XXXVI. A NEW METHOD FOR PREPARING ESTERS OF AMINO ACIDS. COMPOSITION OF CASEINOGEN.

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IN a preliminary paper [Foreman, 1912] reasons were given for attempting to improve the methods commonly in use for separating the products of protein hydrolysis, and a new method for preparing the esters of the amino acids was briefly described.

The method has been applied to the products of two further hydrolyses of caseinogen with the following objects in view:

A. Hydrolysis by sulphuric acid. To determine the distribution of the nitrogen in the various fractions, and to test the completeness of the esterification.

B. Hydrolysis by hydrochloric acid. To determine the effect of the process upon the yields of the monamino acids, and to endeavour to throw some light upon the nature of the unidentified substances making up the deficit in protein analysis.

Before describing the experiments, it will be advisable to set forth briefly the steps involved. Full details of the working will be found in the account of Hydrolysis *B*.

(*a*) The amino acids are converted into their dry lead salts.

(*b*) The lead salts are suspended in absolute alcohol, and the esterification brought about by saturating with dry hydrochloric acid gas.

(*c*) The free hydrochloric acid is removed, partly by reducing the liquid to half its bulk at 40° and 15 mm., and the remainder by the addition of absolute alcohol saturated with dry ammonia gas.

(*d*) Alcohol is removed by evaporation at 40° and 15 mm., and the ester hydrochlorides taken up in dry chloroform.

(*e*) The esters are liberated from their hydrochlorides, and any remaining traces of esterification water removed by shaking the chloroform solution with anhydrous baryta.

(*f*) The chloroform is evaporated at 40° and 15 mm., and the esters taken up in anhydrous ether.

Hydrolysis A.

Nitrogen distribution. 320 g. "Hammersten casein," containing 45.056 g. N equal to 288.4 g. pure caseinogen calculated on the basis of 15.62 % N, was hydrolysed by boiling for 48 hours with three parts of sulphuric acid and six parts of water. The sulphuric acid was removed by means of baryta before making the lead salts.

The following table shows the percentages of the total nitrogen found in the fractions:

In the crude tyrosine removed	...	...	...	...	...	...	...	1.98
In the barium sulphate precipitate after very thorough washing	...	...	...	...	...	...	...	7.13
In the aqueous solution of amino acids	...	...	...	...	...	...	...	88.38
Loss	...	...	...	...	...	...	...	2.51
								<hr/> 100.00

From the aqueous solution of amino acids:

Ammonia lost when making the lead salts (calculated from the percentage for caseinogen given by Osborne and Guest [1911])	...	...	...	...	...	...	...	8.48
In the undissolved lead oxide and hydroxide	...	...	...	...	...	...	...	1.36
In the dry lead salts	...	...	...	...	...	...	...	77.08
Loss	...	...	...	...	...	...	...	1.46
								<hr/> 88.38

From the lead salts:

In the washed lead chloride removed after the esterification	...	...	...	...	...	...	...	1.63
In the filtrate containing the ester hydrochlorides	...	...	...	...	...	...	...	75.02
Loss	...	...	...	...	...	...	...	0.43
								<hr/> 77.08

The baryta residue obtained when the esters were liberated from their hydrochlorides contained 13.42 %, and a small quantity of esters soluble in chloroform but insoluble in ether 2.06 % of the total N. When removing the ether by distillation *in vacuo* from about half the ester solution, the flask was slightly fractured by accident and water gained access to the solution. The esters were recovered as far as possible by ether extraction, but small losses undoubtedly occurred. The nitrogen determinations were therefore discontinued at this stage. After removing the ether the esters weighed 244.7 g.

The ester process was not applied a second time to the material taken from the chloroform solution of the ester hydrochlorides by the anhydrous baryta. The quantity of esters obtained therefore represents the yield of a single preparation.

The esters were fractionally distilled in the usual way, and the fractions partially worked up. The quantities of amino acids separated, so far as these operations were carried, are given in the following table:

Amino acids				Total amounts obtained from 288.4 g. caseinogen. Obtained by one esterification
Glycine	...	...	...	0.5 g. triglycylglycine ester
Alanine, leucines and valine	...	...	...	52.09 g.
Proline	...	...	...	20.12 g.
Phenylalanine	...	...	...	8.96 g. of the hydrochloride
Aspartic acid	...	...	...	4.2 g.

The proline was estimated by van Slyke's method [1911].

The 8.96 g. phenylalanine hydrochloride represents the first crop of the crystals. The mother liquor would have yielded a further quantity. Phenylalanine ester was undoubtedly extracted to some extent by the water which gained admission to the ethereal solution of the esters by accident (p. 379).

The aspartic acid was separated as barium aspartate from the highest boiling ester fraction.

The 0.5 g. triglycylglycine ester separated from Fraction I while it was standing in the ester condition in a corked bottle for about three weeks. The substance was easily soluble in water, alkaline to litmus, and gave a pronounced biuret reaction. When its melting point was taken, it commenced to turn brown at 200°, sintered on further raising the temperature, blackened and finally decomposed at 267°. Fraction I always contains ether. The conditions were therefore identical with those under which Curtius [1904] prepared his biuret base, triglycylglycine ester. The composition, properties and reactions of the substance were also in agreement. Found 20.6 % N; calculated for triglycylglycine ester 20.44 % N.

The efficiency of the esterification method.

The dry lead salts weighed 558 g. and contained 50.27 % Pb. The lead chloride removed by filtration after esterifying, dried at 100°, weighed 335.7 g. The filtrate was found to yield a precipitate on adding absolute alcohol, which settled to the bottom in the form of a brown syrup. Extraction with a little cold water gave a brown solution containing 2.727 g. N leaving 36.2 g. lead chloride. The brown solution invites further investigation. The total weight of dry lead chloride recovered was therefore 371.9 g., which corresponds to 277 g. lead. The lead salts contained 280.5 g. lead. The lead salts were therefore practically completely transformed in the esterification.

Hydrolysis B.

YIELDS OF MONAMINO ACIDS. INVESTIGATION OF THE RESIDUES.

In the previous sulphuric acid hydrolysis, no less than 7 % of the total N was retained by the washed barium sulphate precipitate formed when the acid was removed. In order to avoid the effect of this loss upon the yields of amino acids, this hydrolysis was brought about by means of hydrochloric acid. Glutamic acid was removed in the form of its hydrochloride, as far as possible, before the esterification.

320 g. of the same sample of "Hammersten casein" as used in Hydrolysis A (= 288.4 g. pure substance) was hydrolysed in the usual way by boiling with hydrochloric acid for 48 hours.

Removal of glutamic acid as the hydrochloride. The liquid arising from the hydrolysis was evaporated on the water-bath to a volume of about 700 cc., saturated with hydrochloric acid gas, and left in the ice chest. The crystals

were filtered off on an asbestos pad, and washed several times with aqueous hydrochloric acid saturated with the gas at 0°. The crude hydrochlorides thus obtained weighed 88.1 g. after drying to constant weight. This material was taken up with water, the solution decolorised by warming with animal charcoal, reduced to about 300 cc., and saturated with hydrochloric acid gas. The perfectly white crystals were filtered off on asbestos, and washed many times with ice cold saturated aqueous hydrochloric acid. The free hydrochloric acid was removed in a desiccator over strong potash. 57.1 g. resulted after drying to constant weight at 100°. The filtrate yielded a further 0.16 g. of the hydrochloride when reduced in bulk and again saturated. The total weight was therefore 57.26 g., which corresponds to 45.87 g. glutamic acid. Portions weighed out from the whole specimen gave the following results on analysis:

	Found	Calculated
	%	%
C	32.61	32.70
H	5.80	5.45
N	7.64	7.62
Amino N (van Slyke)	7.74	7.62

It will be noted that the recrystallised material only accounted for about two-thirds of the crude dry hydrochlorides.

Preparation of the lead salts. The filtrate from the recrystallised glutamic acid hydrochloride was added to the main hydrolytic liquid, and hydrochloric acid removed as far as possible by evaporation on the water-bath. A current of steam was then passed through the heated fluid for some time to remove further hydrochloric acid. The volume was made up to 2 litres with water, a current of steam passed through, and 100 g. precipitated lead hydroxide added. The steaming was continued for a few minutes, and the hot solution filtered by suction. The insoluble material was dark in colour, and contained the greater part of the humin matter. 400 to 500 g. ordinary litharge was added to the filtrate in portions during a further 45 minutes' steaming. The excess of litharge was filtered off rapidly by suction, and washed with hot water.

The preliminary treatment with lead hydroxide was introduced because the compound formed with the humin matter remains in suspension, and can be easily filtered off. When litharge was used from the commencement, however, the humin compounds tended to collect at the bottom in the form of a troublesome cake. The use of litharge after lead hydroxide appears to be necessary for the complete transformation of the amino acids into lead salts, and a good excess should be added as the bigger particles are not readily dissolved.

The solution containing the lead salts was evaporated on the water-bath to a volume of about 500 cc. and cooled. The semi-solid mass of salts which resulted was placed on the water-bath and stirred until it became too viscous. After standing in the steam-oven all night and then being cooled the material can be easily reduced to a powder. The grinding however is quite unnecessary,

as the dry cakes will esterify readily when treated in the manner to be described. The dry lead salts weighed 475 g. The undissolved lead hydroxide and litharge contained 3.1 % of the total N of the 320 g. caseinogen.

Esterification. The lead salts were suspended in 1500 cc. absolute alcohol, and saturated with dry hydrochloric acid gas. The liquid was then cooled by means of a freezing mixture of ice and salt, and again brought to saturation. The lead chloride was filtered off and washed with absolute alcohol.

Removal of the free hydrochloric acid. The greater part of the free hydrochloric acid was removed by reducing the volume to one-half at 40° and 15 mm. The fluid was transferred to an enamelled jug, and the flask washed out with absolute alcohol. The jug was surrounded by a freezing mixture of ice and salt, and the remaining free hydrochloric acid removed by means of a saturated solution of dry ammonia gas in absolute alcohol, which was added slowly, with continual stirring, until the liquid remained only faintly acid. The further addition of alcoholic ammonia would liberate esters from their hydrochlorides, and the fluid would darken in colour. It was considered advisable, however, to stop at the faintly acid stage in order to avoid loss which might result from decomposition of the free esters in this as well as subsequent operations. By the means described the free acid was eliminated without involving the formation of water. The ammonium chloride was filtered off by suction, and washed thoroughly with absolute alcohol.

Removal of the alcohol. The alcohol was removed as completely as possible by evaporation at 40° and 15 mm. The specific gravity of the distillate was found to be higher than that of absolute alcohol. Assuming this increase to be due to the presence of water, the distillate contained a quantity which corresponded very well with the calculated amount of water produced during the esterification. The content of lead in the lead salts of the previous sulphuric acid hydrolysis provided a basis for making this calculation, after an allowance had been made for the glutamic acid removed in the case of Hydrolysis B.

The ester hydrochlorides taken up in dry chloroform. The syrup was taken up in dry chloroform, in which the ester hydrochlorides dissolved readily. A little ammonium chloride, which had been retained in solution by the alcohol, was filtered off and washed with chloroform. The total amount of ammonium chloride formed from the free hydrochloric acid was about 250 g.

Liberation of the esters from their hydrochlorides. The chloroform solution of the ester hydrochlorides occupied a volume of 1000 to 1500 cc. 300 to 350 g. anhydrous baryta were added in successive portions of about 30 g., and the mixture well shaken after each addition. After all the baryta had been added, the mixture was allowed to stand for two minutes, by which time the temperature at the bottom where the solid material had settled had risen to about 35°. A temperature of about 30° throughout the whole fluid resulted on further shaking. The baryta by this time had become granular, and settled instantly the shaking was discontinued. The mixture was cooled under the tap, and a thermometer placed so that its bulb was covered by the solid material at the

bottom. After standing two or three minutes, a rise of 2° was recorded. The mixture was shaken again, cooled, and the test repeated, but no further increase of temperature resulted. It was concluded therefore that the reactions were complete. It may be found sometimes, however, that the clear fluid contains a little chloride at this stage. In this case the mixture should be allowed to stand, with an occasional shake, until no chloride remains. In one experiment the addition of a little more anhydrous baryta was necessary before the last traces of chloride could be removed. The solid material was filtered off by suction and washed thoroughly with dry chloroform. The chloroform was removed from the filtrate by evaporation at 40° and 15 mm., and the esters shaken with anhydrous ether. Practically the whole dissolved immediately, only a very small quantity of syrup which collected on the sides of the flask remaining insoluble. The latter was found to contain substances precipitated by phosphotungstic acid after hydrolysis. The esters weighed 235.9 g.

Recovery of a second portion of esters. The baryta residue obtained when the esters were liberated from their hydrochlorides contained 9.6 g. N. This was suspended in water, and the barium all converted into sulphate at 40° by adding a slight excess of dilute sulphuric acid. After heating for some time on the water-bath, the barium sulphate was removed and washed. The solution was steamed with litharge in the manner already described, and 116 g. dry lead salts obtained. Esterifying these lead salts as before, using equivalent amounts of the various reagents, only 25.6 g. of esters resulted.

The two lots of esters, together amounting to 261.5 g., were combined and distilled in the apparatus described by Fischer and Harries [1902]. For reasons which will be explained later (p. 393) it is regretted that these two quantities of esters were not investigated separately.

Fractional distillation of the 261.5 g. esters.

Fraction	Temp. of bath °C.	Temp. of vapour °C.	Pressure mm.	Weight of fraction, g.
I	Up to 88	Up to 65	11	28.07
II	88 to 110	65 to 84	10	73.71
III	110 to 132	84 to 94	10	20.00
IV	152 to 165	100 to 125	10	13.57
Distillation residue	—	—	—	82.03
In liquid air condenser	—	—	—	10.45
Total				227.83

Fraction IV did not commence to distil over until the temperature of the bath was 152° and of the vapour 100° . A very slow current of carbon dioxide, too small to increase the pressure, was passed through the liquid during the distillation. The fractions were thus assisted in their passage to the condenser, and air was excluded. An accident to the Dewar's flask containing the liquid air supply just before the reduction of pressure was required prevented the usual fractions at 0.5 mm. from being obtained. The distillation was continued

at 10 mm. as described, and a very satisfactory separation of the fractions containing glycine, alanine, valine, leucines and proline was effected.

Phenylalanine. Fraction IV was treated with water and extracted with ether in the usual way, and the ether-soluble ester evaporated to dryness with hydrochloric acid. The crystals obtained were dissolved in water, decolorised with animal charcoal, and recrystallised from hydrochloric acid. The specimen was washed thoroughly with saturated aqueous hydrochloric acid, and the free acid removed in a desiccator over potash. This fraction of the phenylalanine hydrochloride, dried completely at 100°, weighed 1.36 g. It contained 7.07 % N (Kjeldahl).

The distillation residue was treated in exactly the same way as Fraction IV, yielding 11.71 g. of the dry recrystallised hydrochloride. This contained 6.87 % N; calculated for phenylalanine hydrochloride 6.95 % N.

The total quantity of hydrochloride separated was 13.07 g. equal to 10.70 g. phenylalanine.

The liquids from which these separations of the hydrochloride had been made were combined, and an attempt made to obtain a further crop of the hydrochloride. A little more was separated, but its content of nitrogen was 0.45 % too high. It was returned therefore, and the amino acids liberated by quantitatively removing the free as well as the combined hydrochloric acid. A total weight of 7.89 g. dry substance resulted, from which 0.25 g. tyrosine was separated, leaving 7.64 g. in which nothing was satisfactorily identified.

Glycine and alanine. The esters of Fraction I, obtained up to a temperature (vapour) 5° higher than usual, were hydrolysed by boiling with water, and the solution evaporated to dryness. After extracting proline in the usual way, the amino acids, dried at 100°, weighed 13.28 g. By fractional crystallisation from water, one fraction was obtained which melted at 250°, and contained 13.05 % N. This was sub-fractionated, but no portion contained a much higher percentage of N, and no alanine could be separated. The other original fractions gave much higher melting points, and judging by their nitrogen content consisted of mixtures of valine and leucine.

The mother liquor on evaporation yielded 6.58 g. dry amino acids, which melted at 239°, and contained 16.03 % N. This was examined for glycine by the picrate method of Levene and van Slyke [1912], and 2.38 g. glycine picrate melting at 200°, and decomposing at 202°, was separated. The picric acid was removed from the filtrate by means of *N* sulphuric acid and ether and the sulphuric acid by baryta. The amino acids recovered, dried at 100° C., weighed 4.77 g. This material was crystallised from water, leaving a small mother liquor which on evaporation yielded 1.45 g. dry substance melting at 238°, from which a further 0.756 g. glycine picrate with the correct melting point was separated. The two portions of glycine picrate, having identical melting points, were mixed, and the amino nitrogen in a portion weighed out from the whole quantity was determined by the nitrous acid method: found 7.33 % (corrected); calculated for glycine picrate 7.39 %.

The total quantity of the picrate thus separated from the amino acids of Fraction I was 3.136 g., which corresponds to 1.24 g. glycine.

The contents of the liquid air condenser, obtained when the esters were distilled, were freed from ether and hydrolysed by boiling with water. On evaporation 0.36 g. dry substance was obtained which melted at 239°, and contained 16.07 % N. This yielded 0.1 g. glycine picrate with the correct melting point, showing that losses were falling upon glycine ester during the distillation. The ether distilled from the ethereal solution of the esters before they were fractionated yielded by similar treatment 0.17 g. dry substance, from which no trace of glycine picrate could be separated.

The substance separated by crystallisation from the 4.77 g. amino acids recovered after removing the first lot of glycine picrate was submitted to analysis:

	Found	Calculated for alanine
C	40.82 %	40.45 %
H	7.97	7.86

It was concluded therefore that the 6.58 g. portion consisted of a mixture of 1.24 g. glycine and 5.34 g. alanine. This statement is supported by the fact that a mixture of glycine and alanine in these proportions would contain 16.15 % N, whereas 16.03 % was actually found.

Glycine from pure caseinogen. The examination of the first fraction obtained by distilling the esters prepared from the hydrolysis products of "Hammersten casein" by the method advocated in these pages revealed the presence of glycine. Evidence had been given which showed that losses of glycine occurred during the distillation owing to the low boiling point of its ester. The products of pure caseinogen therefore very probably contain considerably more than the 0.45 % isolated as the picrate. The specimen of picrate was excellent in quality. The 0.5 g. triglycylglycine ester separated in the previous hydrolysis (p. 380) provides confirmatory evidence.

If the absolute purity of "Hammersten casein" be doubted, it would be reasonable to suspect contamination with small quantities of the other protein constituents of milk. So far as I can ascertain, no glycine has been found amongst the hydrolytic products of lactalbumin, and the ester hydrolysis of lactoglobulin has not been done. A yield of 3.5 % glycine, however, has been obtained from serum globulin, which closely resembles lactoglobulin. Assuming lactoglobulin to contain 3.5 % glycine, and the 0.45 % glycine isolated from the products of caseinogen to be derived from lactoglobulin as impurity, the presence of 13 % of the latter in the caseinogen must be admitted. Emmerling [1888] states that milk contains not more than about 0.005 % lactoglobulin. Crowther and Raistrick [1916] however isolated 0.03 %, which is equivalent in quantity to only about 1 % of the caseinogen. The glycine separated therefore cannot be attributed to protein impurity.

Valine and leucines. The esters of Fractions II and III were hydrolysed in the usual way by boiling with water, and the solutions combined. The dry

amino acids obtained on evaporation were thoroughly extracted with absolute alcohol to dissolve proline, and dried at 100°. The following results were obtained on analysis: C 53.28, 53.22 %; H 9.67, 9.52 %; N (Dumas) 11.27, 11.28 %.

From these figures the proportion of leucine isomers to valine in the mixture was calculated as follows:

$$\frac{53.28 - 51.24}{54.92 - 51.24} \times 100 = 55.4 \% \text{ leucine isomers.}$$

$$\frac{11.27 - 10.68}{11.97 - 10.68} \times 100 = 45.7 \% \text{ valine.}$$

It will not be far from the mark therefore to conclude that the mixture consisted of 55 % leucine isomers and 45 % valine. To verify this further the lead method of Levene and van Slyke [1909] for the separation of these substances was employed. The lead salt of the leucines which separated was found to contain 44.64 % Pb. Calculated for lead leucine, 44.34 % Pb.

The leucine and valine were recovered from the lead precipitate and from the filtrate respectively, and their content of nitrogen determined.

Nitrogen in the leucine isomers:

0.1995 g.; (Kjeldahl) NH_3 neutralising 15.4 cc. $\text{N}/10$ acid, 10.80 % N. Calculated for leucine, 10.68 %.

Nitrogen in the valine:

0.2292 g.; (Dumas) 22.6 cc. N at 14.5° and 768 mm., 11.90 % N. Calculated for valine, 11.97 %.

The dry amino acids insoluble in alcohol obtained from the ester Fractions II and III, together with the small portions from Fraction I which showed a percentage of N between that of leucine and valine, amounted to 50.85 g. 55 % equals 27.97 g. leucines, leaving 22.88 g. valine.

Proline. The united alcoholic extracts of the dry matter obtained from ester Fractions I, II and III, already in such condition that the amino acids they contained would completely dissolve when well stirred in cold alcohol, were freed from alcohol, and the content of proline calculated from the non-amino nitrogen content according to van Slyke [1911]. The following results were obtained:

Total N	3.44 g.
Amino N	0.761
Proline N	2.679

This amount of proline N corresponds to 22.005 g. proline.

The material was dried to constant weight in the steam-oven, and weighed 28.05 g. It contained therefore 12.26 % total N, which is in accord with the experience of Osborne and Guest [1911]. A 2 g. portion was treated with phenyl isocyanate, and a moderate yield of the proline derivative was obtained. This was converted into the hydantoin, which melted at 143°.

Assuming all the non-amino N to be in the form of proline, 6.05 g. amino acids of the straight chain type were also present in the alcoholic extracts.

Aspartic acid. The esters of Fraction IV, from which phenylalanine ester had been extracted, were hydrolysed by boiling with baryta. A small quantity of barium aspartate separated which yielded 0.48 g. aspartic acid. This was converted into the copper salt which crystallised from plenty of water in the form of light blue needles. The barium was removed quantitatively from the filtrate from the barium aspartate, and the solution evaporated to dryness. After drying at 100°, this material weighed 3.71 g. By means of the titration method of Osborne and Liddle [1910] the presence of a further 0.51 g. aspartic acid was shown. The liquid which had been titrated was concentrated, and a small crystal fraction separated from dilute alcohol, melting at 245°. This substance was found to contain 39.39 % C, 7.82 % H, and 11.25 % N. An attempt to make a β -naphthalenesulphonic derivative gave no satisfactory result. The amino acids obtained from ester Fraction IV, which were not identified, weighed 3.2 g.

THE DISTILLATION RESIDUE.

The esters remaining in aqueous solution after extracting phenylalanine ester with ether from the distillation residue were hydrolysed by boiling with baryta for several hours. The deposit which formed on long standing contained no aspartic acid. The cold filtrate was made neutral to litmus with sulphuric acid as a preliminary to the complete removal of barium. The precipitate which resulted was flocculent and had not the appearance of barium sulphate. It was filtered off, shaken with cold water, decanted several times, and washed many times on the filter with cold water. On investigation this precipitate was found to contain 3.5 g. amino acid material consisting of two unidentified portions, one of which was precipitated by phosphotungstic acid, and aspartic acid which was separated easily and of high purity.

The flocculent precipitate was extracted with successive quantities of boiling dilute sulphuric acid until the extract came through colourless. The solution was reduced in volume, and while at the boiling point sufficient baryta was added to reduce the content of sulphuric acid to 5 %. After heating on the water-bath and filtering, the fluid was cooled and treated with 25 % phosphotungstic acid in 5 % sulphuric acid solution until precipitation was complete. The precipitate was decomposed in the usual way, and yielded 0.64 g. material brown in colour, not hygroscopic, and strongly acid to litmus. The filtrate was freed from phosphotungstic and sulphuric acids, and evaporated to dryness on the water-bath. The dry material weighing 2.88 g. was extracted and washed with a small quantity of ice cold water, yielding a very soluble slightly coloured portion, and a perfectly white insoluble part, weighing 0.85 g. which proved to be aspartic acid. It was found to contain 36.13 % C and 5.27 % H; calculated for aspartic acid, 36.09 % C and 5.26 % H.

A portion was converted into the copper salt, which separated from a dilute aqueous solution in the characteristic form.

The fraction soluble in the ice cold water was converted into the copper

salt, which would not crystallise from water. It dried to a dark blue brittle material, pale blue when powdered, and insoluble in alcohol. Its identity was not established. The analytical figures, given below, are not unlike those obtained for some of the copper salt fractions described on page 389.

Copper ...	...	25.86 %
Nitrogen ...	...	8.0
Carbon ...	...	33.14
Hydrogen ...	...	4.14
Water lost at 125°		3.84

From the foregoing it appears that considerable losses of aspartic acid may occur when barium is removed quantitatively as the sulphate from amino acid solutions containing this substance. In all probability the considerable amount of nitrogen rigidly retained by the large barium sulphate precipitate removed in the early stages of the previous sulphuric acid hydrolysis (p. 379) is partially due to aspartic acid.

The solution from which the flocculent barium precipitate had been removed was made to contain 5 % sulphuric acid, and 25 % phosphotungstic acid dissolved in 5 % sulphuric acid added until the supernatant fluid had lost its brownish colour. This precipitate was removed, and a second fraction obtained by continuing the treatment with phosphotungstic acid until the precipitation was complete. These precipitates were decomposed in the ordinary way. The first one yielded 9.18 g. brown material acid in reaction to litmus. The second gave 10.1 g. amino acids alkaline to litmus from which 3.31 g. lysine picrate was obtained by adding an alcoholic solution of picric acid.

The amino acids not precipitated by phosphotungstic acid. On working up the filtrate from the phosphotungstic acid precipitates, a syrup weighing 24.7 g. very acid to litmus was obtained. This was extracted with alcohol. The insoluble part yielded nothing crystalline from water.

A concentrated aqueous solution of the extract gave two small successive fractions on long standing in a desiccator over sulphuric acid. The first, nodular in form, weighed 0.49 g., contained 62.54 % C, 6.93 % H, and 8.74 % N. Calculated for phenylalanine: 65.5 % C, 6.66 % H, and 8.48 % N. The odour of phenylacetaldehyde developed on warming a portion with dilute sulphuric acid and potassium bichromate.

In order to obtain dry matter suitable for analysis so that a general idea of the composition could be formed, the syrupy materials and their fractions were converted into copper salts, and the copper salts fractionated as described below.

Syrupy material insoluble in alcohol:

- A. Copper salt crystallised from water.
- B. Copper salt precipitated from concentrated aqueous solution by alcohol.
- C. Copper salt, derived from material more soluble in water than that which gave copper salts A and B, precipitated from strong aqueous solution by alcohol.

Syrupy material dissolved by alcohol:

D. Copper salt, made from material which had crystallised from water as already described, precipitated by alcohol out of strong aqueous solution.

E. Copper salt precipitated from strong aqueous solution by alcohol.

F. Copper salt soluble in alcohol, precipitated by ether from alcoholic solution.

The analytical data for these copper salts are given in the following table:

	A	B	C	D	E	F
Carbon	—	32.90	31.54	—	35.15	39.79
Hydrogen	—	4.90	4.88	—	4.64	5.35
Nitrogen	8.0	8.51	8.80	—	8.0	8.83
Copper	26.11	26.18	28.74	27.57	23.63	18.90
Amino nitrogen	—	—	6.84	—	3.45	1.34

These copper salts swelled up considerably during ignition, and gave very disagreeable smells. The first four were blue when moist, and light blue in colour when dry. E gave a deep blue water solution, and a pale greenish blue dry salt precipitated by alcohol. F gave the deepest possible blue alcoholic solution, and a very pale blue dry salt when precipitated by ether.

Copper salt B. The copper was removed from this salt, and the aqueous solution concentrated to the syrupy stage. A very small quantity of nodular crystals separated on long standing, which were removed and recrystallised. The dried material weighed 0.18 g., softened at 180°, completely melted at 215°, gave a strong pyrrole reaction when warmed with zinc dust, and contained 44.42 % C, 6.78 % H, and 11.08 % N. These figures correspond approximately to a calculated formula of $C_5H_9O_3N$. The specimen was too small for conclusive identification.

Copper salt C. The copper was removed and 0.0493 g. crystals resembling glutamic acid were obtained from the concentrated aqueous solution. M.P. 208°. Contained 9.58 % N, calculated for glutamic acid 9.52 % N.

Copper salt E. Gave the pyrrole reaction on heating with zinc dust, but not so strongly as copper salt F.

Copper salt F. Weight = 2.5 g. Strong pyrrole reaction. Approximate formula for the free substance calculated from the analytical figures was $C_5H_9O_3N$. Impurity represented by the amino nitrogen (15.8 % of the total N) probably present. The properties and conditions strongly favour the view that the main constituent was the copper salt of pyrrolidonecarboxylic acid, the copper content differing by only 1 % from theory.

The syrupy material. Discussion.

No way was found for isolating a chemical individual from this viscous matter. Salt F was probably the impure compound of pyrrolidonecarboxylic acid. The ratio N : O in salt E was practically 1 : 3.56, and about 1 : 3 in B and C. A trace of glutamic acid was isolated from C. The composition of the free acids calculated from the data given for the copper salts resembles that of the "gummy substance" obtained by the lime-alcohol method for dibasic

acids [Foreman, 1914]. There can be no doubt that the same material is represented. The total weight of unidentified matter obtained from the distillation residue, omitting diamino acids, was 43.7 g. A considerable quantity, probably of peptide nature, was acid to litmus although precipitated by the ordinary phosphotungstic method for separating diamino acids.

THE UNESTERIFIED RESIDUE.

The baryta residue obtained when the second lot of esters were liberated from their hydrochlorides, completely freed from esters by washing with chloroform, was suspended in water, and the baryta all neutralised with sulphuric acid at 40–50°. After heating on the water-bath, the barium sulphate was removed and washed. The liquid was reduced in volume, sulphuric acid added until 5 % was present, and treated with 25 % phosphotungstic acid in 5 % sulphuric acid solution until precipitation was completed. The precipitate decomposed in the usual way yielded 12.96 g. dry matter. The filtrate was freed from phosphotungstic and sulphuric acids, and as much of the free hydrochloric acid as possible removed on the water-bath. The syrup which resulted was taken up in water, made up to 200 cc., and by estimating in aliquot portions, the whole solution was found to contain 1.568 g. total N, 1.043 g. amino N (van Slyke), and 0.042 g. N as NH_3 . The hydrochloric acid remaining was quantitatively removed and the liquid again treated with phosphotungstic acid as before. A small gummy precipitate was obtained, and the whole of the colour disappeared from the solution. The reagents were completely removed, the liquid evaporated, and made up to 200 cc. The amino acid content was estimated by evaporating an aliquot and drying the residue to constant weight at 100°; 10 cc. gave 0.503 g. Allowing for the portions removed in the nitrogen estimations, the unesterified residue therefore contained only 10.06 g. amino acids not precipitated by phosphotungstic acid.

The 0.5 g. obtained from the aliquot was returned to the main solution, and the volume reduced to about 50 cc. on the water-bath. 2.76 g. tyrosine crystallised.

Serine. The filtrate from the tyrosine was evaporated down to 10–15 cc. On standing over night, crystals in the form of thin hard transparent plates separated. This substance was recrystallised from water after removing a trace of colour from the solution by animal charcoal, and yielded crystals of the same character, which weighed 0.998 g. M.P. 231°, with decomposition. Analysis gave the following results:

	Found	Calculated for serine
	%	%
C	34.49	34.29
H	6.87	6.67
N	13.48	13.33

A syrup obtained from the remaining material. An attempt was made to obtain a second crop of serine, but when the concentration was carried further than before, the solution became viscous, and although crystals nodular in form appeared on standing, they could not be separated by suction. A very small quantity from which most of the liquid had been removed by suction gave a copper salt, deep blue in colour, insoluble in alcohol, which contained 19.56 % Cu. The viscous matter resembled cheese in consistency after standing a long time *in vacuo* over sulphuric acid. After treatment with a little cold water, a few large hard crystals were picked out, washed, and recrystallised. This specimen weighed only 0.2 g., and melted at 213°.

Material separated from the syrup by means of basic lead acetate and alcohol. A satisfactory precipitate was obtained by means of alcohol after adding basic lead acetate solution to the aqueous solution of the syrup. The addition of alcohol was continued until the separation was complete. The lead was removed from the washed precipitate, and the resulting solution reduced to very small volume. Large white spherical nodules separated from the viscous solution on standing over night. These were removed, and a second fraction of the same type obtained in the same way. The fractions were recrystallised from water.—No. 1 gave 0.123 g., M.P. 182°; No. 2, 0.05 g., M.P. 181°. A trace heated with zinc dust gave a very strong pyrrole reaction. The material, which appeared to be definite in character, gave the following results on analysis:

C.	39.97 %
H	6.33
Amino N	9.94

Although the percentages do not differ widely from those of glutamic acid, further work showed that the substance would not give a hydrochloride insoluble in saturated aqueous hydrochloric acid. The total weight of material precipitated by basic lead acetate and alcohol was 2.23 g.

The amino acids not precipitated as lead compounds. The filtrate from the lead precipitate was freed from lead. Spherical nodules separated from the concentrated aqueous solution on standing. The mother liquor on further concentration yielded crystals of the same kind; M.P. 263° after recrystallisation from water. These gave a strong pyrrole reaction. The quantity obtained was too small for analysis. (Hydroxyproline melts at 270°.)

Discussion.

The unesterified residue contained only 10.06 g. monamino acids. The identifiable part consisted of tyrosine and serine, which are both hydroxy acids. The remainder was syrupy. No traces of known amino acids containing two oxygen atoms in the molecule were found. Further work may show that only the lead salts of hydroxy acids resist esterification. The two small specimens derived from the lead-alcohol precipitate seemed to be definite in character, and the analytical data are not inconsistent with the view held that the substance was probably a hydroxy acid.

THE YIELD OF MONAMINO ACIDS.

The amount of each monamino acid separated in the course of the foregoing investigations will be found in the following table:

Amino acid	Reference page	Weight in g.		%
Glycine	385	1.24		
"	385	0.04	1.28	0.45
Alanine	385		5.34	1.85
Valine	386		22.88	7.93
Leucines	386		27.97	9.70
Proline	386		22.01	7.63
Phenylalanine	384	10.70		
"	388	0.49	11.19	3.88
Serine	390		1.0	0.35
Aspartic acid	387	0.48		
"	387	0.51		
"	387	0.85	1.84	0.64
Glutamic acid	381		45.87	15.91

Discussion.

With the exception of aspartic acid and serine, the percentages are all appreciably higher than those given by Osbrne and Guest [1911] in their compounded table which shows the highest reliable figures for each amino acid obtained from caseinogen up to that date. The yield of aspartic acid is much lower than that obtained in Hydrolysis A (p. 379), owing to the fact that no liquid air was available when the esters were distilled.

THE NEW METHOD FOR PREPARING ESTERS.

The older methods for preparing the esters of the amino acids leave much to be desired from the point of view of yield. This is chiefly due to the fact that water is added to the ester hydrochlorides and the esters are subjected to the saponifying influence of aqueous alkali during their liberation and extraction. Levene and van Slyke [1909] brought about a considerable reduction in the content of free alkali in the aqueous solution by substituting anhydrous baryta for the soda or potash previously employed and better yields resulted. The esters however are exposed, in their method, to aqueous baryta during the extraction which occupies three-quarters of an hour, and a certain amount of saponification occurs. Their original syrup contains a considerable amount of free hydrochloric acid as well as the ester hydrochlorides, and on adding the baryta a large amount of heat of neutralisation as well as further water develop within a thick mass which in a few minutes becomes semi-liquid and alkaline. In spite of the cooling by means of a freezing mixture, local overheating in such material appears likely. The mass eventually breaks up into granules, remaining in this condition until the operations are concluded. A complete extraction of such material, for the greater part of the time in a wet condition, by stirring with successive additions of ether, seems highly improbable. Saponification and incomplete extraction probably account for the

results obtained by the authors, expressed by them as follows: "Extraction as described...yields a second smaller crop of esters, and by repeating the process a third and even a fourth crop in decreasing yields may be obtained."

Esterification and saponification are both influenced in degree by the amount of water present. The new method for the preparation of esters was designed to eliminate the adverse effect of water upon the yield. As the extraction of esters from large bulks of moist material with ether is probably very incomplete, the esters are liberated in a large volume of a more suitable solvent which protects them from saponification, and from which the residue can be filtered and washed. The following statements will explain the procedures adopted:

(1) The water of the original solution of amino acids is eliminated by obtaining the amino acids in the form of dry lead salts.

(2) In the esterification the reverse reaction is prevented by a large excess of alcohol and the favourable influence of the lead chloride formed.

(3) Excess of free hydrochloric acid is removed from the ester hydrochloride solution without the formation of water.

(4) In the absence of free hydrochloric acid, the water of esterification can be removed with the alcohol from the alcoholic solution of the ester hydrochlorides.

(5) The ester hydrochlorides readily dissolve in chloroform, which can be very easily dehydrated by means of anhydrous baryta.

(6) The amount of water produced by the action of the baryta upon the hydrochloric acid part of the ester hydrochloride molecules is small, as two molecules of hydrochloride are required to give one of water. Water is very sparingly soluble in chloroform (100 cc. dissolve 0.152 cc. water at 22° [Herz, 1898]), and the mass of anhydrous baryta relative to that of the water produced is very large. The water is therefore rapidly taken up. The esters on the other hand are very readily soluble in chloroform, and consequently are removed from the spheres of action immediately. There is practically no exposure of the esters to the influence of aqueous baryta, and saponification is prevented. The increase in temperature which occurs produces little if any ill effect upon the yield, and cooling by means of freezing mixtures is unnecessary. The esters are completely separated from the residue by the simple method of filtering and washing.

The efficiency of the method. The second crop of esters obtained in Hydrolysis *B* weighed only 25.6 g. and probably included a little ether. The opinion was formed that this quantity consisted of the esters of dibasic material, and was practically free from the monobasic acids whose esters appear in the first three fractions of the ester distillation. It is regretted that this portion was not investigated separately. The opinion was based upon the following facts. In Hydrolysis *A*, the yield of glycine, alanine, valine and leucines, obtained from a single ester preparation, together amounted to 18.2 %, and the proline was 6.98 %. The corresponding figures for Hydro-

lysis *B*, in which the second crop of 25.6 g. esters was combined with the first before distillation, were 19.93 and 7.63 respectively. As yield was the chief object of Hydrolysis *B*, the experiments were more carefully conducted and the caseinogen was hydrolysed by means of hydrochloric acid, whereas in Hydrolysis *A* sulphuric acid was employed. The yields are generally not so good when sulphuric acid is used, probably because the hydrolysis is not carried so near to completion and loss of amino acids occurs on removing the large barium sulphate precipitate (p. 379).

In another hydrochloric acid hydrolysis of a kilo of "Hammersten casein," equal to 901.3 g. of the pure substance, one preparation of esters by the new method was made, and the first three ester fractions were distilled in the same manner and up to the same temperature as in Hydrolysis *B*. After extracting the amino acids of these three fractions with hot alcohol, 163.03 g. of a mixture of glycine, alanine, valine and leucines resulted, which corresponds to a total yield of 18.1 %. The small quantities of these amino acids which separate in the ordinary way from the proline extract before it will enter completely into solution in cold alcohol are not included in the calculation. The freedom of the unesterified residue of Hydrolysis *B* from amino acids of the leucine type supports the view that the small second crop of esters contained practically none of these substances.

The very large distillation residue obtained, and the very small quantity of monamino acids found in the unesterified residue, are facts which also point to the high efficiency of the new ester method.

The fractionation of the esters. In distilling the esters of Hydrolysis *B*, four fractions were obtained without the aid of liquid air. After Fraction III was complete, a rise of 6° "vapour" temperature and 20° in the "bath" occurred before further esters commenced to distil over. Fraction IV yielded only 1.36 g. phenylalanine hydrochloride, while nearly nine times as much was obtained quite as easily from the distillation residue. The other material derived from Fraction IV was apparently free from amino acids of the leucine type, and, so far as the estimations were concerned, no advantage was gained by distilling this fraction. Now that the dibasic acids can be easily and completely removed before esterification, their estimations do not depend upon the working up of the higher boiling esters, a method which usually gave very poor yields, owing to the large amount of decomposition. If serine is present in the distillation residue, it will probably crystallise quite readily in the absence of dibasic acids, syrupy products and phenylalanine. It appears, therefore, that there is no need to continue the ester distillation further than Fraction III if the work is carried out on the lines suggested, and the esters of the distillation residue will not be subjected to the influence of the higher temperatures.

IMPROVEMENTS SUGGESTED BY THE RESULTS OF LATER EXPERIMENTS.

The foregoing experiments were completed in 1913. Further work showed that the syrupy material found in such large quantity in the distillation residue, as well as to some extent in the unesterified fraction, can be removed from the original hydrolytic mixture by the "lime-alcohol" method for separating dibasic amino acids [Foreman, 1914].

Further investigations on the nature of the new syrups prepared under certain temperature precautions, and on the possibilities of the "lime-alcohol" method for simplifying the mixture of amino acids before esterification, were carried out in the latter part of 1914. The work was suspended early in 1915 owing to war conditions. The experiments will be described in a further paper, and the results compared with those obtained by Dakin [1918] who has since identified a new amino acid, β -hydroxyglutamic acid, in the syrups which the "lime-alcohol" method enabled him to obtain.

The further investigations showed that on working up the alcoholic filtrate from the insoluble calcium salts, the monobasic amino acids can be obtained in the form of a light brown powder, proving that all the viscous matter can be removed by the "lime-alcohol" method. Impure proline was obtained by extracting the powder with alcohol. The ester process however will probably yield purer proline.

The advantages likely to be gained by removing the alcohol-insoluble calcium salts before applying the new method for preparing esters are given in the following summary:

(1) A single ester preparation will contain the whole of the esters of the monobasic monamino acids, with the possible exception of those of the hydroxy type.

(2) The esters will fractionate more easily, and the difference between "bath" and "vapour" temperatures will be reduced, thus decreasing the risk of decomposition.

(3) Substances of syrupy nature will not be present to complicate the issue at almost every stage. The separations will therefore be more complete, and the amino acids yielded in purer condition.

(4) The residues will be very much simpler in composition, and the amino acids they contain thrown into much greater relief.

COMPOSITION OF CASEINOGEN.

The sum of the percentages of the known amino acids separated from the products of caseinogen by the older methods showed a very considerable deficit. The method for estimating diamino acids gives very satisfactory results, and consequently it is generally believed that the loss falls almost entirely upon the monamino acids during their isolation and estimation. The preparations of esters by the older methods were incomplete, and definite information in regard to the nature of the amino acids making up the de-

iciency could not be gained by examining the residues. The methods of preparing and distilling the esters of Hydrolysis *B* made a better examination of the residues possible. The distillation residue yielded material probably of peptide character (p. 390), indicating that a complete hydrolysis of caseinogen is not effected by boiling for 48 hours with hydrochloric acid, as well as a large amount of syrupy material of unknown constitution. The amount of mon-amino acids in the unesterified residue was very small and consisted of hydroxy acids together with further syrups. Neither of these residues gave any indication that amino acids of the leucine type were present. The syrupy material was afterwards separated directly from the hydrolytic products, together with glutamic and aspartic acids, in the form of alcohol-insoluble calcium salts [Foreman, 1914], and as much as 37·88 % of the caseinogen was accounted for by this method.

Our present knowledge of the quantitative proportions of the products obtained by the acid hydrolysis of caseinogen, including the results of these investigations, may be summarised as shown in the accompanying table. The percentage of lysine was calculated from the figures given by van Slyke [1913] for his estimation of this substance by the Kossel method. The figures for the other amino acids represent the highest reliable results as selected by Osborne and Guest [1911] in their review of the information then available. A total of 67·85 % of caseinogen was accounted for by them in this manner, including the figures for elementary sulphur and phosphorus.

Results of Hydrolysis <i>B</i> (1913)	...	...	Glycine	...	...	...	...	0·45 %
			Alanine	...	...	...	...	1·85
			Valine	...	...	...	...	7·93
			Leucines	...	...	...	...	9·7
			Proline	...	...	...	...	7·63
			Phenylalanine	...	...	...	...	3·88
Results obtained by the method for separating dibasic amino acids [Foreman, 1914]			Glutaminic acid	...	...	...	...	21·77
			Aspartic acid	...	...	...	...	1·77
			New Syrups	...	...	...	...	14·34
Van Slyke	...	...	Lysine	...	...	...	...	7·62
Results selected by Osborne and Guest	...		Histidine	...	...	...	...	2·5
			Arginine	...	...	...	...	3·81
			Tryptophan	...	...	...	...	1·5
			Serine	...	...	...	...	0·5
			Cystine	...	...	...	...	—
			Tyrosine	...	...	...	...	4·5
			Hydroxyproline	...	...	...	...	0·23
			Diaminotrihydroxydodecanic acid	...	...	...	...	0·75
			Ammonia	...	...	...	...	1·61
			Sulphur	...	...	...	...	0·76
			Phosphorus	...	...	...	...	0·85
								93·95
Obtained from Hydrolysis <i>B</i> , probably of peptide nature (p. 390)	...	...						3·41
								97·36

The products include the water of hydrolysis, and the total should be greater than 100. The application of the new ester method after removing the

dibasic material will very probably throw considerable further light upon the composition of caseinogen, and further investigation is proceeding on these lines.

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