171. ANALYSIS OF PROTEINS

12. DEPHOSPHOCASEOSE OR DEPOCASEOSE

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The phosphorus of caseinogen is completely separated by the action of 1% NaOH at 37° in 24 hr. [Plimmer & Bayliss, 1906; Rimington & Kay, 1926]. Acidification of the solution precipitates a product called dephosphorized casein by Rimington & Kay [1926; Rimington, 1927] and dephosphocasein or depocasein by Plimmer & Lawton [1939], who made various analyses of its component amino-acids. Dephosphocasein represents about 60% by weight of the caseinogen (55% of the N). The remainder has now been found to consist mainly of primary proteose, dephosphocaseose or depocaseose with much smaller amounts of secondary proteose and peptones. This communication deals with the composition of depocaseose with some further analyses of depocasein.

Experimental

Distribution of the nitrogen after 24 hr. alkaline action

Hammarsten casein has been used in these experiments.

In preparing depocaseose it was deemed essential to avoid the presence of sodium acetate in the solution. Repetition of the conditions for maximal precipitation of depocasein by acetic acid showed that this occurred at *p*H 4·2. The precipitation is effected with HCl, using as indicator bromophenol blue which is just blue at this *p*H. It was first desired to study the nitrogen distribution of the products.

10 g. casein (8·93 g. dry protein) were dissolved and brought to 502 ml. with 1% NaOH (*N*/4). 2 ml. contained 5·23 mg. N. The remaining 500 ml. (1·32 g. N) were kept at 37° for 24 hr. and air was drawn through the solution to remove NH_3 originating from amide-N. To the solution, brought to 535 ml. (1·284 g. N) conc. HCl was carefully added with constant stirring to *p*H 4·2. Next day the depocasein was filtered off and washed with water. The filtrate and washings were brought to 890 ml. (0·61 g. N), warmed and saturated with recrystallized $MgSO_4,7H_2O$ (60 g. per 100 ml.) and kept overnight. The precipitate of primary proteose, depocaseose, was filtered off and the filtrate, made up to 1375 ml. (0·178 g. N), was saturated with crystals of $ZnSO_4$ with slight warming. After standing, the small precipitate of secondary proteose was filtered off. The filtrate (1370 ml., 0·145 g. N) was treated with 50 ml. of 20% phosphotungstic acid in *N*/2 HCl. The white flocculent precipitate of peptone was filtered off; the residual nitrogen in the solution was 0·027 g. In a second determination the depocaseose was thrown out by half-saturation with $ZnSO_4$. The results of these two experiments

with the N removed at each precipitation calculated as percentage of the total N taken are as follows:

	Exp. I		Exp. II		Mean
	g.	%	g.	%	%
Total N	1·320	—	1·330	—	—
NH_3-N	0·036	2·9	0·070	5·2	4·0
Depocasein-N	0·674	52·0	0·704	53·4	52·8
Depocaseose-N	0·433	33·0	0·403	31·2	32·1
Secondary proteose-N	0·033	2·5	0·051	4·7	3·6
Peptone-N	0·118	8·3	0·085	6·5	7·4
Residual N (amino-acids or reagents)	0·027	2·0	0·017	1·3	1·7

The action of 1 % NaOH on caseinogen at 37° thus yields two chief products, depocasein and depocaseose. Only 1/10 of the original protein-N consists of secondary proteose and peptone, mainly the latter.

Prepration of depocasein and depocaseose

Several smaller scale experiments were made to investigate the isolation of depocaseose free from contaminating salt and in a granular form. Half-saturation of the filtrate from depocasein with $(NH_4)_2SO_4$ was preferred on account of its easier removal by means of $Ba(OH)_2$; $BaSO_4$ was filtered off and NH_3 removed by evaporation *in vacuo*. Excess Ba ions were removed exactly with H_2SO_4. As first precipitated depocaseose dissolves slowly, yet completely, in water. It is insoluble in absolute alcohol, but about 60 % of the material dissolves in 75 % alcohol. When a concentrated solution is poured into 10 vol. of absolute alcohol a large proportion is thrown out in granular form which can be filtered off and dried by successive changes of absolute alcohol.

Large scale preparations were carried out with several batches of 40 g. casein which were placed in Winchester bottles with 250 ml. of 2*N* NaOH and diluted to 2 litres with water. On shaking for a short time the casein dissolved. After warming to 37° the bottles were put in an incubator for 24 hr. The alkaline solutions were transferred to beakers and conc. HCl slowly run in with vigorous stirring until a precipitate just appeared; the depocasein was completely thrown out by adding small quantities of *N* HCl to *p*H 4·2. The precipitate, at first white and flocculent, agglutinated and adhered to the beaker. Next day the combined precipitates from 12 or 16 portions were filtered off, redissolved in *N* NaOH and reprecipitated at *p*H 4·2 by cautious addition of *N* HCl. The precipitate was washed with water and dried with successive changes of absolute alcohol and finally *in vacuo* over H_2SO_4. The combined filtrate and washings (about 30 litres) were concentrated *in vacuo* to about 1/3 vol., at which concentration frothing became troublesome, and the solution was half-saturated with the required amount of $(NH_4)_2SO_4$ crystals (400 g. per litre) and kept overnight. The depocaseose first settled out as a white flocculent precipitate, but on standing became a crusty mass slightly brown in colour. It was filtered off, redissolved in the minimum of water and reprecipitated by half-saturation with $(NH_4)_2SO_4$. The filtration of the ammonium sulphate solutions was always slow.

The impure precipitate of depocaseose was dissolved in water and $Ba(OH)_2$ solution was added with thorough stirring until the solution was distinctly alkaline. The precipitate of $BaSO_4$ was filtered off and thoroughly washed with hot water. The combined filtrate and washings were evaporated *in vacuo* to remove ammonia and the excess Ba ions removed quantitatively with H_2SO_4. The filtrate and washings were concentrated *in vacuo* to a thick syrup (about

200 ml.) and poured with thorough stirring into at least 10 vol. of absolute alcohol. The depocaseose, thus thrown out in granular form, was ground up and dried with successive changes of absolute alcohol. A further quantity was obtained by evaporating the alcoholic liquors, pouring into absolute alcohol and drying as above. The combined crops were finally dried *in vacuo* over H_2SO_4 and consisted of a fine almost white powder. The yields of material were:

	Batch I g.	Batch II g.
Casein taken	480	640
Depocasein	170 = 35 %	280 = 44 %
Depocaseose	90 = 19 %	100 = 16 %

The rather low yields of depocaseose are due to loss in removing $(NH_4)_2SO_4$ and incomplete precipitation by alcohol.

The analyses of these preparations compared with the casein, all dried at 105°, were:

	N %	P %	S %	Ash %
Casein	15·08	0·82	0·63	1·10
Depocasein batch I	13·84	0·03	0·45	0·49
Depocasein batch II	13·78	0·03	0·42	0·35
Cf. Plimmer & Lawton [1939]	13·50	0·03	0·43	0·70
Depocaseose batch I	14·46	0·04	0·69	0·81
Depocaseose batch II	14·57	0·02	0·67	0·98

Depocaseose has the same low P content as depocasein, but slightly higher N and S contents. Both depocasein and depocaseose have lower N contents than caseinogen owing to removal of amide-N by the action of alkali.

Determination of arginine, histidine and lysine

The basic amino-acids in depocasein and depocaseose and casein for comparison were determined by the micro-method of Block [1934] with the modifications proposed by Plimmer & Lowndes [1938] and by Block [1938] using 2·5 g. protein. The main alteration was in the use of silver sulphate added as Ag_2O and H_2SO_4 to avoid loss of lysine. The introduction of excess Ag ion in this way was not altogether satisfactory as the operation was lengthy and a positive test for excess Ag ion was not always obtainable. On this account the results for histidine may be low. The use of limited amounts of $AgNO_3$ [Tristram, 1939] is probably preferable. These analyses were completed before the publication of his thorough and critical review of the method. The finding by Ayre [1938] that ammonium phosphotungstate is not decomposed by dilute H_2SO_4 in the presence of amyl alcohol-ether, rendering the removal of NH_3 with NaOH unnecessary, was not followed. The isolation of arginine flavianate always gave between 70 and 80 % of the yield expected from the N value of the solution, though the salt crystallized readily from the hot solution. Histidine diflavianate was also too low in amount partly on account of the small quantity present and partly from solubility loss in washing free from excess flavianic acid. The sulphur contents of both arginine and histidine flavianates were higher than the theoretical values, which agrees with the observations of Tristram and others. The use of nitranilic acid for isolation of histidine as suggested by Block and proved quantitative [1940] should be an improvement of the method. The isolation of lysine picrate proved the most difficult, probably owing to the formation of some more soluble dipicrate. In agreement with the findings of other workers we found that lysine picrate gives a decomposition point with slight explosion only after recrystallization. A new indicator "Sofnol Purple" which changes from green to blue at

*p*H 7·4 and from blue to purple at *p*H 9 was used in later analyses. As it does not contain N it can be added to the solutions if required. Solubility corrections of the salts were not applied to the results. They have been studied very fully by Tristram with simple mixtures of the three amino-acids, but their application to protein hydrolysates should be accepted with reserve. The data of our analyses were:

Amino-acid: % of dry protein

	Arginine		Histidine		Lysine	
	By N	From flavianate	By N	From flavianate	By N	From picrate
Depocaseose	3·53	2·34	0·73	—	8·65	4·91
	3·02	2·01	0·99	0·90	7·33	5·30
	3·44	2·70	—	—	7·44	—
	2·94	2·22	0·88	0·76	(9·45)	6·10
Mean	3·23	—	0·87	—	7·81	—
Depocasein	4·87	3·23	0·90	0·71	5·76	2·34
	4·31	3·17	—	—	5·43	4·00
	4·66	2·56	1·05	0·62	5·86	—
Mean	4·61	—	0·98	—	5·70	—
Cf. Plimmer & Lawton	—	3·32	—	1·58	—	4·49
Casein	3·3	2·1	1·33	1·05	7·0	6·2
Cf. Vickery & White [1933]	—	3·85	—	1·83	—	6·25

The figures for depocasein differ considerably from those of Plimmer & Lawton, who used the macro-method of Vickery & Leavenworth [1926–8]. The differences are explicable in part by the actual *p*H taken in precipitating histidine silver, the shade of the blue-green of the bromophenol blue being easily capable of considerable variation in interpretation by different eyes. Lysine picrate in small quantities is difficult to precipitate quantitatively. We regard the figures by N determination as more accurate than those by isolation.

On the present figures depocaseose contains less arginine and more lysine than casein, whereas depocasein has more arginine and less lysine. Histidine is less in both than in the original protein.

Expressed in terms of N as % of total N the results are:

% of total N

	Arginine-N	Histidine-N	Lysine-N
Depocaseose	7·2	1·62	10·6
Depocasein	10·7	1·95	8·2
Cf. Plimmer & Lawton	7·9	3·1	6·5
Casein	7·0	2·4	9·1
Cf. Vickery & White	8·2	3·2	7·7

The molecular ratios of the amino-acids calculated by dividing the percentage figures by the mol. wt. and taking the nearest probable whole number are:

	Histidine	Arginine	Lysine	Histidine	Arginine	Lysine
Depocaseose	0·0056	0·0185	0·0535	1	3	9
Depocasein	0·0063	0·0265	0·0390	1	4	6
Cf. Plimmer & Lawton	0·0102	0·0191	0·0310	1	2	3
Casein	0·0086	0·0190	0·0480	1	2	5
Cf. Vickery & White	0·0117	0·0221	0·0430	1	2	4

The ratios for casein agree nearly with those from Vickery & White's analyses. For depocasein the ratios from our analyses agree with those by Plimmer & Lawton for arginine and lysine, but not for histidine.

Determination of cystine and methionine

These amino-acids were determined by the method of Baernstein [1936] with the alteration of Plimmer & Lowndes [1937]. The alterations suggested by Kassell & Brand [1938] were considered unnecessary as their results were the same in the case of casein as those of Plimmer & Lowndes. The results of our analyses of casein and depocaseose compared with those of depocasein and cow's caseinogen were:

		Casein %	Depocaseose %	Depocasein %	Cow's caseinogen %
Cystine		0·27	0·23	—	—
		0·31	0·25	—	—
	Mean	0·29	0·24	0·30	0·36
Methionine		2·59	2·84	—	—
		2·54	2·74	—	—
	Mean	2·57	2·80	2·09	2·89
S as H_2S etc.		0·04	0·14	—	—
		0·05	0·13	—	—
	Mean	0·04	0·13	—	—

If the percentage of S in the protein be calculated from these data (cystine, 26·7 % S; methionine, 21·5 % S) the total S is slightly higher than that determined directly by Pirie's method [1932]:

	Casein	Depocaseose	Depocasein	Cow's caseinogen
Cystine-S	0·077	0·064	0·080	0·096
Methionine-S	0·553	0·602	0·449	0·621
H_2S-S	0·040	0·130	—	—
Total	0·670	0·796	0·529	0·717
Total S direct	0·63	0·69	0·45	0·65

The small differences are probably within the range of experimental error magnified by the calculations involved. They do not justify the small factor corrections of Kassell & Brand which would increase the differences still further.

Expressed in terms of amino-acid-N as percentage of total N the data are:

	Casein	Depocaseose	Depocasein	Cow's caseinogen
Cystine-N	0·224	0·193	0·24	0·28
Methionine-N	1·60	1·81	1·35	1·78

On calculating the molecular ratios of $\frac{\text{methionine}}{\text{cystine}}$ the differences in the proteins are striking:

Casein	Depocaseose	Depocasein	Cow's caseinogen
14·3	18·8	11·2	12·9

For every molecule of cystine there are 14 molecules of methionine in casein, 13 in cow's caseinogen, 19 in depocaseose and 11 in depocasein.

Determination of tyrosine and tryptophan

The combination of the colorimetric methods of Folin & Looney [1922] and Folin & Ciocalteu [1927] as recommended by Plimmer & Lawton [1939] was used to determine tyrosine and tryptophan. Tryptophan was also determined by the bromination method of Plimmer & Lowndes [1938]. The determinations were

almost completed before the appearance of Lugg's [1938] method of hydrolysis with alkaline stannite. The results of these determinations were:

	Tyrosine			Tryptophan		
	By Millon reagent %	By phenol reagent %	Mean %	By bromination %	By phenol* reagent %	Mean %
Casein	5·95	5·16	—	1·44	1·24	—
	5·74	—	5·62	—	—	1·34
Depocasein	8·28	7·75	—	1·21	1·12	—
Batch II	8·33	—	8·12	—	—	1·17
Cf. Plimmer & Lawton	8·15	7·20	7·68	1·39	1·24	1·31
Depocaseose	3·01	3·67	—	1·01	0·92	—
Batch I	3·07	3·21	3·24	—	—	0·97
Batch II	3·41	3·75	3·57	0·99	0·84	0·92

* Mean of determinations by HgS and NaCN methods.

The Millon reagent gave slightly higher results than the phenol reagent for casein and depocasein, but the reverse was the case with depocaseose. As previously observed the bromination method for tryptophan gives higher results than the phenol reagent, probably owing to precipitation of traces of histidine. It is noticeable that the higher tyrosine content of depocasein than of casein is compensated by the lower content in depocaseose. Tryptophan is slightly lower in depocaseose.

The data are very similar if the results be expressed in terms of tyrosine-N:

N as % of total protein-N

	Casein	Depocasein	Depocaseose (batch II)
Tyrosine-N	2·88	4·55	1·90
Tryptophan-N	1·22	1·20	0·90

Determination of phenylalanine

The colorimetric method of Kapeller-Adler [1932] and its modification by Block [1938], in which phenylalanine is nitrated to 3:4-dinitrobenzoic acid and the reduction of this compound to di-aci-dihydro-dinitrobenzoic acid which has a deep purple colour, was not successful with our solutions of protein hydrolysates. Dark brown or dark-coloured solutions were always obtained which were impossible to match in a Duboscq colorimeter with standard phenylalanine solutions which gave a typical purple colour. The method is evidently satisfactory with solutions containing fewer amino-acids obtained during fractionation of the amino-acids as in the experiments by Baptist & Robson [1940]. Arnaud [1939] does not give details of the modification by which he determined the phenylalanine content of hen's egg albumin.

Determination of glycine

Patten's colorimetric method [1935] for estimation of glycine with *o*-phthalic dialdehyde, which had proved unsatisfactory in the trials by Plimmer & Lawton was not again attempted. Town's semi-micro-method [1936] of precipitating with nitranilic acid, reported as specific in the absence of NH_4, Na, K, Ca and Ba ions, was used.

Numerous experiments were made in which pale yellow precipitates, not always crystalline, presumably of glycine nitranilate, slowly settled out. After

washing with alcohol, drying *in vacuo* over H_2SO_4 and weighing, their purity was examined by dissolving in water (in several experiments a portion remained insoluble) and estimating the nitranilic acid by precipitation with $BaCl_2$ and the N (glycine) in the filtrate. The results indicated that the precipitate was a mixture. The method tested on pure glycine, using 11–20 mg., gave a recovery of only 64–80 %. With larger amounts of glycine such as are contained in gelatin and other proteins the recovery would be greater. Our figures gave 2·1 and 2·5 % of glycine in casein and 2·1–2·6 % in depocaseose, values which are considerably higher than those of 0·5 % [Patten, 1935] and 0·45 % [Foreman, 1919] and 1 % [Plimmer & Lawton, 1906] for casein. With the small quantities of glycine nitranilate obtained from casein and depocaseose it was not possible to make 3:5-dinitrobenzoyl derivatives as was done with gelatin hydrolysates by Town, who obtained a crude yield of 82 % and a yield of 27 % of pure substance. Town gave no proof that *only* glycine was precipitated by nitranilic acid.

Our investigations were resumed later after Block [1940] had shown that histidine nitranilate was quantitatively precipitated. Our high figures would thus be explained. It may also be noted that histidine nitranilate contains 59·8 % nitranilic acid compared with 60·5 % in glycine nitranilate, values so close that the nitranilic acid content of the precipitate does not indicate whether glycine or histidine or both are present.

New estimations were carried out in which NH_4 ions and basic amino-acids were removed by precipitation with phosphotungstic acid. 2 g. protein were hydrolysed by boiling with 40 ml. of 20 % HCl for 24 hr. The solution was evaporated several times *in vacuo* to remove excess HCl. The syrup was dissolved in water, heated nearly to boiling and about 20 ml. of 40 % phosphotungstic acid in $N/2$ HCl were added with stirring till precipitation was complete. Next day the precipitate was filtered off and washed with $N/2$ HCl. The combined filtrate and washings were shaken twice with 20 ml. amyl alcohol-ether mixture to remove excess phosphotungstic acid and the clear aqueous solution evaporated *in vacuo* and brought to 25 ml. 10 ml. portions were evaporated to smaller bulk and transferred to centrifuge tubes so that the volume was 3–4 ml., and absolute alcohol added to make 80 %. Any precipitate which formed was filtered off and 30 mg. or known excess of nitranilic acid dissolved in a few ml. absolute alcohol were added. The pale yellow precipitate of glycine nitranilate slowly formed and settled out. It was centrifuged, washed and analysed for nitranilic acid as barium salt. The results were:

	Glycine nitranilate		Glycine (mg.)		% glycine in protein		
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	Mean
Casein 1·788 g.	10·0	7·0	2·5	2·0	0·35	0·28	0·32
Depocasein 1·80 g.	3·6	6·1	1·2	2·0	0·17	0·27	0·22
Depocaseose 1·80 g.	8·0	5·2	2·0	1·3	0·28	0·19	0·24

The value for glycine was calculated from the Ba nitranilate precipitate; in each case the nitrogen of the filtrate (glycine) from barium nitranilate corresponded.

The values thus obtained are lower than the accepted value for casein. As shown above the recovery of glycine with such small quantities is between 60 and 80 %. The true values of glycine in the three proteins are probably between 0·5 and 0·6 %. More satisfactory results would be obtained by using larger quantities of protein.

Determination of glutamic acid by enzyme micro-method

Cohen [1939] introduced a method of determining glutamic acid consisting in its oxidation with chloramine-T to cyanopropionic acid which is then hydrolysed to succinic acid and extracted from the solution with ether. The succinic acid is dissolved in phosphate buffer and estimated by determination of oxygen uptake under the action of succinic dehydrogenase in a Warburg apparatus. The method is specific for glutamic acid and its derivatives, glutamine and glutathione. The adaptation of the method to the estimation of glutamic acid in protein hydrolysates was not actually tried by Cohen. Dr Cohen was interested in this development of his method, kindly carried out an analysis and gave a demonstration.

Before carrying out estimations of glutamic acid in protein hydrolysates it seemed advisable to make experiments with glutamic acid, under the conditions used in the actual analyses:

Glutamic acid			
Taken mg.	Found mg.	% recovery	Time of ether extraction
20	13·0	65	3 hr.
12	8·9	75	5 ,,
16	12·4	78	5 ,,
20	18·0	90	5 ,,
16	13·3	83	5 ,,
16	12·6	80	5 ,,
20	15·9	80	Overnight
24	20·6	85	Overnight

The recovery was much less than the 95 % recorded by Cohen, who regarded the loss as largely due to over-oxidation. In these experiments a larger quantity of chloramine-T was used: 4 ml. of 20 % solution as compared with 2 ml. of 10 % by Cohen. The higher quantity here taken was necessary to allow for oxidation of the other amino-acids in the protein hydrolysates. According to Dakin [1917] the molar concentration of chloramine-T should be at least twice that of glutamic acid. In the case of the hydrolysates the loss due to over-oxidation would be much reduced or eliminated as may be noticed from the comparative results from isolation (below).

10 g. of each protein were hydrolysed by boiling with 20 % HCl for 24 hr. The HCl was partially removed from the solutions by evaporation *in vacuo* and completely by treatment with Ag_2O and H_2SO_4. Ag was removed with H_2S and the latter by evaporation *in vacuo*. The acid solutions were made up to 250 ml. and aliquots taken for N estimation and acidity.

Volumes (4–5 ml.) of the solutions (=150–200 mg. protein) containing 20–25 mg. glutamic acid were placed in small conical flasks, brought to *p*H 4–5 with approx. 0·5 ml. dil. NaOH and 3 ml. citrate buffer added which brought the *p*H to 4·7; 4 ml. of 20 % chloramine-T solution were added drop by drop with thorough shaking (a precipitate of *p*-toluenesulphonamide appeared) and the flasks placed in a rack and shaken at 40° for 10 min. The flasks were then placed in an ice bath for 20 min. so as to precipitate most of the *p*-toluenesulphonamide or excess reagent. The solutions were filtered and the precipitates washed with several small volumes of water and the combined filtrate and washings, 25–30 ml., collected in large boiling tubes. Conc. HCl was added to give a concentration of not less than 12·5 % and the tubes placed in boiling water for 15 min. to hydrolyse the cyanopropionic acid to succinic acid. After allowing to cool 40 % NaOH was added dropwise until the solutions became hot again, at which point

1 ml. of 5% NH_4Cl was added to decompose traces of chloramine-T. Several drops of phenol red were then added and the solutions made alkaline to a purple colour, avoiding a large excess of alkali. The cool solutions were transferred to a continuous ether extractor with small quantities of water used to wash the boiling tubes and extracted with freshly distilled peroxide-free ether for 2 hr. to remove traces of *p*-toluenesulphonamide. The solutions were then acidified strongly to phenol red with 20% H_2SO_4 (usually 3–4 ml.) and extracted for 4–5 hr. into a clean extraction flask during which time the phenol red and succinic acid were removed. 2–3 ml. *M*/10 phosphate buffer were added to the ether solutions in the flasks, followed by dropwise addition of 2*N* NaOH until the indicator showed neutrality or slight alkalinity (red-purple). The ether was distilled off and the aqueous solutions concentrated on a steam bath to 1–2 ml. and transferred to small graduated cylinders. The extraction flasks were rinsed two or three times with 1 ml. *M*/10 phosphate, the washings added to the solutions in the cylinders and the total volume of each solution recorded. They were kept within 5 ml., as it was necessary to have approximately 4 mg. succinic acid per ml. for satisfactory readings in the subsequent manometric estimation. The apparatus used for this purpose was the Warburg type of constant volume with simple reaction flasks with which Keilin cups were employed. 4 ml. suspension from pigeon breast muscle, prepared as described by Cohen, were placed in the main compartment and the volume of succinic acid taken was between 0·2 and 0·5 ml., depending on the capacity of the Keilin cup. The oxygen uptake was measured.[1] The amount of glutamic acid in the hydrolysate was calculated from

$$\mu l.\ O_2 \times \frac{1{\cdot}47}{112} = \text{mg. glutamic acid}$$

or 1 mg. glutamic acid = 76·2 μl. O_2 uptake. The results were:

Hydrolysate	Glutamic acid in 4 ml. mg.		% in protein	Cf. by isolation
Casein 3·56 %	(23·4)		(16·6)	—
	31·2		21·6	—
	32·6		22·9	—
		Mean	22·3	21·0 [Dakin, 1918]
By Cohen	—		20·6	—
Depocasein 3·70 %	34·7		23·6	—
	32·9		22·0	—
	35·7		24·1	—
		Mean	23·3	3·6 (Plimmer & Lawton)
By Cohen	—		15·4	—
Depocaseose 3·75 %	28·6		19·1	—
	(25·4)		(16·8)	—
	28·9		19·5	—
		Mean	19·2	17·5 (see below)
By Cohen	—		19·9	—

The figure for casein corresponds well with the figure by isolation; it is higher than the isolated figure in the case of depocaseose. The isolated figure for depocasein is undoubtedly too low. The general agreement of the figures shows the value of the enzyme method of Cohen for estimating glutamic acid. Depocasein has a slightly greater amount of glutamic acid than casein. As might be expected depocaseose has a relatively smaller quantity. In terms of glutamic acid N as % of total protein N the figures are: casein 14·1; depocasein 16·2; depocaseose 12·6.

[1] These experiments were carried out in the Biochemistry Dept., Oxford. We desire to express our thanks to Prof. Peters for allowing the use of his laboratory.

Determination of hydroxyamino-acids

A. *Threonine.* Block & Bolling [1939] introduced a micro-method of determining threonine, consisting in its oxidation with lead tetra-acetate to acetaldehyde, which is aerated into concentrated H_2SO_4 and condensed with *p*-hydroxydiphenyl to give a red-violet colour.

In preliminary experiments to test the method certain difficulties were encountered and some modifications had to be introduced. It was advantageous to insert an extra tube or Drechsel wash-bottle containing purified acetic acid between the spray tube (2) and the reaction tube (3). By this means the volume of solution in tube (3) was kept approximately constant, thus correcting for acid carried over into tubes (4) and (5). Tube (5) containing pellets of solid NaOH often became clogged with sodium acetate which stopped the air current and held back traces of acetaldehyde. It was replaced by a second empty tube placed in ice water with tube (4). Tube (2*a*) or Drechsel bottle and the reaction tube (3) were kept covered with black paper to stop effects believed to be due to sunlight on lead tetra-acetate and acetic acid. In sunlight it was found that with only the reagents present a blue colour developed in the absorption tube (6) different in colour from the red-violet produced by acetaldehyde. Even with purified acetic acid a blank aeration of at least 1 hr. was necessary to remove traces of acetaldehyde from reagents. With threonine solution (standard or unknown) the aeration had to be continued for 2–3 hr.; 1 hr. was insufficient with 1 mg. threonine. The colour comparison was made with a Duboscq type of colorimeter with a blue filter (Ilford) to cut out red and yellow shades as recommended by Block & Bolling.

In practice, a blank aeration was carried out for 1 hr. and if no colour developed in tube (6) a known quantity of standard threonine solution (0·5–1 mg.) was placed in reaction tube (3) containing 1 g. $Pb(Ac)_4$ in purified glacial acetic acid and air drawn through at a steady slow rate for about 2·5 hr. during which time the colour attained maximum density. Tube (6) was then disconnected, placed in boiling water for 2 min. to remove excess of *p*-hydroxydiphenyl and the clear solution after cooling in ice was kept at room temperature. A fresh absorption tube (6) was attached and aeration continued to ensure that all acetaldehyde had been removed. The same procedure was repeated with the unknown solution.

0·5–1 ml. of a 0·1 % solution of threonine synthesized by the method of West & Carter [1937] was used as standard.

The protein solutions were prepared by boiling 1–2 g. protein with 10–20 ml. 20 % HCl for 24 hr., evaporating several times *in vacuo* and treating with Ag_2O suspension to remove Cl ions. Ag was removed with H_2S and the filtered solution evaporated to dryness on a water bath. After standing *in vacuo* over H_2SO_4 the residue was dissolved in purified glacial acetic acid and brought to 50 ml. Total N was determined in an aliquot; 2 ml. gave colours comparable with 1 mg. threonine. The results were:

		% threonine in dry protein	Threonine-N % of total N
Casein		2·8	—
		3·7	—
	Mean	3·3	2·58
Depocasein		1·92	1·64
Depocaseose		1·80	—
		2·10	—
	Mean	1·95	1·60

B. *Total β-hydroxy-α-amino-acids.* Van Slyke *et al.* [1940] pointed out that β-hydroxy-α-amino-acids were oxidized by potassium periodate in alkaline solution with quantitative liberation of their N as NH_3. This reaction was studied by Nicolet & Shinn [1939]. Investigations by Mr Lowndes in this department with threonine, serine and hydroxyglutamic acid gave results fully agreeing with Van Slyke's statement.

The same hydrolysates were used as for the threonine estimations above. The operation is carried out in three aeration tubes as commonly employed for NH_3 estimations. First, 10 ml. hydrolysate were made strongly alkaline to phenolphthalein with K_2CO_3 and NH_3 from amide groups aerated into standard acid. Powdered KIO_4 was then added to the tube and NH_3 aerated into a fresh tube with standard acid for 1 hr. The results were:

	NH_3 from amide groups as N	NH_3 from β-hydroxy-amino-acids as N	% of total protein-N	
			Amide-N	β-Hydroxy-amino-acid-N
Casein 10 ml. hydrolsate = 22·4 mg. N	2·41	1·80	10·75	8·05
Depocasein 10 ml. hydrolysate = 40·9 mg. N	2·92	2·06	7·20	5·05
Depocaseose 10 ml. hydrolysate = 54·3 mg. N	2·82	3·68	5·20	6·80

The amide-N for casein corresponds with the usual figure of 10–12 %. The figures for depocasein and depocaseose are lower on account of liberation of NH_3 during the alkaline digestion of casein. The figures for hydroxyamino-acids in both depocasein and depocaseose are lower than in casein. Unless alkaline digestion of casein produces some special action on these hydroxyamino-acids the greater amount in depocaseose suggests that depopeptone will contain still higher amounts, which corresponds to their order of solubility in water.

Deducting threonine-N from the total hydroxyamino-N, the difference represents serine-N and hydroxyglutamic acid-N:

In casein, 8·05 − 2·58 = 5·47.
In depocasein 5·05 − 1·64 = 3·41.
In depocaseose 6·80 − 1·60 = 5·20.

Our estimations of serine (below) were not successful. The amount of serine in casein determined by isolation is quoted as 0·5 = 0·44 % serine-N of total N. This would leave 5·0 % N as hydroxyglutamic acid-N = 8·7 % amino-acid. Dakin's figure was 10·5 %. In contrast Rapoport [1935] gave 8·1 % of glycine and serine in casein calculated as glycine. Assuming 0·5 % glycine calculation shows that there would then be little or no hydroxyglutamic acid in casein. Harington & Randall [1931] failed to isolate this amino-acid and Gulland & Morris [1934] only obtained 0·33 %.

C. *Serine.* Rapoport's method [1937] of estimating serine, consisting in deamination with nitrous acid to glyceric acid and condensation with naphthoresorcinol in conc. H_2SO_4 to give a blue colour, has been tried with the hydrolysates of these proteins. In the final stage a dry residue containing the glyceric acid is suspended in 2 ml. conc. H_2SO_4 containing 0·1 % reagent and heated on the water bath for 1 hr. In all cases a brown charred solution was obtained without any blue colour. We are unable to account for the lack of success with this method.

Isolation of monoamino and dicarboxylic acids in depocaseose

The amino-acids other than those estimated by special methods were determined as far as possible by isolation.

50 g. depocaseose were hydrolysed by boiling with 1500 ml. 20 % HCl for 24 hr. and the solution evaporated *in vacuo* several times to remove excess HCl. The solution of the resulting syrup, filtered from humin material, was brought to a known volume for determination of total N. This figure corresponded to 42·2 g. depocaseose in 1 litre used for isolation.

Removal of basic amino-acids. A test portion of 50 ml. was found to be precipitated by 22 ml. phospho-24-tungstic acid [Wu, 1920] solution. The remainder of the solution was heated and precipitated with 418 ml. The actual quantity of phosphotungstic acid required was 4·16 g. per 1 g. depocaseose. After several days at 0° the precipitate of phosphotungstates was filtered off and washed three times with $N/10$ HCl. The clear filtrate and washings were combined and shaken with about 200 ml. of amyl alcohol-ether mixture to remove excess phosphotungstic acid and evaporated to about 30 ml.

Precipitation of glutamic acid HCl. The concentrated solution was saturated with HCl gas and kept for several days at 0°. The crystalline precipitate G_1 was filtered off, washed with fuming HCl and dried *in vacuo* over NaOH and CaO. The filtrate was evaporated to much smaller volume and again saturated with HCl gas. Two further precipitates G_2 and G_3 were obtained and dried *in vacuo* over NaOH and CaO.

G_1 weighed 0·4 g., contained 1 % N and consisted mainly of inorganic material.

G_2 and G_3 together weighed 14·2 g. and contained 8·6 % N and 22·4 % Cl. On recrystallization from sat. HCl two crops G_a of 7·8 g. with 7·74 % N and 19·9 % Cl and G_b of 0·31 g. with 7·5 % N and 18·8 % Cl were obtained (glutamic acid HCl has 7·63 % N and 19·3 % Cl). The yield corresponds to 6·6 g. glutamic acid.

The filtrates from the recrystallized crops of glutamic acid HCl were evaporated *in vacuo* to remove HCl. Cl ions were removed with sat. Ag_2SO_4 solution, Ag with H_2S, and after evaporation *in vacuo*, SO_4 and NH_4 ions with $Ba(OH)_2$ solution and finally Ba ions quantitatively with H_2SO_4. On evaporating to a small bulk two crops of glutamic acid (calc. N = 9·5 %) mixed with leucine (calc. N = 10·69 %) crystallized out, G_c of 2·84 g. with 10·16 % N and G_d of 0·4 g. with 10·26 % N. During recrystallization some of the solution was lost, but 0·8 g. glutamic acid with N = 9·3 % and 0·7 g. leucine with N = 10·27 % were isolated. From one of the mother liquors a precipitate of 0·06 g. with N = 8·0 % and M.P. 230°, possibly phenylalanine (calc. N = 8·4 %), was obtained. The yields were thus 7·4 g. glutamic acid, 0·7 g. leucine, 0·06 g. phenylalanine.

Precipitation of proline and hydroxyproline as reineckates. The main filtrate of amino-acids from the crops of glutamic acid HCl was evaporated *in vacuo* several times. The residual syrup was dissolved in 100 ml. water and treated with 200 ml. 10 % aqueous solution of ammonium reineckate [Kapfhammer & Eck, 1927; Bergmann, 1935]. After 2 days at 0°, the reddish brown precipitate R_1 was filtered off. On concentration of the filtrate and addition of more reineckate two further crops R_2 and R_3 were obtained on standing for 2 days at 0°. The combined precipitates weighed 4 g. and were suspended in about 100 ml. water and treated with 10–20 ml. pyridine. The dark oily residue which settled out was filtered off and washed with difficulty. The solution was made alkaline with $Ba(OH)_2$ solution and evaporated *in vacuo* to remove NH_3 and pyridine. A small

insoluble residue was filtered off and Ba ions removed quantitatively with H_2SO_4. The filtrate and washings were acidified with HCl and a solution of ammonium rhodanilate in MeOH added. No crystalline precipitate was obtained, nor could any hydroxyproline or proline be isolated from the solution. Only dark gummy residues settled out.

The dark coloured solution from the reineckate precipitate containing HCl and pyridine was heated and treated with hot saturated Ag_2SO_4 solution until precipitation was complete. Most of the colour was removed. The clear filtrate was evaporated *in vacuo* to 200 ml., Ag removed with H_2S and the solution again evaporated. It was made alkaline with NH_4OH and evaporated *in vacuo* to remove pyridine and excess NH_3. The almost neutral solution, about 75 ml., was kept *in vacuo* over H_2SO_4 for 7 days. A crystalline precipitate of impure tyrosine T_1 slowly formed. The filtrate and washings on further evaporation gave a second crop T_2. These crops, weighing 1·1 g. with 9·0 % N and 0·5 g. with 10·9 % N, were recrystallized from very dilute NH_4OH, followed by evaporation and addition of small quantities of alcohol. Four crops of crystals resulted: (1) 0·45 g. with 7·6 % N, almost pure tyrosine (calc. N = 7·73 %); (2) 0·28 g. with 9·15 % N, a mixture of equal parts of tyrosine and leucine; (3) 0·3 g. with 10·15 % N, mainly leucine (calc. N = 10·69 %) and (4) 0·31 g. with 13·3 % N, possibly valine (calc. N = 12·97 %). The yields of tyrosine and leucine would be 0·6 g. and 0·45 g.

Separation of calcium aspartate. The filtrate from crop T_2 was treated with hot $Ba(OH)_2$ solution to remove SO_4 ions and the alkaline filtrate evaporated *in vacuo* to remove NH_3. Excess Ba ions were removed quantitatively with H_2SO_4 and the filtrate and washings, about 100 ml., were made strongly alkaline with a 10 % suspension of $Ca(OH)_2$ and filtered from excess. The remaining colour was thus removed from the solution which was evaporated to a small volume and to which 10 vol. of absolute alcohol were slowly added with careful stirring. A white precipitate of Ca salts settled out and was filtered off after 24 hr. The precipitate weighing 4·5 g. was dissolved in warm water, filtered from a trace of insoluble matter and treated with the exact amount of oxalic acid to remove Ca ions. After evaporation to a small volume it was saturated with HCl gas. The small precipitate consisted of inorganic matter. HCl was removed by evaporation and with Ag_2SO_4, Ag with H_2S, SO_4 ions with $Ba(OH)_2$ and the latter quantitatively with H_2SO_4 and the clear neutral solution was boiled with excess $CuCO_3$. The dark blue solution on evaporation to 25 ml. gave crystals of Cu aspartate, which were filtered off, washed with cold water and air-dried. The yield was 1·28 g. The crystals lost 0·959 g. at 100° *in vacuo* over P_2O_5 = 24 % (Cu aspartate + 3·5 H_2O = 24·4 % H_2O). The anhydrous salt contained 32·1 % Cu (calc. Cu = 32·5 %). The yield corresponds to 0·67 g. aspartic acid. Addition of small quantities of alcohol failed to produce further crystallization. The solution was freed from Cu with H_2S and evaporated to 10 ml. No crystalline precipitate formed and alcohol produced a gummy residue. The alcoholic solution evaporated *in vacuo* gave a very deliquescent residue on standing over H_2SO_4, which weighed 0·67 g. and contained 10·9 % N and some Cl. Cl was removed and ultimately 0·4 g. of white crystals with 11·8 % N was obtained. This was believed to be proline (calc. N = 12·1 %). The residue insoluble in alcohol was dissolved in water and the solution poured into alcohol. A powder, slightly coloured, weighing 0·8 g. was obtained. A white residue, insoluble in water, weighing 0·21 g., was obtained from it. Its N content was 7·4 % and it gave Millon's reaction and was tyrosine. From the insoluble Ca salts 0·67 g. aspartic acid, 0·21 g. tyrosine and 0·4 g. proline (?) were thus obtained.

The alcoholic filtrate from the insoluble Ca. salts was evaporated, filtered from $CaCO_3$ and the Ca removed quantitatively with oxalic acid. On further evaporation crystalline plates appeared which were redissolved on heating the solution and came out again on cooling M_1. The filtrate and washings on evaporation gave a series of further crops M_2–M_7 and more crops M_8–M_{13} were obtained by adding small quantities of alcohol. The final solution contained only 0·17 g. N from which no more crystals could be obtained. The yields and N contents of the crops were:

M_1	0·33 g. with	10·67 % N	Correspond to 2·9 g. leucine (calc. N = 10·69 %)
M_2	0·65	10·40	
M_3	0·20	10·22	
M_4	0·54	10·54	
M_5	0·26	10·70	
M_6	0·56	10·90	
M_7	0·36	10·45	
M_8	0·50	11·2	Corresponds to 0·3 g. leucine + 0·2 g. valine.
M_9	0·33	11·9	Correspond to 1·8 g. valine (calc. N = 11·96 %) and traces of alanine or lower amino-acids.
M_{10}	0·25	12·65	
M_{11}	0·76	11·9	
M_{12}	0·15	11·8	
M_{13}	0·31	11·4	

The total yields of amino-acids isolated and with their N expressed in terms of total depocaseose-N were:

	Isolated g.	% of depocaseose	Amino-acid-N	% of total depocaseose-N
Glutamic acid	7·4	17·5	0·703	11·5
Tyrosine	0·81	1·9	0·063	1·03
Leucine	4·35	10·3	0·466	7·6
Valine	2·0	4·9	0·24	4·0
Aspartic acid	0·67	1·6	0·07	1·14
Proline?	0·4	1·0	0·048	0·8
Unidentified N	—	—	0·17	3·5
			0·042	—

The quantity of glutamic acid is nearly the same as that determined by the enzyme method, but the amount of tyrosine is much lower than that by colorimetric determination. The values for leucine are probably near the actual amounts in depocaseose, but the amount of aspartic acid is probably too low.

The optical activity of isolated amino-acids

In view of Dakin & Dudley's observations [1912; 1913] on the racemization of the amino-acids produced by the action of $N/2$ NaOH at 37° for 18–20 days on casein it was of interest to examine the rotations of the amino-acids obtained from depocaseose. The crops of leucine and valine were recrystallized in two main fractions and used for these experiments.

Glutamic acid. 5·35 % in 9 % HCl in 2 dm. tube gave $\alpha = 2·68°$, $[\alpha]_D = +25·0°$; pure *l*-acid in 9 % HCl = +31·0°.

Leucines. 3·45 % in 20 % HCl in 2 dm. tube gave $\alpha = 1·80°$, $[\alpha]_D + = 26·1°$; *l*-leucine in 20 % HCl = +15·9°; *l-iso*leucine in 20 % HCl = +37·3°.

Valine. 2·72 % in 20 % HCl in 2 dm. tube gave $\alpha = 1·02°$, $[\alpha]_D = +18·5°$; *l*-valine in 20 % HCl = +28·8°.

Both valine and glutamic acid have thus undergone a small amount of racemization by the action of $N/4$ NaOH at 37° for 24 hr. No conclusion can be drawn from the leucine specimen as it is probably a mixture of the isomers.

Summary and Discussion

The compositions of caseinogen, depocasein and depocaseose are compared in the following tables:

g. of amino-acids as % of dry protein

	Caseinogen	Depocasein	Depocaseose
Cystine	0·29	0·30*	0·24
Methionine	2·57	2·09*	2·80
Tyrosine	5·62	8·12	3·57
Tryptophan	1·34	1·17	0·94
Arginine	3·30	4·61	3·23
Histidine	1·33	0·98	0·87
Lysine	7·00	5·70	7·81
Glutamic acid	22·30	23·30	19·20
Threonine	3·30	1·92	1·95
β-Hydroxyamino-acids other than threonine	7·65	4·0	6·8
Glycine	0·32	0·22	0·24
Ammonia	1·94	1·20	0·91
Aspartic acid	4·1*	3·0*	1·6
Leucines	9·4*	11·5*	10·3
Valine	7·9*	0·3*	4·9
	77·36	68·41	65·36

The figures marked * under caseinogen are from Dakin's results and those under depocasein from Plimmer & Lawton's.

The figure for β-hydroxyamino-acids is an approximate estimate based on the N content (next table) assuming an average molecular weight of these amino-acids (serine and hydroxyglutamic acid) = 130.

The last three amino-acids have not been estimated by the same methods. No importance can be attached to the figures from a comparative point of view.

Amino-acid-N as % of total N

	Caseinogen	Depocasein	Depocaseose
Cystine	0·22	0·25	0·19
Methionine	1·60	1·42	1·81
Tyrosine	2·88	4·55	1·90
Tryptophan	1·32	1·20	0·90
Arginine	7·00	10·70	7·20
Histidine	2·40	1·95	1·62
Lysine	9·10	8·20	10·60
Glutamic acid	14·10	16·20	12·61
Threonine	2·58	1·64	1·60
β-Hydroxyamino-acids other than threonine	5·47	3·41	5·20
Glycine	0·40	0·31	0·31
Ammonia	10·75	7·20	5·20
Aspartic acid	2·85	2·30	1·14
Leucines	6·90	9·30	7·60
Valine	6·30	0·20	4·00
	72·87	68·83	62·08

There is great similarity in the compositions of caseinogen, depocasein and depocaseose. A marked difference only appears in tyrosine and threonine and other β-hydroxyamino-acids. Small differences are observable in methionine, lysine and glutamic acid. They may however be significant as they may indicate an unequal distribution of the amino-acids between depocasein and depocaseose. The differences are shown more clearly in the molecular ratios obtained by

dividing the % amount of the amino-acid by its molecular weight and then taking cystine as unity, or from the N figures making allowance for the number of N atoms in each molecule.

Ratio of amino-acids from g. mol. per 100 g. protein

	Caseinogen	Depocasein	Depocaseose
Cystine	1	1	1
Methionine	14	11	19
Tyrosine	26	36	20
Tryptophan	6	5	5
Arginine	16	21	19
Histidine	8	5	6
Lysine	40	32	54
Glutamic acid	126	127	132
Threonine	24	13	16
Other β-hydroxyamino-acids	50	26	54
Glycine	4	3	4
Ammonia	100	56	54
Aspartic acid	26	18	12
Phosphorus	22 atoms		

The principle of Bergmann & Niemann [1936] to calculate from the analytical figures the frequencies of the amino-acids from the formula:

$$F = \frac{100 \times W}{G \times A}$$ where W = mol. wt. of the amino-acid,
G = % wt. of the amino-acid in the protein,
A = average mol. wt. of all amino-acids,

requires the assumption of the mean mol. wt. A. The value of F depends on the value assigned to A and on the accuracy of the value of G. Small changes in these figures affect F considerably. When applied to depocaseose the frequencies could not be expressed in powers of 2 or 3. As an absolute value it has little significance as a characteristic of the protein. The simple ratios in the above table show the variations in the compositions of the three proteins more clearly.

Certain of the amino-acids now appear to be distributed unequally between the two products from caseinogen:

Methionine and lysine show marked differences; there is a greater amount in depocaseose and less in depocasein than in casein.

β-Hydroxyamino-acids including threonine are more abundant in depocaseose than in depocasein. It is possible that the larger number of OH groups which are hydrophilic accounts for the greater solubility of the proteose.

The tyrosine content of the products may also be of significance with regard to solubility. Tyrosine is present in greatest amount in depocasein. All the tyrosine of casein is accounted for in the two fractions, depocasein and depocaseose, and thus the still more soluble peptone should not contain this amino-acid (see below). The 24 hr. alkaline digestion of casein splits off almost exactly half of the amide groups. The NH_3 from proteins is generally assumed to be present as amide groups on the dicarboxylic acids. The extent to which free carboxyl groups are combined in casein appears to be $\frac{100}{126+26}$ (assuming the aspartic figure to be correct) = 65 %. Damodaran [1931] found it to be 55 %.

The other amino-acids, tryptophan, arginine, histidine, glutamic acid etc., occur in almost equal proportions in depocasein and depocaseose.

Molecular weights of the proteins. Assuming that each molecule of protein contains one molecule of cystine the molecular weights calculated from the g. mol. of cystine per 100 g. protein are:

$$\text{casein } \frac{100}{0{\cdot}0012} = 83{,}300\text{: depocasein } \frac{100}{0{\cdot}00125} = 80{,}000\text{: depocaseose } \frac{100}{0{\cdot}0010} = 100{,}000.$$

The view of Sörensen [1930] that caseinogen is a reversibly dissociable system of closely similar components in a state of equilibrium depending on the conditions of the solution, reached also by Svedberg & Carpenter [1930] by ultracentrifugal methods (the components having mol. wt. 75,000–100,000), is strengthened by these analyses. The action of dilute NaOH produces a change in the complex system with the formation of two main components depocasein and depocaseose.

The action of 1 % NaOH on casein may be summarized as follows:

(*a*) Hydrolysis of the ester linkages by which the phosphoric acid is believed to be bound.

(*b*) Hydrolysis of approximately half the dicarboxylic acid amide groups during 24 hr.

(*c*) Some change as a result of which the complex system of casein is separated into two main components, depocasein and depocaseose, which may or may not be homogeneous. These products from chemical data appear to be of the same molecular size between 75,000 and 100,000, and they exhibit small yet significant differences in amino-acid composition.

It is believed that the differences in properties, such as solubility between the metaprotein and proteose are dependent on the amino-acid composition and not on molecular size.

(*d*) It appears probable from mol. wt. and other data that dilute NaOH does not attack peptide linkages to any great extent if at all. It may be noted that Dakin & Dudley found that after separation of racemized casein and caseose only 1/10 of the N remained in solution, i.e. after 20 days the peptide chains have not been broken more than after 1 day.

Composition of depopeptone. A speculative view based on the above conception of the casein molecule would be that the peptone consisted of molecules of the same size as depocasein and depocaseose, but having a greater proportion of hydrophilic groups (hydroxyamino-acids) to account for its greater solubility. Estimations of the amino-acids in the peptone can be calculated from the figures of the analyses of the three proteins. There should be no tyrosine and hydroxy-amino-acid-N should be high. A preliminary experiment with 1·3 g. impure peptone containing 14·1 % N showed the absence of tyrosine, but the hydroxy-amino-acid-N was only 3·7 % of the total N. A special series of experiments will be needed to prepare sufficient of the peptone to make full analyses.

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