THE DIACETYL REACTION FOR PROTEINS.

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In the course of an investigation into the nature of the Voges and Proskauer reaction, Harden[1] showed that the colouration was due to the presence in the culture medium of acetylmethylcarbinol. This substance, which is a product of the growth of certain bacteria on sugars, is readily oxidised to diacetyl which then combines in the presence of alkali with some part of the protein molecule, the complex thus formed imparting to the solution a pink colour and green fluorescence.

As it is of interest to ascertain which part of the protein molecule is concerned in this reaction further experiments have been carried out with this end in view, and the results of these are embodied in the present communication. For this purpose a large number of tests have been carried out with various proteins and other substances. Beginning with more complex bodies and then testing the products of hydrolysis of those which gave a positive result, it has been found possible to trace the reaction to several bodies of simple constitution.

The following description of the examination of the protamine clupein will serve as an illustration of the methods employed. Clupein itself gives the reaction in the typical manner with a well marked fluorescence, but the mixed products obtained by heating for 14 hours with 50 % H_2SO_4 only give a pink colour, unaccompanied by fluorescence.

The products of hydrolysis were separated according to the method used by Weiss[2]. Each fraction was then tested and it was found that arginine alone of the degradation products gave the reaction.

[1] *Proc. Roy. Soc.* B, 1906, LXXVII. p. 424.

[2] *Ztsch. physiol. Chemie*, 1907, LII. p. 107.

As a confirmatory test another sample of arginine, which was kindly given by Dr Hedin, was examined and this also gave a positive result, a pink colour unaccompanied by fluorescence.

The test was carried out by mixing a dilute solution of the protein or other substance in question with a small quantity of 10 % KOH and then adding one drop of a 1 % solution of diacetyl. In the case of simple substances it is essential to avoid both excess of alkali and of diacetyl, otherwise the diacetyl and alkali react, giving a brown colouration which in some cases may completely mask the true reaction.

Each substance appears to have its own optimum conditions; for example, using a 0·25 % solution of arginine, the best results were obtained by mixing 1 c.c. of this solution with 0·5 c.c. N. KOH, making the total volume up to 5 c.c. with water and then adding 0·05 c.c. of a 1 % solution of diacetyl. Using complex proteins such as gelatin stronger alkali is necessary, though excess of diacetyl is still to be avoided.

The reaction was usually done in the cold though heating in boiling water for one minute hastens the effect.

By these means the following substances of known structure were found to give the reaction.

Arginine $NH : C(NH_2) . NH . CH_2 . CH_2 . CH_2 . CH(NH_2) . COOH$.
Agmatine $NH : C(NH_2) . NH . CH_2 . CH_2 . CH_2 . CH_2 . NH_2$.
Creatine $NH : C(NH_2) . N . (CH_3) . CH_2 . COOH$.
Dicyanamide $NH : C(NH_2) . NH . CN$.
Guanidineacetic acid $NH : C(NH_2) . NH . CH_2 . COOH$.

With the exception of dicyanamide all the above are known to contain the guanidine grouping $NH : C(NH_2) . NHR$. In the case of dicyanamide the formula may be written $NH : C(NH_2) . NH . CN$ and here again is the guanidine structure. The reaction is however dependent to some extent upon the nature of R, for methylguanidine and aminoguanidine give decisively negative results. Free guanidine and the nitrate and hydrochloride only give the very faintest tinge of colour and this cannot be detected until the solutions have stood for several hours. The reaction is somewhat more marked with the carbonate and is quite normal with guanidineacetic acid.

It should here be noticed that no fluorescence is obtained with these simple bodies and it is possible that the fluorescence produced when complicated proteins are used may simply be due to the large molecular weight of the resulting complex. In favour of this may be cited the experiment with clupein already mentioned. Before hydrolysis this protamine gives a typical green fluorescence in addition to

the pink colouration; after hydrolysis with sulphuric acid, although the pink colour due to the arginine still persists, the fluorescence is entirely lost.

The delicacy of the reaction in the case of proteins is dependent on the amount of alkali, the strength of caustic potash which will give good results with the simpler substances being altogether insufficient in the case of proteins. The following table shows this clearly in the case of Witte's peptone:

0·5 % solution of Witte's peptone	N. KOH	H_2O	1 % diacetyl	Strength of peptone in total volume	Result
4·5 c.c.	0·5 c.c.	—	0·05 c.c.	1 in 220	–
2·0 ,,	0·5 ,,	2·5 c.c.	0·05 ,,	1 in 500	–
0·5 ,,	0·5 ,,	4·0 ,,	0·05 ,,	1 in 2000	–
0·1 ,,	0·5 ,,	4·6 ,,	0·05 ,,	1 in 10000	–

Similar results were obtained with gelatin when this strength of KOH was used.

2·5 N. NaOH was next tried and the following results obtained:

0·5 % solution of Witte's peptone	2·5 N. NaOH	H_2O	1 % diacetyl	Strength of peptone in total volume	Result
5·0 c.c.	5 c.c.	—	0·05 c.c.	1 in 400	+
4·0 ,,	5 ,,	1·0 c.c.	0·05 ,,	1 in 500	+
3·0 ,,	5 ,,	2·0 ,,	0·05 ,,	1 in 700	+
2·0 ,,	5 ,,	3·0 ,,	0·05 ,,	1 in 1000	+
1·8 ,,	5 ,,	3·2 ,,	0·05 ,,	1 in 1150	+
1·6 ,,	5 ,,	3·4 ,,	0·05 ,,	1 in 1300	+
1·5 ,,	5 ,,	3·5 ,,	0·05 ,,	1 in 1400	+
1·4 ,,	5 ,,	3·6 ,,	0·05 ,,	1 in 1500	–
1·2 ,,	5 ,,	3·8 ,,	0·05 ,,	1 in 1700	–
1·0 ,,	5 ,,	4·0 ,,	0·05 ,,	1 in 2000	–

The positive results in the above table indicate a pink colour together with a green fluorescence. This last series was repeated, using 0·3 c.c. of 1 % diacetyl instead of 0·05 c.c. The positive reaction ceased at the same point of dilution as before but was somewhat more intense in colour and fluorescence.

It will be seen that a typical reaction is obtained up to 1 part in 1400 of Witte's peptone under the above conditions and that after this the results are negative.

Some experiments were then made with 60 % KOH and here there is a striking difference in the results obtained. A pink colour can be detected with 1 part in 20,000 of Witte's peptone but there is no

fluorescence, and this holds even in a solution of 1 part in 1000. Taking gelatin instead of Witte's peptone a pink colour can be detected with only 1 part in 30,000. The following tables summarise these results:

0·5 % Witte's peptone	60 % KOH	H_2O	1 % diacetyl	Strength of peptone in total volume	Result
0·2 c.c.	3·5 c.c.	1·3 c.c.	0·05 c.c.	1 in 5000	+ no fluorescence.
0·1 ,,	3·5 ,,	1·4 ,,	0·05 ,,	1 in 10000	+ ,,
0·075 ,,	3·5 ,,	1·4 ,,	0·05 ,,	1 in 15000	+ ,,
0·05 ,,	3·5 ,,	1·4 ,,	0·05 ,,	1 in 20000	+ ,,

0·5 % gelatin	60 % KOH	H_2O	1 % diacetyl	Strength of gelatine in total volume	Result
0·1 c.c.	3·5 c.c.	1·4 c.c.	0·05 c.c.	1 in 10000	+ no fluorescence.
0·075 ,,	3·5 ,,	1·42 ,,	0·05 ,,	1 in 15000	+ ,,
0·05 ,,	3·5 ,,	1·45 ,,	0·05 ,,	1 in 20000	+ ,,
0·03 ,,	3·5 ,,	1·5 ,,	0·05 ,,	1 in 30000	+ ,,

It appears probable that in these very dilute solutions the protein is hydrolysed by the strong alkali used. This would account for the loss of fluorescence, the pink colour being due to the liberated arginine.

The same thing occurs in stronger solutions if time is allowed for the hydrolysis to take place. This is illustrated by the following experiments.

I. 5 c.c. of 0·5 % solution of Witte's peptone were mixed with 5 c.c. of 10 % KOH and 0·3 c.c. of 1 % diacetyl added; a typical reaction was obtained.

II. 5 c.c. of 0·5 % solution of Witte's peptone were mixed with 3·5 c.c. of 60 % KOH and this mixture was then allowed to stand over night, and then 0·3 c.c. of 1 % diacetyl added; a pink colouration only was obtained, no fluorescence.

A few experiments have been carried out using other diketones in the place of diacetyl. As yet these have yielded negative results though the γ-diketones still remain to be tried. Those actually tested were—benzil, acetylacetone, benzoylacetone, dibenzoylmethane, dibenzoylethylene. Acetone, acetaldehyde, dihydroxyacetone and glycerose also failed to give the reaction.

A list of the substances tested with diacetyl is given in the table on p. 336.

Substance	Result	Substance	Result	Substance	Result
Gelatin	+	*l*-Alanyl-*d*-alanine	–	Serine Anhydride	–
Egg Albumin	+	Di-alanyl-*l*-cystine	–	Glycine Anhydride	–
Fibrin	+	*l*-Leucinimide	–	Putrescine	–
Edestin	+	Glycyl-*l*-tyrosine	–	Cadaverine	–
Casein	+	Phenyl-alanine-*d*-alanine anhydride	–	Asparagine	–
Nuclein from Yeast	+	Glycyl-glycine	–	Urea	–
Yeast Juice	+	Biuret base	–	Uric Acid	–
Pancreatin	+	Histidine	–	Dicyanamide	+
Pepsin	+	Amine from Histidine	–	Creatine	+
Protein from Rice	+	Cystine	–	Creatinine	–
Normal Rabbit Serum	+	Amine from Cystine	–	Guanidine	?
Serum Globulin	+	Tryptophane	–	Methylguanidine	–
Globulin	+	Glycinetryptophane	–	Aminoguanidine	–
Egg Globulin	+	Amine from Tryptophane	–	Guanidine Hydrochloride	?
Clupein	+	Arginine	+	Guanidine Nitrate	?
Witte's Peptone	+	Agmatine	+	Guanidine Carbonate	?
Chapotraut's Peptone	+	Valine	–	Guanidineacetic Acid	+
Peptone Roche	–	Tyrosine	–	Semicarbazide hydrochloride	–
Peptone dry without salt	+	Aminotyrosine	–	Formamidine	–
Peptone pptd. by alc.	+	Tyrosine Anhydride	–	Succinimide	–
Peptone ex albumin	+	3·4 Dihydroxyphenyl-alanine	–	Succinamide	–
Peptone Riedel	+	Glycocoll	–	Acetamide	–
Peptone ex carne	+	Alanine	–	Sulphanilic Acid	–
Normal Urine	–	Phenylalanine	–	Potassium Cyanide	–
Broth	+	Leucine	–		
Meat extract	+	Glutamic Acid	–		
Spleen Autolyses	+				
Trypsin digestion of Casein	+				

SUMMARY.

The diacetyl reaction for proteins depends on the presence in the protein in question of the group $NH:C(NH_2).NH.R$.

The exact significance of R has not yet been worked out.

Only complex protein substances give any fluorescence, and this is lost if time is allowed for hydrolysis to take place before the diacetyl is added.

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