per cent. of the total nitrogen of caseinogen was in the non-amino form. If the whole of this were due to proline, ignoring the small amount of hydroxyproline it would be equivalent to 9·2 per cent. proline. Abderhalden obtained only 3·1 per cent. of proline by the ester method, while Foreman in unpublished experiments is quoted by Plimmer [1917] as having obtained 7·6 per cent., a figure in close agreement with that found by me.

By the direct extraction method just described, there is no evidence of any racemisation of the proline as judged by the solubility of copper salts in methyl alcohol and by the preparation of other derivatives, but on the other hand, the impurities which accompany the proline appear to be fairly strongly dextro-rotatory, so that a direct polarimetric estimation of proline is not possible. The observed rotations correspond to about three-fourths of the proline believed to be actually present.

Preparation of l-*prolylhydantoin.* The preparation of uramido-acids and the hydantoins derived from them by the action of mineral acids has not been made as much use of for the identification and separation of amino-acids as the method deserves. In many cases the products are more easily purified than the more generally known phenyl derivatives, obtained by the use of phenyl isocyanate, on account of well-marked solubility differences. Proline affords a good example of the utility of the method. On warming crude proline on the water bath with a moderate excess of potassium cyanate, it is converted smoothly into the uramido-acid, and the same is true of any contaminating amino-acids. On liberating the uramido-acids by making the solution just decidedly acid to congo-red and extracting with ether for a few hours in a continuous extractor, practically none of the proline compound is extracted, but the whole of any leucine, isoleucine, valine, alanine, phenylalanine and tyrosine derivatives are removed. On heating the extracted aqueous solution with 10–20 per cent. sulphuric or hydrochloric acid for an hour and then re-extracting with ether, prolylhydantoin is obtained in excellent yield and possesses convenient properties for purposes of identification. The following gives details of a typical experiment.

Crude *l*-proline (5 g.), containing as judged by van Slyke's analytical methods about 16 per cent. of impurity, was evaporated on the water bath with potassium cyanate (5 g.) and water (50 cc.). On taking up with water, acidifying to congo-red with sulphuric acid and extracting with ether for seven hours, 0·55 g. of uramido-acids, chiefly derived from valine and leucine, were obtained. The extracted aqueous solution was then heated for 1–2 hours on the water-bath after the addition of one-fifth volume of 50 per cent. sulphuric acid and then again extracted with ether for 12 hours. On evaporation of the ether 4·3 g. prolylhydantoin was obtained in the form of transparent shining thick prismatic needles. The substance is very easily soluble in hot water and readily crystallises from the concentrated solution. The yield of pure products amounted to about 85 per cent. of the theoretical amount, after allowing for the known impurity in the crude proline.



l-Prolylhydantoin has a high optical rotation, as shown by the following observations on two different preparations:

(1) $c = 2{\cdot}0$, $l = 1{\cdot}0$, $\alpha = -4{\cdot}77°$ hence $[\alpha]_D^{20°} = -238{\cdot}5°$,

(2) $c = 2{\cdot}0$, $l = 1{\cdot}0$, $\alpha = -4{\cdot}64°$ hence $[\alpha]_D^{20°} = -232{\cdot}0°$.

On analysis, the two specimens were found to contain 19·80 and 19·95 per cent. nitrogen (Kjeldahl) compared with a calculated value of 20·0 per cent. *l*-Prolylhydantoin, after recrystallisation from water, melts at 165–167°. When prepared from partially racemised proline obtained by the ester method, the melting point is materially lower, *e.g.* 153–156°.

It is not without interest to note that *l*-prolylhydantoin is racemised by alkali at room temperatures much less readily than most other hydantoins observed by the writer. A 1 per cent. solution in 0·4 *N* NaHO still possessed about one-third of its initial rotation at the end of a week. The writer has advanced the view that racemisation of hydantoins by alkali at low temperatures is due to keto-enol tautomeric changes and as the CH group adjacent to the CO in prolylhydantoin, unlike the other hydantoins examined, forms part of the cyclic structure, it is not improbable that this fact is related to its slower rate of racemisation.

It is believed that *l*-prolylhydantoin may serve as a useful derivative for the characterisation of proline in impure mixtures, when successively converted into uramido-acid and hydantoin. It has been already stated that under these conditions, contaminating alanine, valine, leucine, isoleucine, phenylalanine and tyrosine are removed as uramido-acids on extraction with ether. On the other hand, glycine, serine, proline, hydroxyproline, aspartic and glutamic acids, are not extracted by ether as uramido-acids, but are extracted after conversion into their hydantoins. The practical utility of these facts under various conditions, is sufficiently obvious and will be studied in further detail.

The monamino-acids extracted by butyl alcohol.

The amino-acids extracted by butyl alcohol other than proline, separate out as previously described as a cream-coloured granular deposit. They dissolve in water to give a clear light yellow solution and contain only insig-

nificant traces or none of the diamino-acids or dibasic acids. The reaction of the aqueous solution is barely acid to litmus.

The yield of these monamino-acids from caseinogen is indicated as follows:

48 g. caseinogen gave 18 g. dry monamino-acids = 37·5 per cent.
150 ,, ,, ,, 51 ,, ,, ,, = 34·0 ,,
232 ,, ,, ,, 86 ,, ,, ,, = 37·3 ,,

The above figures do not include about 2–3 per cent. of tyrosine filtered off prior to extraction, so that the total yield is approximately 40 per cent. of the caseinogen taken. It is not unlikely that slightly higher figures might follow greater care in avoiding loss in the removal of sulphuric acid following hydrolysis. The amino-acids thus separated contain about 11·5 per cent. of nitrogen and are chiefly made up of alanine, valine, leucines and phenylalanine, with a little tyrosine. Hydroxyproline and serine appear to be present, but the complete analysis of the mixture awaits further investigation. The product is in a most convenient form for application of the ester method, but it is believed probable that preliminary separations can be advantageously made. Apart from the traces of valine and leucine which accompany the crude proline, it is believed that the whole of these acids are contained in the fraction under consideration and most careful search by various methods has failed to detect them in the other fractions, provided the preliminary extraction with butyl alcohol has been adequate. Glycine, which appears to be absent or only present in traces in caseinogen, is only extracted quantitatively with difficulty, as experiments with gelatin showed. Traces of serine have also been detected among the unextracted amino-acids. A further report on the composition of this monamino fraction will be made at an early date.

Experiments on the rate of extraction by butyl alcohol of individual amino-acids from pure aqueous solution will also be made.

It is perhaps not out of place to refer to the possible use of such a readily obtainable dry, almost neutral, amino-acid mixture for furnishing a basis for nutrient media, with or without the addition of tryptophan. The fact that most of the amino-acids which furnish glucose in the diabetic organism are absent from this material suggests dietetic possibilities.

The amino-acids not extracted by butyl alcohol. Separation of aspartic, glutamic and other acids.

As has already been stated, the amino-acids which are not extracted by butyl alcohol from aqueous solution, are the strongly ionised diamino-acids and dicarboxylic acids. Quantitative experiments, in which the mixture was

precipitated with phosphotungstic acid, showed that the whole of the diamino-acids could be recovered in this way, but the filtrate yielded less of the dicarboxylic acids than could be obtained by other methods, so that for purposes of quantitative analysis of proteins, it is decidedly preferable to use an aliquot portion for the examination of the bases and the rest for the dicarboxylic acids.

The diamino-acids. The precipitation with phosphotungstic acid and subsequent separation of histidine, arginine and lysine requires no comment other than the desirability of removing dissolved butyl alcohol from the solution by heating before precipitation, for the inhibitory action of alcohols on the precipitation is well known.

The dicarboxylic acids. The degree of purification effected by the removal of the mono-amino acids by extraction, permits a much more nearly quantitative separation of glutamic acid as hydrochloride than is usually the case. Precipitation moreover is usually more rapid and after removing the first crop of crystals, concentrating and re-saturating with hydrogen chloride in the usual way, the second crop of crystals is very small. It may be noted here that in the analysis of gelatin and other proteins containing much glycine, the precipitation of glycine hydrochloride along with the glutamic acid hydrochloride is possible on long standing in concentrated solution. Caseinogen gave the following yields of glutamic acid hydrochloride. In each case, the glutamic acid hydrochloride was filtered off on asbestos and well washed with cold hydrochloric acid. It was then dissolved in a small volume of water and again precipitated by saturation with hydrogen chloride. The only impurities to be feared were ammonium chloride and barium chloride and these were excluded by controlling the purity of the hydrochloride by analysis, using the Kjeldahl or van Slyke methods, after evaporation of the portion used for analysis with calcined magnesia. The values obtained by either method were all within 0·1 per cent. of the theoretical value of 7·65 per cent. of nitrogen.

I.	16 g.	caseinogen	gave	4·3 g.	glutamic	acid	hydrochloride	= 21·6 %	glutamic	acid
II.	50 „	„	„	13·2 „	„	„	„	= 21·2 „	„	„
III.	150 „	„	„	36·7 „	„	„	„	= 19·6 „	„	„
IV.	232 „	„	„	42·6 „	„	„	„	= 14·7 „	„	„

The first three experiments quoted above gave reasonably concordant results indicating an average of about 21 per cent. of glutamic acid. The last experiment, IV, differed from the others in that previous precipitation of the diamino-acids with phosphotungstic acid was carried out and the filtrate was then freed from sulphuric and phosphotungstic acids by means of barium

hydroxide. There was undoubtedly considerable loss connected with the handling of the bulky precipitates, and it seems reasonable to exclude this low result in computing the average. The proportion of glutamic acid in caseinogen, as estimated above, is materially higher than that previously recorded, with the exception of Foreman's estimations [1914]. Foreman obtained about 17 per cent. of glutamic acid as such, together with pyrollidone-carboxylic acid, determined analytically and presumably derived from glutamic acid, indicating a total yield of 21·8 per cent. glutamic acid. These figures are in excellent agreement with those now presented and are six to 12 per cent. higher than those previously recorded.

The next problem was the separation of aspartic acid from the filtrate from the glutamic acid hydrochloride. Various obvious methods were tried with unsatisfactory results, partly due to the presence of another dicarboxylic acid, to be described in the following section. Levene and van Slyke [1910] showed that aspartic acid was almost completely precipitated on boiling its aqueous solution with excess of lead oxide, the apparent solubility of the salt being about 1 in 4700. This fact was utilised successfully for the separation of aspartic acid as follows. The filtrate from the glutamic acid hydrochloride was concentrated under reduced pressure to remove as much of the hydrochloric acid as possible and the resulting syrup dissolved in about ten parts of hot water. It was then treated with calcium hydroxide and the calcium salts precipitated by alcohol as described by Foreman [1914]. The sticky deliquescent calcium salts after washing with alcohol were dissolved in water and the calcium removed exactly with oxalic acid. Freshly precipitated and well washed lead hydroxide was then added by degrees while the solution was kept boiling under a reflux condenser. The addition of lead hydroxide was continued until the reaction of the solution was distinctly alkaline to litmus and heating was then continued for a further 15 minutes. The mixture was then allowed to cool and stand overnight. The precipitate containing the aspartic acid and much lead chloride was filtered off and washed with cold water; the filtrate being reserved for the separation of the acid to be described in the following paper. The lead precipitate is somewhat difficult to decompose with hydrogen sulphide, so that it is more convenient to boil it with dilute sulphuric acid in moderate excess. Almost all the lead is thus removed and the filtrate contains aspartic, hydrochloric and sulphuric acids. The filtrate is then concentrated with excess of precipitated barium carbonate, filtered and made up to a convenient volume such as 1 cc. per gram of protein taken. A nitrogen estimation on a small aliquot part gave an indication of

the possible aspartic acid concentration. A hot concentrated solution of normal copper acetate was then added in amount equal to 1·5 molecules per molecule of aspartic acid. On allowing the solution to stand for a couple of days in a cool place, the characteristic crystalline copper aspartate separated out almost completely and was filtered off, washed, dried and weighed.

In two experiments, 150 g. of caseinogen gave 9·7 and 11·6 g. of crystalline analytically pure copper aspartate. The salt, after drying in the air, contained more nearly 3 mols. of water than the more customary $4\frac{1}{2}$, similar observations having been made by Curtius and Koch and by Skraup. The anhydrous salt dried at 130° contained 32·6 per cent. copper and 7·2 per cent. nitrogen, compared with calculated values 32·7 and 7·2 respectively.

The yields of crystalline copper aspartate noted above correspond to 3·5 and 4·1 per cent. aspartic acid respectively, in caseinogen, and are two to three times as large as the figures previously recorded.

The extraction of tryptophan by means of butyl alcohol.

The hydrolysis of caseinogen by sulphuric acid completely destroys the tryptophan, as is well known, and none of it was encountered in the preceding separations of the products of acid hydrolysis. It appeared desirable to determine whether tryptophan if present in a solution could be extracted, and accordingly the following experiment was made.

Caseinogen (187 g.) in two litres of 1 per cent. sodium carbonate solution was digested for seven days with active pancreas extract. The solution was then treated as described by Hopkins and Cole [1902] and precipitated *once* with mercuric sulphate solution. The precipitate was decomposed with hydrogen sulphide, sulphuric acid removed exactly with barium hydroxide and the solution concentrated to about 30 cc. under diminished pressure. It was then extracted with butyl alcohol in a small extractor. A separation of tryptophan on the sides of the extraction flask was soon noted, and at the end of two hours' extraction 1·5 g. had separated out. The extraction was practically quantitative at the end of 12 hours and the residual aqueous solution gave only a doubtful reaction with Hopkins' sensitive glyoxylic acid reaction. The tryptophan crystallising from the butyl alcohol extract weighed 3·15 g. and was filtered off and washed with ether. The mother liquor (50 cc.) on evaporation gave a sticky residue mostly soluble in hot absolute alcohol and containing but a few centigrams of tryptophan.

The main portion of tryptophan (3·15 g.), corresponding to a yield of 1·7 per cent. on the caseinogen was remarkably pure, considering its simple

method of separation. Apart from a little yellow pigment and a minute trace of tyrosine it seemed to be pure, as judged by its melting point, optical rotation and analysis.

A 1 per cent. solution, without needing clarification, showed a rotation of $-0{\cdot}68°$ in a 2 dm. tube, hence $[\alpha]_D = -34°$. The recorded values vary from -30 to $-40°$, but the above figure agrees well with Hopkins and Cole's estimate of $-33°$. On analysis (Kjeldahl) 13·9 per cent. nitrogen was found, compared with 13·72 per cent. calculated.

The preparation of tryptophan in a pure state in considerable quantities is an operation requiring some skill and experience in order to avoid loss of much of the product in mother liquors which crystallise with some difficulty. The extraction method it is believed will simplify the process to some extent and probably requires less careful manipulation. The crude product is already fairly pure and a single recrystallisation from dilute alcohol, as recommended by Hopkins, furnishes a completely pure substance. The method could probably be operated on a considerable scale and may be of service in obtaining tryptophan for bacteriological and other purposes.

II. A new Dibasic Amino-Acid occurring in Proteins.

It was found that after the separation of glutamic and aspartic acid as described above, large amounts of at least one other dicarboxylic acid were still present and could be isolated.

For simplicity in presentation, it is convenient to anticipate the experimental portion of the paper and to proceed at once to the main properties and probable constitution of the new acid.

(1) The acid is optically active and is extremely readily soluble in water, crystallising slowly in thick prisms from its syrupy solution. Most of its metallic salts, with the exception of those of silver and mercury, are also very soluble in water and do not crystallise readily but may be precipitated by alcohol. It forms a diethyl ester which has not yet been satisfactorily distilled without decomposition.

(2) The acid itself appears to possess the formula $C_5H_9O_5N$ and the salts of the heavy metals contain two equivalents of bases, while salts of the alkaline earths contain one.

(3) The acid behaves as a strong monobasic acid on titration with sodium hydroxide, using phenolphthalein as indicator and the results are in accord with a molecular weight of 163. The same molecular weight follows from the analysis of the well characterised silver salt, $C_5H_7O_5NAg_2$. In the presence of

excess of formaldehyde, the substance behaves towards sodium hydroxide as a dibasic acid.

(4) The acid, if not heated too strongly, contains the whole of its nitrogen in the (NH_2) form, as judged by its reaction towards nitrous acid. On prolonged heating over phosphorus pentoxide at 100–110°, a molecule of water is slowly eliminated and the nitrogen is converted into the (NH) form. This reaction is clearly analogous to the formation of pyrollidonecarboxylic acid from glutamic acid and is evidence of one carboxyl group being in the γ position relative to the amino-group.

(5) The acid or its salts on ignition give a definite but faint pyrrole reaction, especially if much charring occurs, thus favouring reduction. On the other hand, on heating with zinc dust, an intense pyrrole reaction is immediately obtained.

(6) On reduction with fuming hydriodic acid at 150°, glutamic acid and other products are obtained.

(7) The acid gives characteristic colour reactions with various phenols and concentrated sulphuric acid resembling aliphatic hydroxy-acids.

(8) On oxidation of the sodium salt of the acid (1 mol.) with chloramine-T (1 mol.) an aldehyde is obtained, presumably $C_4H_6O_4$, which on treatment with *p*-nitrophenylhydrazine gives a characteristic *osazone* containing one carboxyl group, the analysis of which agrees well with the formula $C_3H_3COOH(:N.NH.C_6H_4NO_2)_2$. The formation of the osazone from the aldehyde-acid is indicative of a hydroxyl group adjacent to the aldehyde group and hence contiguous to the amino-group in the original acid[1].

(9) No indication of lactone formation was observed, thus confirming the assumed position of the hydroxyl group as noted under (8) and excluding a γ position.

(10) On treatment with potassium cyanate a uramido-acid is formed which is not extractable from aqueous solution by ether. The uramido-acid, on heating with acids, gives an extremely soluble hydantoin, which is extractable by ether, thus resembling similar derivatives of aspartic and glutamic acid.

(11) The acid gives derivatives with phenyl isocyanate and β-naphthalene-sulphonic chloride, but they are not well adapted for purposes of identification.

[1] On oxidising the sodium salt with two molecules of chloramine-T, under the conditions which the writer used to convert glutamic acid into β-cyanopropionic acid [1917], a substance was obtained which on hydrolysis apparently furnished *malic acid*. The latter acid has only been identified thus far as calcium salt and by colour reactions. The reaction will be studied further.

The above results can hardly be reconciled with any other structure for the acid than that of an α-amino-β-hydroxyglutaric acid, *i.e.* β-hydroxyglutamic acid, $COOH . CH(NH_2) . CH(OH) . CH_2 . COOH$.

An acid of the above structure containing two dissimilar asymmetric carbon atoms can exist in a variety of forms and it is not at all improbable that the substance isolated by me consists of more than one individual. It will be a matter of some difficulty to determine this as the melting point of the acid appears not to be sharp, partly owing to its rapid conversion into hydroxypyrrolidonecarboxylic acid, and its satisfactory recrystallisation has practical difficulties. Recently, however, a series of beautifully crystalline salts of the acid with strychnine, brucine and other alkaloids have been obtained and a further study of these products may prove of service. The formation of an unsaturated acid, $COOH . CH(NH_2) . CH = CH . COOH$, under the influence of alkaline or acid reagents, is obviously possible and such a product can be reconverted into the β-hydroxy-acid with partial loss of asymmetry. A study of the configuration and synthesis of the new acid and some of its biochemical relationships will be continued.

The amount of the new acid in caseinogen is very considerable, for no less than 10·5 g. of an almost wholly crystalline mass has been obtained from 100 g. of caseinogen after hydrolysis by sulphuric acid[1]. Hydrolysis of caseinogen by barium hydroxide in an autoclave at 140°, gave only about 1·5 per cent. of a syrup crystallising with difficulty but apparently containing some of the acid.

An acid of the structure indicated above has a number of interesting biochemical relationships which invite speculation. The formation from it by oxidation *in vivo* of malic and perhaps tartaric acid is clearly possible. Its relationship with serine is obvious and if it should be shown that the new acid may be formed *in vivo* by the oxidation of glutamic acid, an interesting parallelism with the known β-oxidation of fatty acids would be established. The writer [1913] has already drawn attention to the probable similarity in the behaviour in the animal body of glutamic acid and proline and the existence of β- or γ-hydroxyproline and β-hydroxyglutamic acid as protein constituents may well give a clue to the intermediary metabolism of the former. It is interesting in this connection to recall the old observation of Buisine showing that malic acid comprises 2–5 per cent. of the water-soluble matter of sheep

[1] Osborne has drawn attention to the possible occurrence of hitherto unknown dicarboxylic acids in gliadin and glutenin owing to their disproportionately high yield of ammonia on hydrolysis, compared with their yield of aspartic and glutamic acids. These proteins will be examined for the new acid.

sweat. It appears probable that this may originate from β-hydroxyglutamic acid. The biochemical interconversion of malic acid, lactic acid and glucose is well established. The easy formation of cyclic hydroxypyrrolidonecarboxylic acid from β-hydroxyglutamic acid may also be significant in connection with the synthesis of the pyrrolidine acids *in vivo*. But further knowledge of the new acid is clearly more desirable than premature speculation.

The writer believes that the substance under discussion is probably the first example of a hydroxy-dicarboxylic amino-acid occurring among the proteins. Reference must, however, be made to Skraup's statement as to the occurrence of hydroxyaspartic acid in caseinogen. As Fischer has pointed out, Skraup's experiments are not described in a form capable of repetition and no evidence, other than the analysis of a copper salt, is adduced that the minute amount of substance obtained by Skraup was a dicarboxylic acid. The value of this analysis for determining the basicity of the acid is small, since no carbon and hydrogen determinations were made on the free acid. If hydroxyaspartic acid, as described by Skraup, were obtainable from caseinogen, it would almost surely have accompanied the β-hydroxyglutamic acid, but no such acid was detected. Moreover, a search for tartaric or racemic acids among the products of the action of nitrous acid upon the dicarboxylic acids of caseinogen gave negative results, thus indicating the probable absence of hydroxyaspartic acid.

On the other hand, certain experiments carried out in Hopkins' laboratory, at Cambridge, by F. W. Foreman, read in the light of later work clearly indicate that a partial separation of the new acid had been accomplished. Foreman made use of the insolubility of the calcium salts of dibasic amino-acids in alcohol for their separation—a method which the writer has since utilised in a modified form. He obtained from the calcium salts crystalline aspartic and glutamic acid and a syrup soluble in acetic acid but difficult to crystallise, which contained pyrrolidonecarboxylic acid and "other unidentified substances probably of great importance." While the writer's experiments originated from a totally different direction and were not influenced by Foreman's observations, there is little doubt that if the latter had been able to pursue his investigations uninterruptedly, he would have isolated β-hydroxyglutamic acid.

Experimental.

Preparation of β-hydroxyglutamic acid. Four separate preparations of the acid have been made by different methods, and in each case a substance with

similar properties was obtained. It is not proposed to describe the earlier less satisfactory methods, but simply to give an account of the method which hitherto has given the best result.

The material used was hydrolysed caseinogen thoroughly extracted with butyl alcohol in order to remove the monamino-monocarboxylic acids as described in the preceding section. Glutamic acid was next removed as completely as possible as hydrochloride by saturation with hydrogen chloride, taking care to resaturate the concentrated filtrate from the first crop of crystals and allowing the fluid to stand in an ice box for three days. The filtrate from the glutamic acid hydrochloride was then concentrated under reduced pressure, to remove as much hydrochloric acid as possible. The residual syrup was dissolved in water, digested with an excess of calcium hydroxide, filtered, concentrated to a syrup and then the calcium salts of the remaining dibasic acids were precipitated by alcohol. The calcium salts were then dissolved in water, the calcium removed with oxalic acid and the filtrate boiled with lead hydroxide to remove aspartic acid. The conduct of the preceding operations will be familiar to anyone accustomed to working with the amino-acids and are given in greater detail in the preceding section.

The filtrate from the lead aspartate (from 150 g. caseinogen) was freed from lead with hydrogen sulphide, diluted to 500 cc., acidified with 20 cc. of sulphuric acid and carefully precipitated with phosphotungstic acid to remove bases which are invariably present. The filtrate was then freed from sulphuric and phosphotungstic acids with barium hydroxide in the usual way and the excess of the latter removed fairly exactly with sulphuric acid. The volume of the filtrate, which was strongly acid, was brought to about half a litre. In the earlier experiments the new acid was precipitated with mercuric acetate and sodium carbonate, but later it was found that the silver salt was preferable.

Small amounts of hydrochloric acid are invariably present in the solution and this was first removed by adding dilute nitric acid (5 cc.) and then silver nitrate until no more precipitation occurred. The filtrate from the silver chloride was then made neutral or feebly alkaline with sodium hydroxide. The new acid was then precipitated by the alternate addition of strong silver nitrate solution (15 per cent.) and approximately normal sodium hydroxide. A heavy white precipitate was soon produced in the solution and more silver nitrate or sodium hydroxide was added as long as addition of either solution produced a white precipitate. The sodium hydroxide is best added by degrees from a burette and a large excess must be avoided since it decomposes the

precipitate. When excess of silver nitrate was present and precipitation completed, a test portion of the filtrate, on adding a drop of sodium hydroxide solution, gave only brown silver oxide. The end point is easily determined with a little care. The precipitated silver salt, which at first was pure white but on completion of the operation was light brown, from a little silver oxide, was filtered off using suction and washed with cold water. It was then decomposed with hydrogen sulphide and the filtrate concentrated under reduced pressure. It is probably advisable to avoid heating the solution unduly in order to limit any formation of hydroxypyrrolidonecarboxylic acid. A clear yellowish syrup containing the acid was obtained and the separation of crystals only commenced after most of the water had been removed in the vacuum desiccator. Gradually thick prisms begin to separate and eventually the whole is transformed into a solid mass of crystals. The crystalline magma from 150 g. of caseinogen weighed 19·5 g. and contained, as judged by titration with standard alkali and phenolphthalein, 17·5 g. of the acid. The acid is difficult to crystallise so that a convenient amount of mother liquor may be removed and a fairly good plan is to crush the crystals separated from water with a mixture of one part of glacial acetic acid and four parts methyl alcohol and finally to wash with methyl alcohol. Precipitation of the aqueous solution with alcohol gives a syrup which rapidly solidifies, and the same is true on adding alcohol to the concentrated acetic acid solution. The acid is extremely soluble in water, dissolving in about its own weight of water, and is readily soluble in acetic acid, slightly soluble in methyl alcohol but practically insoluble in ethyl alcohol, ether, or ethyl acetate. In aqueous solution the substance shows a low dextro-rotation which is increased on addition of hydrochloric acid. Its optical properties will be examined more carefully and reported later, as variation in concentration appears to influence the specific rotation considerably.

For analysis, the acid was dried in vacuo over phosphorus pentoxide at a temperature not above 40°:

0·1298 g.; 0·1741 g. CO_2, 0·0661 g. H_2O

0·2314 g. neutralised 13·9 cc. $N/10$ NaOH (Kjeldahl)

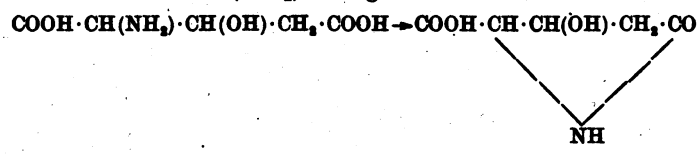
0·0195 g. gave 3·00 cc. N at 23° and 770 mm. (van Slyke)

	C	H	N	
Found	36·6	5·66	8·40	8·45 (van Slyke)
Calculated for $C_5H_9O_5N$	36·8	5·52	8·59	

Other analyses on less pure material gave results varying from 35·8–37·7 per cent. C, 5·6–6·2 per cent. H, 8·4–9·3 per cent. N.

Molecular Weight. 0·2071 g. of the acid dissolved in water and titrated with *N*/10 NaOH required 11·6 cc. when sensitive litmus paper was used as indicator and 12·3 cc. with phenolphthalein. Assuming that the acid reacts like glutamic acid as a monobasic acid, these figures correspond to molecular weights of 171 and 160 respectively, with a mean of 166. Another specimen of the acid (0·240 g.) required 14·2 cc. of alkali using litmus as indicator, equivalent to a molecular weight of 168. Analysis of the silver salt (see later) gave 57·3 per cent. Ag corresponding to a molecular weight of 163. The calculated value for $C_5H_9O_5N$ is 163.

The behaviour of the acid on heating is peculiar. In the neighbourhood of 100° the acid becomes pasty and appears to part with water. Eventually on raising the temperature the whole of the acid is converted into a clear glassy mass. The change is brought about rapidly at a temperature of about 140–150°, but can be effected in a few hours by heating over phosphorus pentoxide at 110°. Most of the nitrogen in the glassy compound, as already stated, is no longer in the (NH_2) form and no longer reacts with formaldehyde or nitrous acid. A sample of the product heated for 6 hours at 110° gave the following result on analysis: C 39·5 per cent., H 5·7 per cent., total N 9·4 per cent., amino-N 4·3 per cent. The results are obviously explainable on the basis of a mixture of β-hydroxyglutamic acid and hydroxypyrrolidone-carboxylic acid. The latter acid contains 41·3 per cent. C, 4·9 per cent. H, 9·86 per cent. total N and no (NH_2) nitrogen.



Silver Salt. This salt was used for the separation of the acid. A specimen for analysis was prepared, as already described, by adding silver nitrate (2 mols.) to the sodium salt of the acid (1 mol.) in about 5 per cent. solution. It separated as a granular very sparingly soluble pure white heavy precipitate showing crystalline structure on standing. It was filtered off and washed with water, alcohol and ether and dried *in vacuo* in the dark. The salt is not excessively sensitive to light. It was analysed with the following result:

0·2568 g.; 0·1495 g. CO_2; 0·0440 g. H_2O

0·1415 g. neutralised 3·6 cc. *N*/10 acid (Kjeldahl)

0·2976 g.; 0·1707 g. Ag.

	C	H	N	Ag.
Found	15·8	1·90	3·56	57·3
Calculated for $C_5H_7O_5NAg_2$	15·9	1·85	3·70	57·3

Copper Salt. The acid forms an extremely soluble copper salt on boiling with precipitated copper oxide and this fact was utilised in preliminary experiments, in order to be assured of the absence of aspartic, glutamic and other acids which form sparingly soluble copper salts. None of these latter salts was ever detected as an impurity in the new acid, and on the whole, the copper salt, for purposes of purification, is much less satisfactory than the silver salt. To prepare the copper salt, the acid was boiled in 2 per cent. solution with a large excess of well washed copper oxide for an hour under a reflux condenser. On filtering and concentrating, the syrup slowly solidified, giving some crystalline product which was hard to separate. It was found more convenient to add methyl alcohol by degrees to a fairly strong solution of the salt, stirring during the addition. The bulk of the copper salt is precipitated as a clear blue powder and a few crystals are apt to separate from the mother liquor on standing. A more complete precipitation is effected by ethyl alcohol, but methyl alcohol is preferred as the copper salt is slightly soluble in it if a little water is present and possibly a certain amount of purification may be so effected. The salt was dried at 110° and contained only traces of water. It was analysed with the following result:

0·1759 g.; 0·1681 g. CO_2; 0·0533 g. H_2O
0·1093 g.; 0·0379 g. CuO
0·1265 g. neutralised 5·8 cc. *N*/10 acid (Kjeldahl)
0·0120 g.; 1·31 cc. N. at 23° and 764 mm. (van Slyke).

	C	H	N	Cu
Found	26·1	3·36	6·42, 6·15	27·7
Calculated for $C_5H_7O_5NCu$	26·7	3·12	6·23	28·3

Mercury Salts. Mercuric chloride added to a solution of the acid produces no precipitate, but on adding excess of sodium acetate, a microcrystalline salt soluble in hot water is precipitated. It has not yet been analysed. The alternate addition of mercuric acetate and sodium carbonate, as practised by Neuberg and Kerb, precipitates the acid practically quantitatively, as a white amorphous salt of complex composition, and use has been made of this property for the separation of the acid. Mercurous salts also precipitate neutral solutions of the acid.

Lead Salt. The lead salt was prepared by boiling the acid in 2 per cent. aqueous solution for one hour with excess of well washed freshly precipitated lead hydroxide. The filtered and concentrated solution did not readily crystallise but dried up to a heavy clear glassy crystalline mass easily soluble in

water but insoluble in alcohol. A sample, dissolved in water and precipitated with alcohol, was obtained as a heavy white granular powder.

0·1350 g.; 0·0824 g. PbO.

Found ... ... 56·6 per cent. Pb

Calculated for $C_5H_7O_5N$ Pb 56·3 ,,

Cadmium and Zinc Salts. These salts were prepared in the same fashion as the lead salt. Neither of them crystallises readily and they are extremely soluble in water but insoluble in alcohol. They appear to be normal salts of the type $C_5H_7O_5N$ M''.

Calcium Salt. The free acid was warmed with excess of lime water and filtered. On concentrating on the water bath calcium carbonate and hydroxide separated out and the operation of filtration and evaporation was twice repeated. It appears that a normal calcium salt is first formed which decomposes on exposure to air to give the acid salt. The concentrated solution of the calcium salt refused to crystallise, and it was therefore precipitated with alcohol. The soft amorphous precipitate soon became hard and on drying *in vacuo* at 110° the substance became crystalline resembling fused sodium acetate. It is extremely soluble in water, insoluble in alcohol.

0·1098 g.; 0·0428 g. $CaSO_4$.

Found ... ... 11·5 per cent. Ca

Calculated for $(C_5H_8O_5N)_2Ca$ 11·0 ,,

Barium Salt. The acid barium salt was obtained by boiling the acid with excess of precipitated barium carbonate. The filtered solution on concentrating dries to a hard gritty mass. The salt is easily soluble in water, insoluble in alcohol. It was dried at 110°.

0·2241 g.; 0·1112 g. $BaSO_4$.

Found ... ... 29·2 per cent. Ba.

Calculated for $(C_5H_8NO_5)_2Ba$ 29·7 ,,

β-Naphthalenesulphonic derivative. One gram of the acid was dissolved in 6 cc. of normal sodium hydroxide and shaken with β-naphthalenesulphonic chloride (2·7 g.) dissolved in ether (15 cc.). Additional sodium hydroxide (30 cc.) was added by degrees with vigorous shaking. The mixture was allowed to stand overnight, and the aqueous portion filtered and precipitated with concentrated hydrochloric acid (6 cc.). A clear oil separated which became sticky on washing with water, and on long standing eventually solidified to a hard crystalline mass. It is sparingly soluble in water but readily soluble in alcohol and acetone, but all attempts to crystallise it satisfactorily failed. It was purified to some extent by redissolving in *N* NaOH

and again precipitating with acid. The substance was found to contain 4·04 per cent. of nitrogen (Kjeldahl) compared with a calculated value of 3·96.

On dissolving the naphthalenesulphonic derivative in a minimum (1 mol.) of warm *N*/10 NaOH and adding copper acetate, a curdy light green copper salt, very sparingly soluble in water, was at once precipitated. It was dried at 110° and analysed as follows:

0·1036 g.; 0·0201 g. CuO
0·1027 g.; 0·0193 g. CuO
0·2098 g. neutralised 5·0 cc. *N*/10 acid (Kjeldahl).

	Cu	N
Found	15·4, 14·9	3·34
Calculated for $C_{15}H_{13}O_7NSCu$	15·3	3·37

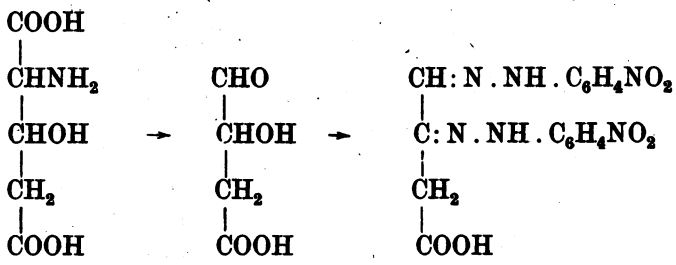
Oxidation of the acid with Chloramine-T. A normal solution (5 cc.) of the acid, containing 0·815 g., was neutralised with normal sodium hydroxide (5 cc.) and then mixed with 1·44 g. chloramine-T (sodio-*p*-toluenesulphochloroamide), dissolved in 5 cc. of water. An immediate reaction took place with precipitation of toluenesulphonamide, but active chlorine did not disappear until after about an hour. The solution was filtered and then warmed on the water-bath with a solution of *p*-nitrophenylhydrazine (2 g.) in dilute alcoholic sulphuric acid. On heating, it appeared that the hydrazone first formed was being converted into a sparingly soluble osazone. After three-quarters of an hour, the precipitate was filtered off with suction and well washed with alcohol. The washed product, weighing about 0·6 g., was recrystallised from boiling nitrobenzene, and separated out as red-brown prismatic needles melting at 297–299°. The substance is sparingly soluble in most neutral solvents but is very freely soluble in pyridine giving a yellow crystalline salt. Alcoholic ammonia gives a red crystalline salt. On adding sodium hydroxide to an alcoholic solution containing a trace of the substance, a deep blue colour is produced. This reaction is apparently characteristic of two adjacent nitrophenylhydrazine groups.

For analysis, the substance was dried *in vacuo* at 120° over phosphorus pentoxide.

0·1142 g.; 0·2140 g. CO_2; 0·0397 g. H_2O
0·1250 g.; 0·2353 g. CO_2; 0·0432 g. H_2O
0·1650 g.; 34·0 cc. N at 26° and 756 mm. (Dumas).

	C	H	N
Found	51·1, 51·3	3·86, 3·84	22·7
Calculated for $C_{16}H_{14}O_6N_6$	51·1	3·72	22·6

Assuming the acid to be correctly represented as a β-hydroxyglutamic acid, the course of the reaction with chloramine-T[1] and the subsequent osazone formation would almost surely be represented as follows:



No attempt to isolate the free aldehyde has yet been made, but this will be tried later, as well as its conversion into malic acid.

Reduction of the Acid with Hydriodic Acid. The reduction of hydroxyglutamic acid with hydriodic acid presents some difficulties. In all the experiments, one part of the acid (0·5–1 g.) was heated with 0·3 part red phosphorus and 10 parts of fuming hydriodic acid (sp. gr. 1·9). At a low temperature, such as 120° on boiling under a reflux condenser, little or no change occurs, whilst in a sealed tube at high temperatures (180–200°) any glutamic acid formed is decomposed with formation of butyric and other products [cf. Kwisda, 1891]. On the other hand for the reduction of β-hydroxyglutaric acid by hydriodic acid, a temperature of 180° is desirable, according to Pechmann and Jenisch [1891]. The best result was obtained by heating the mixture in a sealed tube at 145–150° for six hours. The clear contents of the tube were diluted with water (100 cc.) and boiled with excess of precipitated lead hydroxide until neutral. After filtering, the lead was removed from the filtrate with hydrogen sulphide and the solution concentrated to a syrup. Fuming hydrochloric was then added and the mixture allowed to stand in the ice box. Characteristic crystals of glutamic acid hydrochloride soon separated and they were filtered off and recrystallised again from hydrochloric acid. The crystals melted at 195–197° and a mixed melting point determination with *d*-glutamic acid hydrochloride of other origin melted at the same temperature. The yield of pure product in two experiments was only between 10 and 15 per cent. The substance gave the pyrrole reaction strongly. It was analysed for nitrogen by van Slyke's method:

0·0132 g.; 1·75 cc. N at 21° and 765 mm. = 7·57 per cent. N.
Calculated for $C_5H_9O_4N$ HCl = 7·65 per cent. N.

[1] For analogous oxidations of aspartic and glutamic acids with chloramine-T see Dakin [1917].

Colour Reactions of the Acid. On adding excess of concentrated sulphuric acid to a few drops of a dilute solution of the acid together with a few milligrams of a phenol, a variety of rather definite colour reactions are obtained some of which may be of use for purposes of identification. Perhaps the reactions with resorcinol and α-naphthol are the most striking and sensitive. It should be noted that the absence of any trace of nitrates is essential or misleading results will be obtained.

Resorcinol. Clear reddish purple turning brown on warming.

Catechol. Blue green.

Thymol. Clear bright green.

Pyrogallol. Dark green.

Phloroglucinol. Cherry red.

α-Naphthol. Clear fluorescent green.

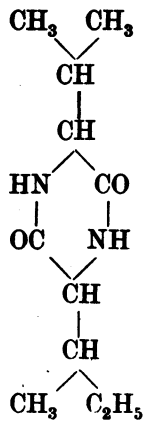
β-Naphthol. On warming, a yellowish green fluorescent solution resembling the similar reaction with malic acid.

The acid also appears to give a red colour reaction with diazobenzenesulphonic acid when warmed with excess of sodium hydroxide. This reaction due to Rosenthaler [1912] is given by various alcohols and especially by malic acid. Minute traces of histidine are apt to complicate the reaction.

III. A new Peptide, Isoleucylvaline anhydride, obtained by the acid hydrolysis of Caseinogen.

In the first of the preceding sections, an account was given of the separation of certain peptide anhydrides from the crude butyl alcohol extract from hydrolysed caseinogen. The peptide mixture is fairly easily soluble in butyl alcohol and was separated from proline by successive crystallisation from absolute alcohol and water—the proline remaining in solution. The two fractions, from alcohol and water respectively, were mixed together and averaged about 5 per cent. of the weight of caseinogen originally hydrolysed. The mixture is evidently composed of a number of different compounds and would repay careful examination. A tyrosine-containing peptide was separated by the action of cold normal sodium hydroxide and subsequent precipitation with acetic acid. Its complete identification still remains to be accomplished. The substance remaining insoluble in cold dilute alkali was recrystallised first of all from boiling absolute alcohol, in which it is sparingly soluble, and subsequently from 50 per cent. acetic acid, in which it dissolves readily. The crystalline product thus obtained was in the form of woolly

masses of fine needles, often over 1 cm. in length, and appeared perfectly uniform under the microscope. The yield was considerable, amounting in one case to slightly over 1 per cent. of pure product, so that the actual amount present must have been considerably more. The substance was almost insoluble in water and in dilute acid or alkali, even on moderate heating. In cold 50 per cent. acetic acid, its solubility was very close to 0·35 per cent., but much greater in the hot acid. It was sparingly soluble in alcohol, acetone or ethyl acetate, and insoluble in ether. It dissolved in concentrated sulphuric acid without developing colour. The substance did not react when boiled with copper oxide to give a copper salt, nor did it evolve nitrogen readily when treated with nitrous acid. These reactions clearly indicated the presence of the diketopiperazine type of linkage. At first the substance was thought to be leucinimide, but this was excluded by both melting point and analysis. The results of the analysis agreed with a *l*-leucyl-*d*-valine anhydride and such a substance has been isolated by Abderhalden and Funk [1907] from caseinogen and has been synthesised by Fischer and Scheibler [1908]. The melting points given for these products agree closely with one another, namely 285° (uncorr.) and 282° (corr.) respectively. On the other hand, the melting point of the substance under investigation was constant at 310–312° (uncorr.), so that there could be no question of identity, though possibly of stereoisomerism. But on hydrolysing the new substance with hydrobromic acid, good evidence was obtained of the presence of both *d*-isoleucine and *d*-valine, so that the original peptide was undoubtedly a *d*-isoleucyl-*d*-valine anhydride, *i.e.* isopropyl-*sec*-butyldiketopiperazine with the following structure:



Analysis. The substance was recrystallised from 50 per cent. acetic acid dried *in vacuo* at 110°.

0·1003 g.; 0·2281 g. CO_2; 0·0884 g. H_2O

0·1030 g.; 0·2343 g. CO_2; 0·0858 g. H_2O

0·1000 g. neutralised 9·45 cc. $N/10$ acid (Kjeldahl).

	C	H	N
Found	62·2, 62·0	9·79, 9·26	13·2
Calculated for $C_{11}H_{20}O_2N_2$	62·2	9·5	13·2

Optical Rotation. A 1 per cent. solution of the substance in glacial acetic acid was examined. Two different specimens gave the following values:

$c = 1·0$, $l = 2$ dm., $\alpha = -0·87°$ hence $[\alpha]_D^{20°} = -43·5°$,

$c = 1·0$, $l = 2$ dm., $\alpha = -0·84°$,, $[\alpha]_D^{20°} = -42·0°$.

Hydrolysis. One gram of the peptide was heated in a sealed tube with 10 cc. hydrobromic acid (sp. gr. 1·48) at 115–120° for eight hours. A little brown insoluble matter was filtered off and the filtrate evaporated to dryness under reduced pressure. The residue was dissolved in water and made just alkaline to ammonia and allowed to crystallise. The crystalline residue was washed with a little water and alcohol and dried at 90°. The mixed amino-acids were analysed for nitrogen and examined optically.

0·1351 g. neutralised 10·7 cc. $N/10$ acid (Kjeldahl) = 11·09 per cent. N.

0·1502 g. dissolved in 10 cc. of 20 per cent. hydrochloric acid, showed a dextro-rotation of + 0·96° in a 2 dm. tube, hence $[\alpha]_D = +32·1°$.

It will be seen that these figures agree closely with the mean of the values for *d*-valine and *d*-isoleucine. *d*-Valine contains 11·97 per cent. nitrogen and has a rotation of + 29·6°, while *d*-isoleucine contains 10·69 per cent. of nitrogen and has a rotation in hydrochloric acid of + 36·8°. The fact that the rotation of the mixed amino-acids from the peptide had as high a rotation as + 32·1°, is almost conclusive evidence of the presence of *d*-isoleucine and *d*-valine, as will be readily seen on consulting a table of optical rotation constants for the amino-acids.

The separation of *d*-isoleucine and *d*-valine is a difficult matter, especially when only small amounts of material are available. A partial separation of the residual amino-acid mixture was accomplished by taking out a fraction containing most of the *d*-isoleucine as a lead salt, according to Levene and van Slyke's method. The filtrate containing most of the valine was treated with hydrogen sulphide and recovered as described by the above authors. Since the optical properties of the two fractions were of greater importance than analytical figures, the rotations were observed first and after reading nitrogen estimations made on aliquot parts of the solution.

The crude *d*-isoleucine, 0·1200 g., dissolved in 10 cc. of 20 per cent. hydrochloric acid, showed a rotation of + 0·82° in a 2 dm. tube, hence $[\alpha]_D = + 34·2°$. It contained 10·90 per cent. of nitrogen. The crude *d*-valine contained 11·31 per cent. of nitrogen and 0·1500 g. in 10 cc. of 20 per cent. hydrochloric acid showed a dextro-rotation of + 0·92°, hence $[\alpha]_D = + 30·7°$. While the comparison of the above figures with the calculated shows that the separation of the isoleucine and valine was incomplete, it also appears to offer satisfactory evidence of the presence of both of them in about equal amounts.

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