LXV. ANALYSIS OF PROTEINS

XI. THE PRODUCTS OF THE ACTION OF SODIUM HYDROXIDE ON CASEINOGEN. THE COMPOSITION OF DEPHOSPHOCASEIN, OR DEPOCASEIN

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(Received 27 February 1939)

PLIMMER & BAYLISS [1906] first showed that the P of caseinogen was completely separated as phosphoric acid by the action of 1 % NaOH at 37° in 24 hr. They also noticed that the solution became very milky in appearance and that acidification precipitated a substance resembling caseinogen. Rimington & Kay [1926] confirmed these and other observations of Plimmer & Bayliss and called the substance thrown down by acetic acid "dephosphorized caseinogen", although it represented only about 40 % of the caseinogen. Rimington [1927] analysed dephosphorized caseinogen by the Van Slyke method and noticed little difference in composition from that of caseinogen except in amide-N. No other work upon this substance has since been published. Meanwhile new and improved methods of analysis of proteins have been devised, and they have now been used in further examination of this substance. Though our results are incomplete, this substance has resemblances with caseinogen, but must be considered different. The term "dephosphorized caseinogen" chosen by Rimington & Kay is not altogether appropriate since caseinogen is converted by 1 % NaOH into this product and a substance or mixture of substances resembling a primary proteose in about equal parts. The latter is a caseose and as it does not contain P is a dephosphocaseose, or shortly depocaseose. The former resembles a meta-protein in solubilities in 0.1 *N* alkali and acid and caseinogen in being precipitated by more acid. It might be called metacasein. This term does not indicate the absence of phosphorus. It is a dephosphometacasein, or for brevity dephosphocasein or depocasein.

It was hoped that chemical examination of the intermediate products of hydrolysis of proteins would show a less complex mixture of amino-acids, so that ultimately it might be possible to fill in the gaps in the amino-acid composition and find out in which order the amino-acids were combined. In the case of caseinogen it appears possible that the whole molecule is made up of two or more very similar portions. Sørensen [1930] adopted the view that caseinogen consisted of many similar molecules of the same solubility.

EXPERIMENTAL

Light white soluble casein. L.W.S. casein

Light white soluble casein supplied by The British Drug Houses, Ltd., has been used for the preparation of depocasein. Dr F. H. Carr kindly informed us that this material is the sodium salt of caseinogen and is prepared by allowing fat-free milk to develop lactic acidity and then precipitating caseinogen by the

addition of very dilute HCl. The caseinogen is washed with water and converted into the sodium salt. Two separate batches supplied at different times gave the following percentage analyses:

Batch	H ₂ O lost at 105°	N	P	S	Ash Na	Corrected for H ₂ O and Na contents		
						N	P	S
1	9.9	12.6	0.72	0.42	3.9 = 1.6	14.2	0.81	0.47
2	10.1	12.5	0.67	0.59	3.5 = 1.4	14.1	0.76	0.66

The computation of the amount of Na in the ash is based upon the figures for P and S as follows:

0.72 % P corresponds with 3.7 % Na ₃ PO ₄	or 3.0 % Na ₄ P ₂ O ₇	or 2.3 % NaPO ₃
containing Na = 1.56 %	or 1.04 %	or 0.52 %
and leaving Na ₂ CO ₃ = 0.2	or 0.9	or 1.56
containing Na = 0.09	or 0.39	or 0.7
Total Na in ash = 1.65	or 1.43	or 1.22
0.42 % S corresponds with 1.78 % Na ₂ SO ₄	containing	0.58 % Na
Remainder of ash as Na ₂ CO ₃ 2.12 %	containing	0.92 % Na
Total Na —	—	1.5 %

If the ash were pure Na₂CO₃ = 3.9 %, it would contain 1.70 % Na. The Na content of the L.W.S. casein is thus about 1.6 %; similarly for batch 2 with 3.5 % ash, the Na is about 1.4 %.

Preparation of depocasein

The conditions for precipitating depocasein from the alkaline digest of caseinogen were not mentioned by Plimmer & Bayliss. Rimington & Kay simply stated that acetic acid was added to give maximum precipitation. In order to determine the optimum amount of acetic acid for precipitation 5 g. of L.W.S. casein were dissolved in water, 62.5 ml. of *N* NaOH added, the solution brought to 250 ml. and placed in an incubator at 37°. After 24 hr. the solution was cooled till it occupied the original volume. 10 ml. were removed to determine the total N, and five lots of 25 ml. were placed in Kjeldahl digestion flasks. Varying volumes of 25 % v./v. (4.32 *N*) acetic acid were added to precipitate the depocasein. The sticky precipitates adhered to the bottom and sides of the flasks. After 24 hr. the contents of each flask were filtered and the precipitates washed with 20 ml. of 0.5 % acetic acid. The filters were returned to their respective flasks and N determinations carried out with the following results:

Total N of the digested solution = 2.49 g./l.

ml. acetic acid added to 25 ml.	4.20	4.30	4.40	4.50	4.60	4.70
N pptd. in g./l.	1.28	1.32	1.36	1.30	1.29	1.28

The maximum yield of depocasein was thus obtained with 4.40 ml. and the precipitate contained $\frac{1.36 \times 100}{2.49} = 55$ % of the total N.

Depocasein was prepared in large quantities by dissolving 40 g. L.W.S. casein by grinding with water, placing in a Winchester bottle, adding 500 ml. *N* NaOH, diluting to 2 l., warming to 37° and maintaining at this temperature for 24 hr. The solution was then transferred to a large beaker and 87 ml. of glacial acetic acid run in from a burette during 5–10 min. with constant stirring. Most of the precipitate adhered to the beaker; the solution was poured off after 24 hr. and subsequent precipitations carried out on top of the first. When sufficient had been collected, usually from four bottles, the precipitates were re-dissolved by grinding with cold *N* NaOH and immediately reprecipitated with dilute H₂SO₄, the acid being added until the precipitate aggregated and the mother liquor gave no further precipitate with acid. The depocasein was filtered, washed with water and dried with successive changes of absolute alcohol and

finally in a vacuum desiccator over H_2SO_4 . The product was a fine white powder and the yield approximately 40 % of the L.W.S. casein taken, some having been lost during purification. Two separate batches of about 250 and 500 g. gave the following percentage analytical figures:

Batch	H_2O	N	P	S	Ash Na	Corrected for H_2O and Na		
						N	P	S
1	19.7	10.90	0.025	0.34	0.7=0.3	13.6	0.03	0.43
2	10.1	11.97	0.030	1.08	0.7=0.3	13.4	0.03	1.20

The S content of batch 2 is erroneous. Analyses of further batches of depocasein by Mr T. J. R. Macara showed 0.42 and 0.45 % S. The low P content compares with the similar figure of Rimington & Kay. The N content is lower than that of caseinogen owing to removal of amide-N by the action of NaOH .

Depocasein dissolves easily in 0.1 *N* NaOH and 0.1 *N* HCl and is precipitated on neutralization and by addition of 2 *N* HCl . The solution in 0.1 *N* alkali made just neutral to incipient precipitation with 0.1 *N* H_2SO_4 gives gelatinous precipitates with silver nitrate, lead acetate, copper sulphate and ferric chloride solutions. With mercuric chloride there was no precipitate till 2 *N* HCl was added. Mercurous nitrate gave a white precipitate which rapidly became black. Calcium chloride, barium chloride and magnesium sulphate solutions did not give precipitates.

The filtrate from depocasein is almost completely precipitated by half saturation with ammonium sulphate, but appears to contain a mixture of proteoses. On evaporating a volume of filtrate containing 15.8 g. N *in vacuo* at 45°, by which some of the acetic acid was removed, a syrup containing sodium acetate remained. The crystals dissolved on diluting with water leaving an insoluble gelatinous mass. The liquid was poured off and the mass washed with water and allowed to dry. It was found to be insoluble in water and alcohol, but soluble in a range of mixtures of water and alcohol. It weighed 28 g. and contained 10.8 % N. The liquid after concentration was fractionated with alcohol. The first fraction weighing 2 g. was insoluble in water. A second fraction of 14.5 g. was thrown out at about 60 % concentration of alcohol and a third fraction of 8.9 g. at over 90 % alcohol. Both the latter were soluble in water and the third fraction was hygroscopic.

Distribution of N

L.W.S. casein, depocasein, its filtrate and the three fractions, also a depocasein made after 144 hr. treatment with 1 % NaOH were examined for distribution of N by Van Slyke's method by the procedure described by Plimmer & Rosedale [1925] using 3 g. (2 g. of fraction 1) of protein in each analysis. The results, the mean of two determinations, are shown in the following table together with the data of Rimington & Kay (R. & K.) for dephosphorized caseinogen:

	L.W.S. casein		Depocasein		Fil- trate	Fractions			144-hr. depo- casein
	Batch 1	Batch 2				1	2	3	
			(R. & K.)						
Amide-N	11.1	11.6	5.3	4.4	1.9	4.4	7.1	5.5	0.2
Humin-N	1.2	2.0	0.9	1.8	3.2	1.6	1.2	1.7	0.1
Arginine-N	8.0	11.6	8.8	6.8	7.4	7.5	6.9	9.0	9.0
Histidine-N	5.1	2.3	8.8	7.0	8.2	10.4	0	4.9	6.3
Lysine-N	7.9	11.9	9.8	13.4	8.8	9.1	13.8	11.0	14.7
Monoamino:									
Amino-N	60.0	56.8	65.0	60.2	49.0	60.7	65.4	67.9	65.8
Non-amino-N	2.5	4.0	0	3.9	14.3	5.7	5.0	0	0.7
Total N recovery	101.8	100.2	98.6	97.5	92.8	99.4	99.4	100.0	96.8
Total N of solid	14.2	14.1	13.6	—	—	10.8	11.4	6.8	—

In the first place our figures do not entirely agree with those of Rimington & Kay. The most noticeable differences are those for lysine-N and the non-amino-N of the monoamino fraction. Our figure is erroneous as proline has actually been isolated (below). The absence or low content of non-amino-N in depocasein is accounted for by its high value in the filtrate, but the fractions isolated did not have such high amounts.

The amide-N of the filtrate is after determination of ammonium salt by distillation *in vacuo* with baryta or sodium carbonate and alcohol and recalculation to a total N of $100 - 19 = 81\%$ of the original total N of the filtrate.

Of the bases, arginine-N is about the same in L.W.S. casein, depocasein and the filtrate. Histidine appears to be greater in depocasein than in L.W.S. casein. The lysine-N, which is dependent on the accuracy of the determination of the other bases, is variable. There is unaccountable variation in the figures of the monoamino fractions.

Determination of arginine, histidine and lysine

Three separate analyses of both L.W.S. casein and depocasein for the amounts of the three basic amino-acids were made by the improved methods of Vickery and his co-workers as described in several separate papers [1926-33]. A final description of the best procedure was not published. The procedure adopted in these analyses was briefly the following.

Hydrolysis. 50 g. of protein were hydrolysed with 240 ml. conc. HCl and 160 ml. water for 30 hr. HCl was partly removed by three evaporations *in vacuo* and then completely by precipitation with Ag_2O and H_2SO_4 at $\text{pH} < 2.8$. The AgCl was extracted with dilute HCl and then with water, and chlorides again removed from the extract. The chloride-free solution and extracts were evaporated, brought to 2 l. and total N estimated in 10 ml. to ascertain the amount of protein present.

Precipitation of histidine and arginine. After concentrating to 1 l. excess of conc. AgNO_3 solution was added, as shown by testing a portion with baryta solution, and hot baryta solution added to $\text{pH} 12$ (alizarin blue S). The silver precipitate of arginine and histidine was filtered off next day, and without washing suspended in water, adjusted to $\text{pH} 2.8$ with H_2SO_4 and decomposed with H_2S . The filtrate and washings from Ag_2S were evaporated *in vacuo*, brought to 500 ml. and N determined on 10 ml.

Histidine. Histidine silver was precipitated by adding Ag_2O and H_2SO_4 alternately until excess of silver was present keeping the reaction below $\text{pH} 2.8$ and then cautiously adding cold baryta solution until the reaction was between $\text{pH} 7.2$ and 7.8 , as shown by the greenish colour of a drop of bromophenol blue on the surface and flocculation of histidine silver. The precipitate was again dissolved and thrown out at a reaction as near as possible to $\text{pH} 7.4$. The histidine silver was filtered off next day and washed once with water. It was suspended in water, made acid with H_2SO_4 and decomposed with H_2S . The solution was evaporated and brought to 250 ml. 5 ml. were used for N estimation and the remainder treated with an equal volume of 10% HgSO_4 in $2N$ H_2SO_4 . The precipitate of histidine mercuric sulphate after washing with 2.5% HgSO_4 in N H_2SO_4 was suspended in water and decomposed with H_2S . The solution and washings were evaporated and brought to 500 ml. N was determined in 5 ml. and the remainder concentrated and treated with a slight excess of flavianic acid (14.96 g. for 1 g. histidine N). Histidine diflavanate crystallized out and a

further crop was obtained from the filtrate. Both crops were recrystallized, the pure crystals dried at 100° and weighed. They had a decomposition point of 235° .

Arginine. The filtrates from the two precipitations of histidine silver were combined, acidified with H_2SO_4 , evaporated *in vacuo*, brought to 1 l. and N determined in 20 ml. The remainder, tested for excess of Ag which was present, was treated with hot baryta solution to pH 12. The precipitate of arginine silver was filtered off, washed twice with baryta solution, suspended in water, acidified with H_2SO_4 and decomposed with H_2S . The solution and washings from Ag_2S were concentrated, brought to 250 ml. and arginine-N estimated in 5 ml. To the remainder, heated to boiling, the requisite amount of flavianic acid (5.61 g. for 1 g. arginine-N) in hot water was added. Arginine monoflavianate crystallized out and a further crop of diflavianate was obtained from the filtrate. The two filtrates at pH 12 were combined, acidified, evaporated to a small volume and precipitation at pH 12 repeated. No further precipitation occurred showing that no arginine had escaped the first precipitation.

Lysine. The filtrates from the two precipitations at pH 12 containing lysine and other amino-acids, also baryta and silver, were acidified with H_2SO_4 , saturated with H_2S and the precipitate removed and washed. The solution was neutralized with NaOH to Congo red and evaporated *in vacuo*. Ammonia was removed by making alkaline to phenolphthalein, adding 2 vol. alcohol and distilling *in vacuo*. After acidifying to pH 2.8, making to 500 ml. and estimating N in 10 ml., the remainder was acidified with H_2SO_4 to 5 % by weight and precipitated with 20 % phosphotungstic acid. The lysine phosphotungstate after 4 days in the dark was filtered off and washed with 2.5 % phosphotungstic acid in 5 % H_2SO_4 , dissolved in a mixture of acetone and water and decomposed with hot baryta solution until the reaction was permanently alkaline to phenolphthalein. The filtrate and washings from Ba phosphotungstate were acidified with H_2SO_4 , filtered from BaSO_4 , concentrated and reprecipitated with phosphotungstic acid as before at a volume of 650 ml. After 24 hr. the lysine phosphotungstate was filtered off and recrystallized from 5 % H_2SO_4 containing 2.5 % phosphotungstic acid. After 4 days the crystalline lysine phosphotungstate was filtered off and decomposed as above with acetone and baryta. Acetone was removed by evaporation and Ba quantitatively with H_2SO_4 . The solution and washings were brought to 250 ml. and N determined in 5 ml. The remainder was concentrated *in vacuo* to a thin syrup, alcohol added till slight precipitation occurred and saturated alcoholic picric acid solution carefully added till lysine picrate was completely thrown down. The precipitate was washed with alcohol and ether, dried in air and weighed.

With allowances made for aliquots removed for N determinations and for solubilities of histidine diflavianate, arginine monoflavianate and lysine picrate the quantities obtained were:

	L.W.S. casein			Depocasein		
	(1)	(2)	(3)	(1)	(2)	(3)
Arginine flavianate	3.20	3.75	3.91	3.20	3.20	3.73
Histidine diflavianate	0.97	2.71	3.46	2.07	3.23	2.66
Lysine picrate	3.84	Lost	4.52	4.49	3.03	3.44

The results of the analyses were thus variable and disappointing. In one case all the bulky inorganic precipitates were examined for N. Small quantities were retained, but extraction with HCl and repetition of the silver precipitations gave no further quantities of the diamino-acids.

The weights of basic amino-acid in the proteins calculated from the highest analytical figure gave the following percentage amounts:

	Depocasein	L.W.S. casein	Pure caseinogen [Vickery & White, 1933]
Arginine	3.32	3.15	3.85
Histidine	1.58	1.53	1.83
Lysine	4.49	4.52	6.25

In comparison with the data of Vickery & White [1933] all the data are lower. Those for lysine are undoubtedly too low. The discrepancy for lysine is attributable to the presence of nitrate in the lysine fractions which had usually to be left over for many days before making lysine phosphotungstate. During this time reduction to nitrite probably occurred with consequent destruction of part of the lysine. In the earlier paper by Vickery & Leavenworth the use of silver nitrate was condemned, but in the later paper by Vickery & Block its use was advised.

The molecular ratios of the amino-acids in the proteins obtained by dividing the percentage figures by the mol. wt. are:

	Histidine	Arginine	Lysine
L.W.S. casein	0.0100	0.0181	0.0272
Depocasein	0.0102	0.0191	0.0300

which work out, more particularly for depocasein, as histidine : arginine : lysine = 1 : 2 : 3.

Depocasein and L.W.S. casein from these analyses thus show no important difference in their contents of basic amino-acids. In comparison with Vickery and White's figure for lysine in pure caseinogen depocasein contains a smaller quantity.

Comparison of these data with those by Van Slyke's method by converting the N figures into weights of protein and amino-acids gives:

	L.W.S. casein		Depocasein	
	Vickery method	Van Slyke method	Vickery method	Van Slyke method
Arginine	3.15	5.08	3.32	3.72
Histidine	1.53	1.25	1.58	4.61
Lysine	3.98	8.75	4.38	6.95

There is little correspondence. Plimmer & Lowndes [1938], who made an examination of the basic amino-acids in the Van Slyke solution, were unable to give an explanation of this discrepancy. In the Van Slyke method there is no attempt at purification of the components in either the mono- or di-amino fractions. Arginine phosphotungstate is definitely not completely precipitated and yet the Van Slyke figure is higher. Monoamino-acids may be thrown down by phosphotungstic acid, particularly proline. The combination of these factors accounts for variability in the Van Slyke figures.

Determination of tyrosine and tryptophan

The method of Folin & Looney [1922] and its modification by Folin & Ciocalteu [1927] have been used for the determination of tyrosine and tryptophan in both L.W.S. casein and depocasein.

(a) *Folin & Looney method.* The results by this method for L.W.S. casein were very variable. Tyrosine values ranged from 1.35 to 3.76 %, and tryptophan values from 1.20 to 1.51 %.

From the consideration that the protein contains three times as much tyrosine as tryptophan, estimations of each amino-acid were made separately. For tyrosine, 2 or 3 ml. of the hydrolysate were precipitated with 2 ml. reagent. Colours comparable with standards of 1 mg. of tyrosine were secured and the results were concordant. For tryptophan, 6 or 8 ml. of hydrolysate were necessary. Complete precipitation of tryptophan mercuric sulphate was not effected with 2 ml. of reagent. Satisfactory results were obtained with 3 ml. reagent, and were not improved with 4 ml. Mercuric sulphate appears to combine with other amino-acids. 2 ml. reagent contain 200 mg. HgSO_4 , a large excess over the amount required to throw down the tryptophan present. This inference is borne out on addition of the Na_2CO_3 solution; with the tyrosine standard there is precipitation of HgCO_3 , with the hydrolysed solution the precipitate was small and sometimes absent. The mercuric amino compounds appear not to be easily decomposed by Na_2CO_3 .

The best results by this modification for L.W.S. casein were:

Tyrosine	7.09 and 7.01 %
Tryptophan	1.77 and 1.76 %

(b) *Folin & Ciocalteu method.* The chief modifications of the Folin-Looney method consist in hydrolysis by means of 20 % NaOH , the use of increased quantities of HgSO_4 and phenol reagents, and the estimation of tyrosine by Millon's reagent. By this method the values for L.W.S. casein were tyrosine 6.7 % and tryptophan 0.36 %. The tyrosine value compares favourably with that by baryta hydrolysis, but the tryptophan value is about 1/5. Hydrolysis by 20 % NaOH seems to produce extensive decomposition of tryptophan. The stability of tryptophan to NaOH hydrolysis was therefore tested. Complete destruction occurred on boiling tryptophan alone, but on boiling with gelatin the destruction amounted to only 35 %.

(c) *Estimation by tryptic hydrolysis.* A solution of L.W.S. casein was submitted to hydrolysis by trypsin in faintly alkaline solution and tryptophan estimated at various times of digestion by the Folin-Ciocalteu procedure. The content of tryptophan was found to be 1.75–1.87 % indicating that baryta hydrolysis gives correct results.

(d) *Combination of the Folin-Looney-Ciocalteu methods.* Determination of tyrosine and tryptophan has been effected by baryta hydrolysis of 1 g. protein, and precipitation of the solution with 4 ml. of HgSO_4 reagent. Tyrosine was estimated both by the Millon reaction and the phenol reagent. Tryptophan was estimated by the phenol reagent and by bromination as described by Plimmer & Lowndes [1938]. The results calculated as percentage of dry mineral-free protein were:

	Tyrosine		Tryptophan	
	Millon reagent	Phenol reagent	Phenol reagent	Bromination
L.W.S. casein batch 1	—	7.09	1.77	—
	—	7.01	1.76	—
batch 2	5.26	5.19	1.21	1.36
Depocasein from batch 2	8.20	7.08	1.24	1.39
	8.05	7.31	1.23	1.39
	8.21	—	—	—
	8.15	7.20	—	—
Mean		7.68		1.31

The two batches of L.W.S. casein gave different values for tyrosine and tryptophan, indicating a difference between them. This may be explained by its

method of preparation which involves production of lactic acidity by bacterial action. Tyrosine and tryptophan may be split off to a different extent for each batch. Further the commercial preparations may contain varying amounts of fat.

Folin & Ciocalteu give 6.41 % tyrosine and 1.4 % tryptophan in pure caseinogen.

Our figures for tyrosine in depocasein are higher by the Millon method than the phenol method. Preference is given to the phenol method, but the mean figure must be taken. Depocasein differs from caseinogen in containing more tyrosine. Tryptophan is the same in both proteins.

Determination of cystine and methionine

These amino-acids were estimated for us by Mr J. Lowndes by the latest method of Baernstein [1936] using the alteration in distillation mentioned by Plimmer & Lowndes [1938]. The results compared with caseinogen were:

	Depocasein	Caseinogen
Cystine	0.288	0.36 %
	0.313 0.30 %	
Methionine	2.077	
	2.110 2.09 %	2.89 %

The cystine contents of caseinogen and depocasein are thus the same, but the methionine content of depocasein is 0.8 % lower.

Determination of glycine

The colorimetric method of Patten [1935] for determination of glycine with *o*-phthalaldehyde was tried with both L.W.S. casein and depocasein. It gave the following results calculated on dry mineral-free protein: L.W.S. casein, 1.0 %; depocasein, 0.2 %. The figure for casein is twice that of Patten. The colour with depocasein was so slight that reproducible readings in the colorimeter were not possible.

The monoamino-acids

The monoamino-acids in depocasein were investigated by the method of Dakin [1918] using 440 g. = 394 g. of dry ash-free protein. After hydrolysis with 21 % HCl for 19 hr., at which period it was found to be complete, as tested by titration with neutralized formalin, the solution was evaporated *in vacuo* several times to remove excess of HCl and neutralized with NaOH. The precipitate of humin and tyrosine was filtered off and 17.9 g. of recrystallized tyrosine with N=7.69 % (calc. 7.73 %) were isolated, equivalent to 4.5 % in depocasein. The concentrated solution was then extracted numerous times by simple shaking with an equal volume of butyl alcohol. This procedure was found by Plimmer & Lowndes (unpublished) to be as effective as a continuous extractor with the advantage that the extracted amino-acids are not heated for a long time with butyl alcohol thus preventing the formation of anhydrides. The butyl alcohol was distilled off *in vacuo* after each extraction and the amino-acids separated off. Shaking with butyl alcohol was continued till only very small amounts were dissolved and at this stage the extract was found to contain small quantities of diamino-acids.

The butyl alcohol extract was examined for proline and simple monoamino-acids. The solid amino-acids were boiled with successive portions of absolute alcohol until extraction was complete. The alcohol was evaporated off and the

residue thoroughly extracted with cold absolute alcohol. The residue from this solution was dissolved in hot water and allowed to cool. The precipitate, probably of peptide anhydrides, contained 1.21 g. N and was not further examined. The solution, clarified with charcoal and brought to 500 ml., contained 4.45 g. total N and 2.89 g. non-amino-N. Two methods were used to isolate derivatives of proline from this solution.

(a) *Proline picrate by Town's method* [1928]. 100 ml. were boiled with CuCO_3 . The dried copper salts were extracted with methyl alcohol and gave 8.8 g. material, from which a solution was obtained containing 0.53 g. of total N and 0.36 g. of non-amino-N. The calculated amount of picric acid was added to 50 ml. of this solution and ultimately 1.4 g. of crystals with m.p. of 146–148°, not altered by recrystallization, resulted. This yield gives 1.14 % of proline in depocasein.

(b) *Proline hydantoin by Dakin's method* [1918]. 100 ml. of the solution were evaporated with 8 g. of KCNO (5.2 g. theoretical). The sticky residue after treatment with H_2SO_4 and ether finally yielded 1.13 g. of opaque white crystals with m.p. 156–158°. Dakin gave 165–167° as m.p. of *l*-proline hydantoin and 153–156° for the racemic compound. The crystals contained 22.2 % N (calc. 20.0 %). The high figure may be due to the presence of some polymerized cyanic acid. The yield corresponds to 1.18 % in depocasein.

Though only 1.2 % proline was isolated the content in depocasein is undoubtedly greater. The solution from the copper salt after boiling with an equal volume of conc. HCl contained 0.0675 g. non-amino-N which is equivalent to 2.8 %. Caseinogen according to Dakin contains 8 % proline.

The separation of the other monoamino-acids insoluble in absolute alcohol was carried out by the ester method. The ester hydrochlorides dissolved in absolute alcohol were treated with the calculated amount of Na in alcohol and NaCl was precipitated with ether. The solution after being shaken with, and filtered through, dry Na_2SO_4 was evaporated *in vacuo*, the alcoholic distillate being collected in dilute HCl to trap low-boiling esters. The esters were then fractionally distilled *in vacuo* using an oil bath and a three-stage mercury diffusion pump, kindly placed at our disposal by Dr Barrow at Birkbeck College. Between the receiver and the pump a trap was inserted immersed in a mixture of solid CO_2 and alcohol to condense vapours escaping the condenser. The following fractions were obtained:

M_1 from alcohol distillate of esters.

M_2 at 58–90° oil bath temp. 60° vapour T . 3.0 mm. Hg pressure.

M_3 from trap in freezing mixture.

M_4 at 90–100° oil bath T . 65° vapour T . 1.5 mm. Hg pressure.

M_5 at 100–120° oil bath T . 0.7 mm. Hg pressure.

No distillate 120–140° oil bath T .

M_6 at 140–180° oil bath T .

M_7 from trap in freezing mixture.

Fraction M_1 was evaporated to dryness and the residue dissolved in water, neutralized and fractionally crystallized. 3.4 g. of crystals with 11.5 % N were obtained and treated by the copper salt method (below). The remainder of the residue was NaCl .

Fractions M_2 , M_3 , M_4 , M_5 were separately boiled with water under a reflux for 15 hr. Except M_5 , which was slightly alkaline and was previously extracted with ether to remove any phenylalanine or serine ester, the solutions were neutral. The amino-acids in these fractions were submitted to extensive frac-

tional crystallization from water and alcohol. Leucine was obtained from each fraction. The final portion had a N content of 10.65 % (calc. 10.69 %). When no further separation of leucine could be obtained, the remainders and fraction M_1 were mixed, dissolved in water and converted into copper salts as described by Brazier [1930]. The copper salts insoluble in cold water gave leucine with 10.64 % N; those soluble in water were separated into copper salts soluble and insoluble in methyl alcohol. Both fractions were found to contain only valine with 11.80 % N (calc. 11.97 %) and leucine with 10.60 % N. The total pure leucine isolated amounted to 38.5 g. The content of leucine in depocasein would thus be 9.8 %. The valine, amounting to 7.9 g., had a rotation of $[\alpha]_D = 20^\circ$ and would consist of 25 % valine, equal to 0.3 % in depocasein. The remainder corresponds to 1.7 % leucine, making leucine at least 11.5 % of the protein.

Fractions M_6 and M_7 were combined, mixed with water and extracted with ether. The ether-soluble part of M_5 was added. The ether extracts were shaken with conc. HCl and gave on hydrolysis, evaporation and crystallization 2.8 g. of phenylalanine hydrochloride with 17.8 % Cl (calc. 17.6 %). 2.1 g. of phenylalanine were prepared from it by solution in water, neutralization with ammonia and treatment with alcohol. The crystals contained 8.92 % N (calc. 8.48 % N). The high value is probably due to the presence of a little NH_4Cl . The yield is equivalent to 0.6 % in depocasein. The aqueous part was hydrolysed with baryta and examined for dicarboxylic acids and serine. Derivatives of these acids were not isolated, though there was some barium salt insoluble in alcohol and some very soluble amino-acid. Alanine was not found in any of the first fractions of esters. From the large amount of N remaining unextracted by butyl alcohol it is probable that it remained in the aqueous solution.

The residual aqueous solution, after removal of butyl alcohol by evaporation *in vacuo*, was brought to 1 l. and contained 29 g. N. An analysis by Van Slyke's method on 15 ml. without further hydrolysis, gave the following distribution of N:

	% N	Calc. to original depocasein
Amide	4.3	—
Humin	0.7	—
Total N of bases	35.2	19.4
Arginine	14.1	7.8
Histidine	0.3	0.2
Lysine	20.8	11.4
Total monoamino	56.3	31.0
Amino	50.7	28.0
Non-amino	5.6	3.0

The second column, obtained by multiplying the first by 29.0/52.7, shows the distribution as a fraction of the total N of the depocasein taken for hydrolysis. Comparison with the figures for depocasein shows no great difference in arginine; histidine has been reduced and lysine is slightly higher. It is possible that histidine, which is less basic than the two other bases, has been dissolved by butyl alcohol. The monoamino filtrate contains nearly half the total monoamino-acids of the depocasein despite the numerous extractions with butyl alcohol until only very small amounts were removed. Dakin [1920] found that the extraction of the monoamino-acids from gelatin was by no means complete, also observed by Plimmer & Lowndes (unpublished), whether the extraction be made with a continuous extractor or by frequent shaking with fresh portions of butyl alcohol. Dakin's description of the extraction of the amino-acids from hydrolysed caseinogen leads to the conclusion that it is complete. The non-amino-N of the monoamino solution amounting to 3 % on the original depocasein may be due to

the presence of peptide anhydrides or hydroxyproline. Proline was extracted by the butyl alcohol.

The solution should contain only the basic amino-acids and the dicarboxylic acids. It was used only for isolation of the latter. Two methods for the isolation of glutamic and aspartic acids were tried.

(1) *Glutamic acid as hydrochloride*. 500 ml. of the aqueous solution were evaporated and saturated with dry HCl gas. The crystalline precipitate consisted almost entirely of NaCl. It was not possible to obtain the insoluble zinc glutamate. The solution of the precipitate contained only 0.08 g. N.

(2) *Barium aspartate and barium glutamate*. 150 ml. of the solution were treated with cryst. $\text{Ba}(\text{OH})_2$ and precipitated with alcohol according to Jones & Moeller [1928]. From the insoluble barium salts the insoluble lead salt of aspartic acid was made. The solution of the lead salt, freed from lead, contained N equivalent to 0.14 M aspartic acid. The preparation of the copper salt by adding 1.5 M copper acetate was not successful though the solution contained 0.157 g. N equivalent to 1.4 g. aspartic acid or 2.5 % of the depocasein.

The filtrate from the insoluble lead aspartate would not yield glutamic acid hydrochloride. Hydroxyglutamic acid was also not found. It is not possible to explain the non-isolation of these dicarboxylic acids, especially glutamic acid.

Further examination for dicarboxylic acids

As these acids could not be isolated from the solution remaining after butyl alcohol extraction a further hydrolysis of depocasein with HCl was carried out with approx. 40 g. material. The hydrolysed solution was evaporated *in vacuo* and found to contain 4.5 g. N = 33.8 g. depocasein.

The basic amino-acids were removed from the solution by precipitation with a slight excess of phosphotungstic acid. The filtrate and washings were evaporated *in vacuo* and the concentrated solution saturated with dry HCl gas. The precipitate, which contained glutamic acid hydrochloride and inorganic matter was first recrystallized as hydrochloride; Cl was removed with Ag_2SO_4 , SO_4 and NH_4 ions with $\text{Ba}(\text{OH})_2$ and after quantitatively removing Ba ions there were isolated 1.2 g. glutamic acid with 9.4 % N (calc. 9.5 % N) which gives a content of 3.6 % in depocasein.

The filtrate from glutamic acid hydrochloride was evaporated *in vacuo* to remove HCl and the residue dissolved in water and treated with ammonium rhodanilate according to Bergmann [1935]. From the rhodanilate 0.4 g. of nearly pure proline with 11.8 % N (calc. 12.2 % N) was isolated which corresponds with 1.2 % in depocasein, comparable with the figure obtained from the butyl alcohol extract. The quantity in depocasein is, however, much larger as the whole of the proline was not crystallized out. The filtrate from proline rhodanilate was treated with pyridine, and the solution precipitated with ammonium reineckate. The reineckate did not give any hydroxyproline in a crystalline state.

Excess reineckate was removed from the solution by means of pyridine, made alkaline with NH_4OH and BaCl_2 added till there was no further precipitation. The filtrate on evaporation *in vacuo* gave 2.0 and then 0.4 g. impure tyrosine with 7.42 % N. It was then treated with solid $\text{Ba}(\text{OH})_2$ according to Jones & Moeller [1928] and poured into 5 vol. of 95 % alcohol. The insoluble Ba salts were filtered off, washed, redissolved and reprecipitated with alcohol. From the insoluble Ba salts there were ultimately obtained 3.4 g. copper aspartate, from which 1.0 g. of pure aspartic acid with 10.25 % N (calc. 10.5 % N) equivalent to 3.0 % in depocasein was isolated. Glutamic acid could not be found in the insoluble Ba salts. The alcoholic filtrates from the insoluble Ba salts were

evaporated *in vacuo*, acidified with H_2SO_4 , filtered from BaSO_4 , Cl being removed with Ag_2SO_4 , Ag with H_2S and finally SO_4 quantitatively with $\text{Ba}(\text{OH})_2$. On evaporation the solution gave 0.4 g. tyrosine. Together with the previous amounts the total tyrosine obtained by crystallization was 2.8 g. Pure tyrosine weighing 1.8 g. with 7.71 % N (calc. 7.73 % N) was prepared from this by recrystallization. The yield corresponds with 5.4 % in depocasein. Further evaporation gave firstly 2.7 g. impure leucine from which 2.1 g. with 10.6 % N was obtained on recrystallization equivalent to 6.3 % of depocasein, and then fractions with 11.2, 12.0, 11.35 % N and finally a fraction of 0.05 g. with 12.84 % N, which indicated that alanine or other lower amino-acids were present in depocasein. The experiment, essentially for the isolation of the dicarboxylic acids, was also a preliminary trial to isolate amino-acids in sequence from the same hydrolysate.

SUMMARY

The substance produced from caseinogen by treatment with 1 % NaOH at 37° for 24 hr. and subsequent acidification is not simply a residue of caseinogen from which only phosphorus and amide groups have been removed, but a product containing 55 % of the original N of the protein. The caseinogen is apparently split into two almost equal halves. This half is preferably called depocasein, as it resembles a metaprotein in solubility in 0.1N acid and alkali, but is precipitated by a higher concentration of acid. Its composition in amino-acids is generally very similar to that of caseinogen, but there are a few marked differences:

	Depocasein	Caseinogen
Glycine	0.2	0.5
Alanine	?	1.5
Valine	0.3	7.2
Leucine	11.5	9.4
Phenylalanine	0.6	3.2
Aspartic acid	3.0	4.1
Glutamic acid	3.6	21.0
Proline	1.5	8.0
Tyrosine	7.7	6.4
Tryptophan	1.3	1.4
Cystine	0.3	0.4
Methionine	2.1	2.9
Histidine	1.6	1.8
Arginine	3.3	3.9
Lysine	4.5	6.3

The differences from caseinogen are in tyrosine which is over 1 % higher, methionine which is 0.8 % lower and lysine which is lower; the figure of 4.5 % is, however, too low. Proline is lower, but is probably at least 3 %. There is a difference in aspartic acid but our figure is probably too low. The most striking difference is in glutamic acid. Further analyses of depocasein are contemplated. Depocasein is under investigation.

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