XXXVI. THE ACTION OF DIAZOMETHANE ON CASEINOGEN (Preliminary Communication).

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Skraup and Krause [1909] methylated caseinogen with methyl iodide and boiling alcoholic potash with the object of showing which of the constituent amino-acids of the protein chain contained free amino- or hydroxyl-groups. Since their product on hydrolysis yielded no tyrosine or lysine and but little histidine and arginine they concluded that such free groups were present in the nuclei of these amino-acids. It is evident that such a vigorous methylation as they employed may be accompanied by an essential hydrolysis of the protein molecule. We have therefore methylated caseinogen with diazomethane in the hope that by this method results of a more conclusive character may be obtained. By the employment of such a mild reagent the possibility of decomposition is reduced to a minimum and it is therefore practically certain that the product obtained is a direct substitution product of caseinogen. By a comparison between the behaviour, on the one hand, of amino-acids and simple polypeptides and, on the other hand, of caseinogen, it is hoped that a direct view may be obtained into the protein molecule as it actually exists. Results have already been obtained which indicate clearly that the presence of carboxyl¹ groups may be proved and their number estimated. It may also be possible to estimate the number of free hydroxyl groups.

It is to be expected that our product, like that of Skraup and Krause, will on hydrolysis fail to give those amino-acids which when combined in the protein chain still contain free groups capable of methylation. It is further hoped that it may be possible to isolate in the form of their methyl

¹ According to T. B. Robertson [1912], for example, the acidity of proteins is not due to carboxyl groups but to groups of the type $C(OH)=N$.

derivatives amino-acids which in the free state may be unable to resist unchanged the vigorous hydrolysis usually employed.

In the present communication we include only the preliminary investigation of our product. The further investigation of this substance, and the study of the action of diazomethane on amino-acids and polypeptides are both in progress and will form the subject of further communications.

If an ethereal solution of diazomethane is poured over dry caseinogen, a moderate evolution of gas occurs. By the continued action of fresh portions of diazomethane a product is finally obtained which can in this way be no further methylated. The substance thus obtained is indistinguishable in appearance from caseinogen itself, but contains 3.5 % more methyl. In properties it differs most markedly in its insolubility. If a portion is added to an excess of dilute (N/50) caustic alkali a clear solution is obtained only after about 20 hours standing whereas caseinogen similarly treated dissolves in a few minutes. Towards acids methylo-caseinogen is even more stable, being dissolved by dilute sulphuric acid only after several days heating on a water-bath. By the addition of acid to the alkaline solution, a precipitate is formed containing less methyl than the original substance; this is readily re-dissolved by excess of acid.

Nitrogen estimations in our methylo-caseinogen by Kjeldahl's method gave consistently lower results than estimations by Dumas' method. It therefore seemed probable that by boiling with concentrated sulphuric acid part of the nitrogen was evolved as such. All attempts to estimate this nitrogen have however yielded only negative results.

Unlike Skraup and Krause's product our methylo-caseinogen readily gives Millon's reaction and it is therefore doubtful whether the tyrosine nucleus has been methylated. It may be pointed out that the failure of Millon's reaction in the case of their product is sufficiently accounted for by the iodine introduced by the action of methyl iodide, since it is known that iodo-proteins do not give this reaction. The Adamkiewicz-Hopkins reaction is more pronounced with methylo-caseinogen than with caseinogen. Skraup and Krause made the same observation but ascribed it to the presence of iodine.

It was found by Skraup and Krause that caseinogen when heated with hydriodic acid according to the Herzig-Meyer method gives a precipitate of silver iodide. We have been able to confirm this but obtain a lower value (0.36 %) than they (1.13 %) for the percentage of N-methyl; in this we are in agreement with the recent observation of Burn [1914]. Our

methylo-caseinogen contains about the same percentage of methyl (4.36 %) as Skraup and Krause's product (4.26 %).

EXPERIMENTAL PART.

Preparation of Methylo-caseinogen. 500 g. of Kahlbaum's "Casein nach Hammarsten" dried in a vacuum at 40° over phosphorus pentoxide were treated with diazomethane in ethereal solution in portions of about 5 g. The first few additions were followed by a vigorous evolution of gas. After each addition of diazomethane the reaction mixture was allowed to stand in the dark until it became colourless. The methylation was continued for four months and about 75 g. (a large excess) of diazomethane were used. The last few portions of diazomethane were only very slowly decolourised and no evolution of gas was observable. Methyl estimations from time to time by Herzig and Meyer's method showed that a maximum methyl content had been reached. The methylo-caseinogen was filtered from the ethereal mother-liquor and well washed with absolute ether. The ether was then removed by exposure to the air in shallow dishes for several weeks. All smell of ether had disappeared in a few days. For analysis the substance was further purified by extracting in a Soxhlet with absolute ether for a week. Only a minimal amount (less than 0.1 %) of fat was thus removed.

The ethereal filtrate and washings on evaporation yielded about 1 c.c. of a semi-solid mass. This probably consisted of fat or fatty derivatives and was not further investigated.

Methylo-caseinogen thus obtained is a light colourless powder indistinguishable in appearance from caseinogen. As has been mentioned above, it is only dissolved by acids and alkalis with difficulty; by the action of alkali methyl groups are lost. The alkaline solution is optically active.

Analysis. For analysis the pure fat-free methylo-caseinogen was allowed to stand for at least twelve hours exposed freely to the air of the balance room.

Water estimations were made whenever portions were weighed out for analysis. The results were calculated on the weight of water- and ash-free protein. [See Geake, 1914.]

It has been mentioned above that nitrogen estimations by Kjeldahl's method gave consistently lower results than by Dumas' method¹. It was

¹ Such differences have been previously observed with proteins. Thus Pittom [1914] working with egg albumin obtained a difference between the two methods of more than 1 % nitrogen.

thought probable that this was due to a portion of the diazomethane having reacted with caseinogen to give a product containing diazo-nitrogen. Such a product when heated with concentrated sulphuric acid would give off part of its nitrogen as nitrogen gas. Estimations of diazo-nitrogen by the usual methods of heating with concentrated hydrochloric acid or with somewhat diluted sulphuric acid yielded negative results. A portion of the substance was therefore heated with the usual Kjeldahl mixture (30 g. conc. H_2SO_4 , 10 g. K_2SO_4 , 2 g. CuSO_4), and the gases evolved passed through a heated combustion tube containing copper oxide and a reduced copper spiral. Even in this way only a minimal amount of unabsorbed gas was collected.

The methylo-caseinogen gave the following results on analysis :

0.5086 g. (10.65 % H_2O) ;	0.0041 g. ash				
0.5049 g. (10.65 % H_2O) ;	0.0034 g. ash				
0.1957 g. (10.04 % H_2O) ;	0.3549 g. CO_2 ;	0.1322 g. H_2O			
0.2118 g. (8.72 % H_2O) ;	0.3869 g. CO_2 ;	0.1429 g. H_2O			
0.1657 g. (7.60 % H_2O) ;	0.3088 g. CO_2 ;	0.1143 g. H_2O			
0.2728 g. (10.07 % H_2O)	neutralised	25.89 c.c. N/10 H_2SO_4 (Kjeldahl)			
0.2497 g. (10.07 % H_2O)	"	23.24 c.c.	"	"	
0.2967 g. (8.48 % H_2O)	"	28.58 c.c.	"	"	
0.2379 g. (8.48 % H_2O)	"	23.08 c.c.	"	"	
0.3066 g. (10.11 % H_2O) ;	36.1 c.c. N over water at 13.8° and 758 mm.				
0.2767 g. (10.04 % H_2O) ;	32.8 c.c.	"	"	12.8°	744 mm.
0.2014 g. (8.48 % H_2O) ;	24.8 c.c.	"	"	11.8°	745 mm.
0.4998 g. (8.48 % H_2O) ;	0.0264 g. BaSO_4				
0.4998 g. (8.48 % H_2O)	neutralised	30.90 c.c. N/10 NaOH (Phosphorus estn. by Neumann's method.)			

$$\text{Ash} = \frac{(0.90)}{(0.76)} = 0.83 \%$$

100 parts water and ash-free methylo-caseinogen contain

Found					
C	H	N		S	P
		Kjeldahl	Dumas		
55.43	7.21	14.90	15.43	0.800	0.755
55.03	7.26	14.62	15.31		
55.46	7.49	14.87	15.73		
		14.97			

Mean composition :

				Methylo-caseinogen	Caseinogen [Geake, 1914]
				%	%
C	..	..	..	55.31	53.20
H	..	..	..	7.32	7.09
N (Kjeldahl)	..			14.84	15.61
(Dumas)	..			15.49	15.63
S	..	..	..	0.800	1.015
P	..	..	..	0.755	0.731

Estimations of methyl were carried out by Herzig and Meyer's method. For convenience all results have been calculated for " CH_3 ," not for " OCH_3 " or " NCH_3 ." It was found by Skraup and Krause that caseinogen contains 0.40 % $(\text{O})\text{CH}_3$ and 1.13 % $(\text{N})\text{CH}_3$. Our results for the latter are considerably lower.

Estimation of methyl in caseinogen :

	AgI		
	HI at 140° C.	HI at 200° C.	HI + NH_4I at 300° C.
(I) 0.4090 g. (11.99 % H_2O)	0.0134 g.	0.0138 g.	0.0215 g.
(II) 0.4265 g. (12.00 % H_2O)	0.0166 g.	0.0161 g.	0.0191 g.
(I) $(\text{O})\text{CH}_3 = 0.24 + 0.25 = 0.49$ % ;	Total $\text{CH}_3 = 0.87$ %		
(II) $= 0.28 + 0.27 = 0.55$ % ;			
	$= 0.33$ %		$= 0.88$ %

Since the completion of this work Burn [1914] has obtained a similar result (0.97 % total methyl).

No great stress can be laid on these values since, as was observed by Skraup and Krause and confirmed by Burn, amino-acids themselves, though free from methyl groups, give a similar precipitation of silver iodide. We have observed that after heating to 140° C. till no further precipitate can be obtained more silver iodide is formed by heating to 200° C. and more again at higher temperatures. The distinction between $(\text{O})\text{CH}_3$ and $(\text{N})\text{CH}_3$ cannot therefore be taken as exact.

Skraup and Krause's methylo-caseinogen contained 0.90 % $(\text{O})\text{CH}_3$ and 3.36 % $(\text{N})\text{CH}_3$, making a total of 4.26 % methyl.

Our methylo-caseinogen gave the following results :

	AgI	
	HI at 140° C.	HI + NH ₄ I at 300° C.
0.3960 g. (9.74 % H ₂ O)	0.1110 g.	0.1301 g.
0.3953 g. (9.76 % H ₂ O)	0.1105 g.	0.1286 g.
0.4340 g. (9.08 % H ₂ O)	0.1381 g.	0.1494 g.
0.4050 g. (9.08 % H ₂ O)	0.1210 g.	0.1185 g.
(O)CH ₃ = 1.99, 1.98, 2.24, 2.10.	Mean 2.08 %	
(N)CH ₃ = 2.33, 2.31, 2.42, 2.06		
Total methyl = 4.36 %		

Since caseinogen gave 0.88 % methyl, the increase of methyl on methylation is 3.48 %.

Colour Reactions. The following reactions are given equally by caseinogen and by our methylo-caseinogen : Biuret reaction, xanthoproteic reaction, lead sulphide reaction, Molisch reaction, Liebermann's reaction, tryptophane reaction with H_2SO_4 , vanillin and KNO_2 or with HCl , vanillin and KNO_2 , Reichl's reaction and Mörner's test for tyrosine. The Adamkiewicz-Hopkins

reaction and Denigé's test for tyrosine are given more strongly by methylo-caseinogen. The reddish violet colouration observed by Cooper [1913] when proteins are immersed in aqueous quinone solutions is also obtained much more rapidly and powerfully with methylo-caseinogen than with caseinogen.

Since the product gives Millon's reaction and the other tyrosine reactions it is doubtful whether the hydroxyl group of the tyrosine nucleus has been methylated¹. As mentioned above, the absence of the reaction in the case of Skraup and Krause's product is sufficiently explained by the presence of iodine. Skraup and Krause also observed the more pronounced Adamkiewicz-Hopkins reaction, but ascribed this to the iodine in their substance.

[Note added July 30th. Since this paper was sent to the press we have noticed that Herzig and Landsteiner [1914] have also made experiments on the methylation of caseinogen by diazomethane. As their paper was only published on 27th April we did not see it until some time after the manuscript had left us on June 3rd.]

REFERENCES.

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¹ Diazomethane has no action on tyrosine in ethereal suspension but in methyl alcohol methylation takes place. Millon's reaction is not given by veratric acid but is given by trimethylgallic acid; the positive reaction is therefore not an absolute proof of the presence of a free hydroxyl group.