CV. THE AZINE AND AZONIUM COMPOUNDS OF THE PROTEOLYTIC ENZYMES. I.

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The immense importance of the establishment of the chemical nature of the proteolytic enzymes has led to many endeavours to purify them. From the earliest attempts of Schwann [1836] to the present day all these have met with obvious failure.

In 1905 it was observed by T. Brailsford Robertson [1907] that if one drop of saturated safranine (Grübler) is added to a solution of trypsin (Grübler) a precipitate forms which flocculates in the course of a few hours and collects at the bottom of the tube. Some years later Holzberg [1913] demonstrated that the precipitate was proteolytically active. With these two facts in view a study of the nature of this precipitate was undertaken.

Preparation of the Safranine Precipitate.

To prepare a sample of the precipitate, 100 g. of commercial pancreatin (Armour) was extracted with 500 cc. of water and the insoluble fraction filtered off. The amber-coloured solution so obtained was treated with an equal volume of 0·5 % safranine (Grübler) and the resulting precipitate allowed to flocculate for half an hour and then separated by centrifugalisation at high speed for twenty minutes. After washing the resulting fine slimy sediment with 0·5 % safranine, excess of the dye and the water were removed by six successive washings with absolute alcohol. The precipitate was then washed with ether and dried at 40°. The yield constitutes about 2 % of the original pancreatin employed. The precipitate is a red, finely grained powder, which in the presence of water emulsifies to form a violet solution which shows a remarkably strong proteolytic activity when the reaction of the solution is adjusted to p_H 8·0. The compound is hydrolysed by dilute acid (0·2 % HCl) with liberation of the free safranine chloride which may be extracted from its solution by butyl alcohol. The treatment with acid, however, greatly impairs the proteolytic activity owing to inactivation of the dissociated trypsin.

The solution from which the safranine precipitate is removed is completely inactive.

A comparison of the activity of the precipitate with that of the solution from which it was removed, and of the original solution from which it was prepared, is shown by the following experiment.

Comparison of the Relative Digestive Activity towards Caseinogen of the Safranine-Trypsin compound and its Equivalent of Unpurified Trypsin.

Solution A. The trypsin employed was the partially purified and very active product prepared by Grübler in Leipzig and sold as trypsin puriss. sicc. A 2 % solution in distilled water was used, the small insoluble residue being separated by centrifugalisation. The solution being freshly prepared before each experiment was preserved in ice until wanted.

Solution B. 4 cc. of Solution A were treated with 4 cc. of safranine 0·5 % in a small centrifuge tube with a conical bottom, and after allowing to flocculate for thirty minutes the resulting precipitate was separated in a high-speed centrifuge. The supernatant fluid was decanted and preserved as Solution C. The sediment was washed twice on the centrifuge with 8 cc. of 0·5 % safranine and then six times with absolute alcohol brought to p_H 6·0 with alcoholic HCl. It was then taken up in 4 cc. $M/15$ phosphate buffer (p_H 7·5). 2 cc. of this solution contain the safranine precipitate from 2 cc. of Solution A.

Solution C. This solution is the residue from the precipitation of the azine compound, 4 cc. being equivalent to 2 cc. of Solution A.

The Substrate. Carefully purified caseinogen, prepared by a modification of the method of Van Slyke and Bosworth [1913], was dissolved, with the aid of a mechanical stirrer, in 80×10^{-5} g. equivalents of NaOH (*i.e.* 8 cc. $N/10$ NaOH per g. caseinogen). To this solution, which is neutral to phenolphthalein (p_H less than 8·0), was added 3×10^{-5} g. equiv. of HCl, the addition of the acid being slow and accompanied by rapid stirring. The solution was then diluted so as to contain 4 g. of caseinogen per 100 cc. This solution was diluted with an equal bulk of $M/5$ phosphate buffer solution, prepared by adding 166 cc. $M/5$ Na_2HPO_4 to 34 cc. $M/5$ NaH_2PO_4, the p_H of which is 7·5. This substrate was preserved by the addition of toluene (0·5 cc.).

Technique of Experiment. 100 cc. portions of the substrate were measured into four long-necked glass-stoppered glass flasks and immersed in a thermostat which was capable of regulating the temperature to within 0·01°. When they had reached the temperature of the bath equivalent portions (2 cc.) of each of the enzyme solutions A, B, C were added to the flasks from which, after shaking, 2 cc. portions were withdrawn by means of a dry pipette and delivered into 2 cc. of $M/6$ CH_3COOH. Subsequently 2 cc. were withdrawn from each at half-hourly intervals and treated similarly. After allowing to flocculate the caseinogen was filtered off and the refractive index of the clear filtrate was determined by means of a Pulfrich refractometer, reading accurately to within 1 minute of the total angle of refraction, the refractive index of the

prism being 1·62098 (experimental error 0·00078 of the refractive index). The amount of caseinogen was determined from the formula:

$$\text{Caseinogen } \% = \frac{2\,(n - n')}{0{\cdot}00152},$$

where n is the refractive index of filtrate,

n' is the refractive index of control (prepared at 0 hour's digestion period),

0·00152 is the change of refractive index due to 1 g. of digested caseinogen in 100 cc. of solvent [Robertson, 1912].

The refractive index method of determining the rate of protein hydrolysis has the objection that the experimental error is large, being ± 0·1 g. of the caseinogen in the digest. However, with this difficulty in view, the velocity constant calculated from the mono-molecular reaction was found to be constant within the limits of the experimental error. The results are shown in Table I.

Table I.

Substrate = caseinogen 2 %. $p_H = 7{\cdot}5$. Refractive index = 1·33743. Temperature 40°.

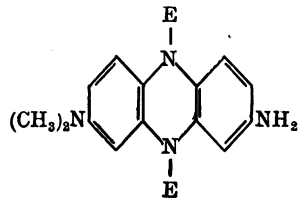
	Time (t) hrs.	Angle of refraction	Refractive index 0 hrs. $= n_1$	Refractive index at (t) $= n$	G. digested $= \frac{2\,(n - n_1)}{\cdot 00152}$	a	x	$K = \frac{1}{t} \log_{10} \frac{a}{a - x}$
Enzyme solution A, 2 cc. (original solution)	0	66° 39′	1·33591	1·33591	0·0	—	—	—
	½	66 36	1·33591	1·33614	0·3	2	0·3	14×10^{-2}
	1	66 33	1·33591	1·33639	0·5	2	0·5	12
	1½	66 31	1·33591	1·33655	0·8	2	0·8	14
	2	66 29	1·33591	1·33671	1·0	2	1·0	15
Enzyme solution B (azine precipitate from 2 cc. of A)	0	66 39	1·33591	—	0·0	—	—	—
	½	66 37	1·33591	1·33606	0·2	2	0·2	9
	1	66 35	1·33591	1·33623	0·4	2	0·4	9
	1½	66 32	1·33591	1·33647	0·6	2	0·6	10
	2	66 31	1·33591	1·33655	0·7	2	0·7	9
Enzyme solution C (residue from azine precipitate B)	0	66 39	1·33591	1·33591	0·0	—	—	—
	2	66 39	1·33591	1·33591	0·0	—	inactive	—

The table explains itself. The azine precipitate which constitutes only approximately 2 % of the preparation has retained at least 70 % of the activity shown by the original enzyme solution. The loss may be due to partial inactivation of the enzyme during the rather rough treatment it receives, but, in all probability it is due to the incomplete dissociation of the enzyme-azine compound. The solution from which the azine precipitate was removed was completely inactive.

With the possibility in view of determining the nature of the linkage between the enzyme and the safranine molecule, the action of the other azine bases (eurhodines, safranines, indulines) towards the proteolytic enzyme trypsin was determined. Of the series tried, all those compounds which were water-soluble and contained the azine nucleus were capable of completely precipitating the enzyme from its solution.

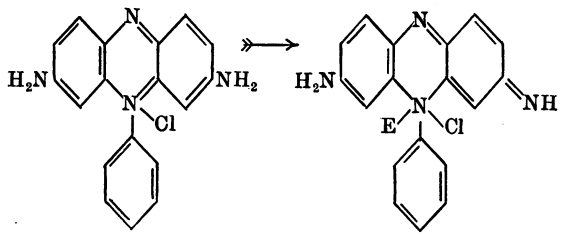
Thus dimethyldiaminophenazine hydrochloride (neutral violet), dimethyldiaminotoluazine hydrochloride (neutral red), diaminophenyltoluazonium

chloride (safranine), dimethyldiaminophenylphenazonium chloride, all quantitatively precipitate the active principle from an impure trypsin solution. Even the complex aminodianilinophenylphenazonium chloride (indamine blue) is active in the precipitation. The type of union between the enzyme and azine base proved to be a direct combination with the azine nitrogen. In the case of dimethyldiaminotoluazine hydrochloride the union would be represented thus, where E represents the Enzyme molecule:



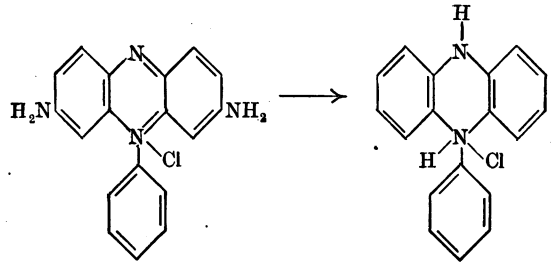
or in the case of the azonium base, safranine

Safranine (red) Safranine-enzyme cpd. (reddish violet)



The mono-enzyme compound would alter the constitution from the ortho- to para-quinoid structure, with a consequent tautomeric rearrangement and accompanying colour change. The colour change, from red to violet, is noticeable in the precipitate when redissolved. This may possibly be due to the occurrence of such a tautomer. The analogous mono-acid salt of safranine, violet in colour, occurs in solutions of moderately high acidity.

Confirmatory evidence of this hypothesis is available. The above reactions would necessitate the enzyme or combining substance to be predominantly acid in character. That this is so, is inferred from the experiment of Bayliss [1906] who showed that trypsin migrated to the anode when subjected to the influence of an electric current. This would be expected if the trypsin were acidic in character. The complete loss of the ability to precipitate the enzyme by the reduced "leuko" compound further supports the hypothesis of its linkage to the azine N.



Dye (red) Reduced leuko cpd.

The enzyme or the substance which carries down the enzyme so completely is precipitable by the azine and azonium bases, the basic N of the azine nucleus being active in this precipitation.

The study of the action of the azine bases was extended to other enzyme preparations. By means of fractional precipitation with alcohol and subsequent dialysis, very active preparations of trypsin may be prepared from extracts of the pancreas. However, the proteolytically active substances, precipitated by the azine bases from these so-called "pure trypsin" preparations, constitute but a very small proportion of the whole material, rarely more than 5 %. No preparation was examined from which the proteolytically active substance could not be precipitated quantitatively by the azine bases.

The azine precipitates from crude gland extracts retain their proteolytic activity, but show no lipolytic or diastatic activity, although both of the enzymes responsible for the latter actions may be demonstrated with ease in the original glandular extracts. The experimental data of this work will be published in a later paper.

The substance precipitable by azine retains the power to clot milk. As this property is common to all the proteolytic enzymes, the action of the azine bases was tried towards proteolytic enzymes from other sources, with the remarkable consequence that in all cases the azine base completely precipitates the whole of the proteolytically active substances from their solutions, thus:

Enzyme	Source	Base	Activity of azine ppt.	p_H
Pepsin	Stomach extract (pig)	Pheno-safranine	+	2 (HCl)
Trypsin	Pancreas (pig)	,,	+	7·5
Erepsin	Succus entericus (pig)	,,	+	7·5
Papain	Paw-paw (Parke, Davis and Co.)	,,	+	5·0
Erepsin	Yeast	,,	+	7·5

Fibrin was used as substrate in the case of the pepsin and trypsins; "Difco" standard peptone being used for the demonstrations of the activity of the erepsins.

Separation of Pepsin from Azine-pepsin Compound.

Owing to the stability of pepsin towards acids the pepsin-azine compound was further studied. At p_H 1·2 the compound is decomposed, and the base may be extracted with amyl or butyl alcohol. When the compound is shaken in a separating funnel with 0·5 % HCl and butyl alcohol, and allowed to stand, three layers are formed. The butyl alcohol layer contains most of the azine. A stable emulsion of butyl alcohol constitutes the second layer. The third or watery layer which should contain the free enzymes, however, is inactivated by this process. The active constituent was found to be adsorbed at the surface of the butyl alcohol droplets in the emulsion. When this emulsion is broken up by the addition of a few drops of ether, the original proteolytic activity is regained.

The pepsin-azine precipitate has been prepared in quantity. It is not widely different in its physico-chemical behaviour, from the trypsin-azine

precipitate. However, it is soluble in 0·5 % HCl. The effect of the quantitative precipitation of the proteolytically active substance from solution by the azine bases may be easily demonstrated with pepsin solutions because of their relative stability.

Discussion.

The power of the azine and azonium bases to remove completely the proteolytic enzymes from solution indicates that a direct union of the enzyme and the precipitating base is established. The linkage has been shown to occur through the basic nitrogen of the heterocyclic azine ring; this union, instituting a tautomeric rearrangement within the azonium base, causes a colour change. The recovery of the proteolytic power of the azine-enzyme compound depends on the conditions which most favour its dissociation. The compound is more highly dissociated by an increase of H ions. At p_H 2·0 the pepsin-azine compound, for example, is decomposed and the red azine dye set free.

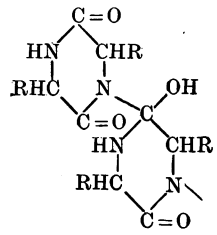
The compound is but slightly soluble in water at neutrality, but complete solution is obtained by the addition of peptone or protein digestion products. This, in all probability, is due to the dissociated enzyme ion combining with the protein digestion products, liberating the azine which reverts to the ortho-quinoid structure, the whole compound thus being decomposed and carried into solution.

The combination of the proteolytic enzyme with the azine nitrogen gives us evidence as to the nature of the combination between this enzyme and its substrate.

From time to time in biochemical literature the occurrence of diketo-piperazines in protein digests has been demonstrated. The presence of leucine anhydride among the cleavage products was first mentioned in 1849 by Bopp [1849]. Salaskin [1901] isolated diketo-piperazines from peptic and tryptic digestion products of oxyhaemoglobin. Abderhalden [1903] showed the presence of about 1 % of leucine anhydride in the products of HCl hydrolysis of caseinogen. He suggested that this anhydride was produced as a secondary product of leucine, and attempted, unsuccessfully, to demonstrate the reversal of the reaction. Water hydrolysis of proteins at high temperature produces about 1 % of leucine anhydride [Graves and Marshall, 1917].

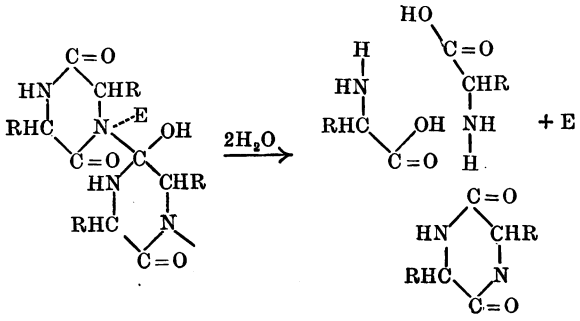
The catenary or chain-like configuration, as accepted in modern protein literature, fails to explain many peculiarities of the chemical actions of proteins. Such a structure would necessitate at least one free amino group at the end of the molecular complex. However, contrary to this, Van Slyke and Birchard [1914], and others, have shown that the nitrogen liberated from native proteins by the action of nitrous acid is equal to one-half of the lysine nitrogen content, that is, it is liberated from the NH_2 groups of the lysine bound up in the protein molecule. Some proteins, salmin and zein for example, when pure do not yield any nitrogen with nitrous acid, thus demonstrably lacking any free NH_2 groups.

These facts, together with other evidence hitherto unpublished, have led the author to put forward the hypothesis of a new form of protein structure. Instead of the conventional structure, the protein molecule may be considered to be built up of a series of amino-acid anhydrides, or, in other words, protein has essentially a poly-diketo-piperazine structure. Taking the type amino-acid $R.CH(NH_2).COOH$ for example, the protein complex is represented by this type of structure as



Such a structural configuration of the protein molecule would make quite clear the absence of terminal NH_2 groups. Peculiarities of protein ionisation and their neutralising power may be easily explained by means of this suggested structure. The similarity of each ring to the azine nucleus of the bases which are functional in combining with the enzyme suggests that the N in these groups is the seat of action of the enzyme.

The proteolytic enzyme E, combining with the nitrogen (N) of the diketo-piperazine ring would necessitate a structural change to supply the fifth valency of the nitrogen atom.

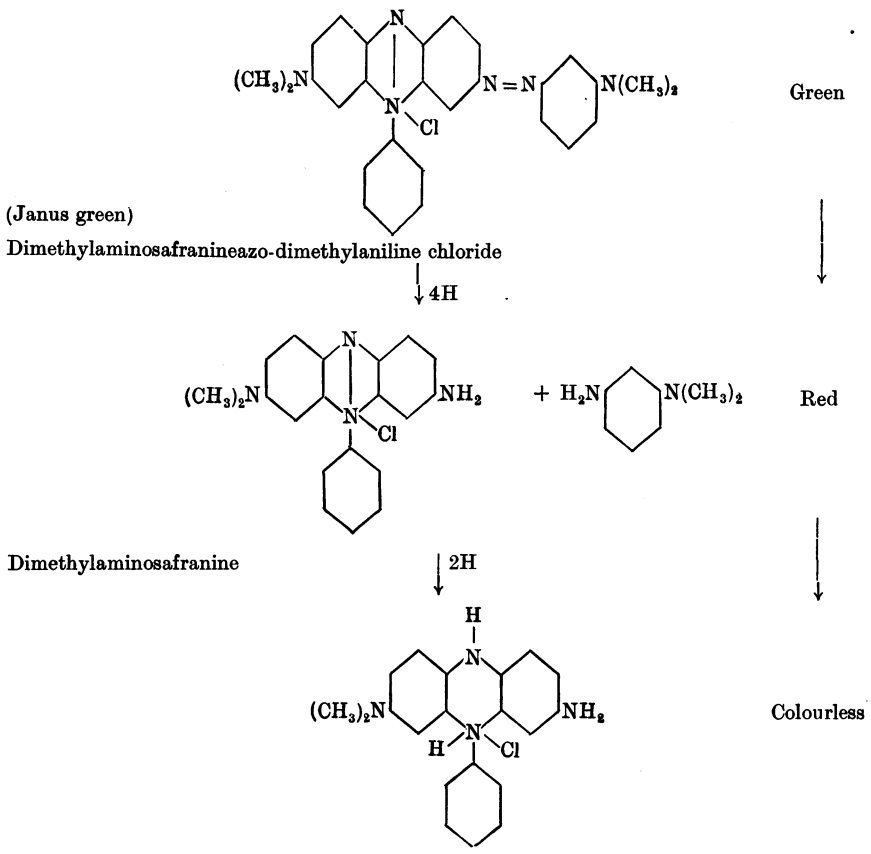


This could be satisfied by the addition of two molecules of water, resulting in the separation of two molecules of amino acid. The enzyme at this point is set free to act on a fresh group.

As the azine and azonium bases are coloured substances the occurrence of the proteolytic enzymes in the cell may be demonstrated by means of their selective staining reactions.

Michaelis, in 1899 [quoted by Cowdry, 1918, p. 86], while making a detailed study of the behaviour and chemical nature of the *intra vitam* dyes, found that dimethylsafranineazodimethylaniline (Janus green) specifically stained certain structures in the living cell. These structures have since been demonstrated

in all cells, from the bacterium to the angiosperm in the plant kingdom, and from amoeba to man in the animal kingdom. Cowdry [1918] showed that ethylsafranine will also stain specifically the mitochondria (as these structures are generally named). He also demonstrated that the preparation stained with Janus green, if kept under anaerobic conditions, will manifest a series of colour changes, the mitochondria changing from green to red and then becoming colourless. This, he points out, is due to the reduction of the dyestuff. If we follow out the reactions involved



leuko-Dimethylaminosafranine.

we see that the point of combination of the dye with the mitochondria could be no other than the N of the azine nucleus. After the stained tissue preparation is bleached by reduction it cannot be restained by application of more dye, so the groups to which the dye is linked are fully saturated with the dye. The modification of the mitochondrial constituents of the cells into zymogen granules during active secretion of the pancreas has been demonstrated by various workers.

The specific staining of the mitochondria with the azine dyestuffs indicates that the proteolytic enzymes are concentrated in these bodies. The mitochondria may be thus the site of syntheses in the cell, the water-poor phases which exist at the surface of the lipoid constituents of the mitochondria instituting favourable conditions for the synthetic activities of the enzyme.

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Note.—The possibility of the existence of diketopiperazine rings in the protein molecule is discussed by Abderhalden [*Z. physiol. Chem.* 1923, **128**, 119] in a paper which appeared in May 1923 but was not accessible in Adelaide at the date when Mr Marston's paper was despatched to England. EDITOR.]