potassium *tert.*-butoxide in *tert.*-butanol on a Δ^4 -3-oxo steroid. This solution method may be more convenient for measuring relatively pure steroids. The paper method, however, has the advantage that additional separation of steroids is obtained during the analytical procedure and the presence of compounds, other than the one to be measured, is revealed; this method may therefore be preferable for the analysis of impure fractions.

It seems likely that the method for measuring fluorescent compounds on paper can be applied to substances other than Δ^4 -3-oxo steroids. During the preparation of this paper our attention was drawn to the description of such an application by other workers (Mourodoneanu, Sanford & Hitchcock, 1955).

SUMMARY

1. A method has been described in which amounts of Δ^4 -3-oxo steroids greater than $0.2 \mu\text{g.}$ can be estimated by measuring their fluorescence after chromatography on paper and development by a solution of sodium hydroxide.

2. The intensity of fluorescence is proportional

to the quantity of steroid if the final spot contains less than $4 \mu\text{g.}$ of steroid and is less than 2 cm. in diameter.

3. If four spots (two of them standards) are used for an analysis, the coefficient of variation of the estimate is between 6 and 10 %.

Financial assistance from the Medical Research Council and the Nuffield Foundation has made this work possible. Dr P. Armitage gave us valuable advice on statistical matters. We have had invaluable technical assistance from Messrs J. Tierney and D. Staples. We wish to acknowledge the encouragement and interest of Professor Sir Charles Dodds, F.R.S., and Professor J. E. Roberts.

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The Reactions of Diazonium Compounds with Amino Acids and Proteins

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(Received 26 March 1956)

The reaction between diazonium compounds and proteins in alkaline solution was first studied in 1904, with sulphanilic acid as the amino compound (Pauly, 1904). Coloured compounds were formed, and the reaction was investigated by allowing the diazonium compound to react separately with all the amino acids then known to be present in proteins. It was found that only tyrosine and histidine gave coloured solutions. Later, Pauly (1915) isolated derivatives from these two amino acids with diazotized *p*-arsanilic acid.

In all azoproteins prepared by treating proteins with an excess of diazotized *p*-arsanilic acid, more arsenic was found on analysis than is to be expected from the combined tyrosine and histidine content (Hooker & Boyd, 1934; Boyd & Mover, 1935; Kapeller-Adler & Boxer, 1936). In an attempt to explain the results it was suggested that diazonium compounds could also react with phenylalanine, tryptophan, proline and hydroxyproline. Kapeller-Adler & Boxer (1936) reported that they had pre-

pared and isolated derivatives from these amino acids with diazotized *p*-arsanilic acid.

The reaction of diazotized *p*-sulphanilic acid with horse serum and compounds with groupings found in proteins has been investigated (Eagle & Vickers, 1936). Indole, the aliphatic amino group of glycine, α -alanine, phenylalanine, ornithine and lysine and the imino group of proline and hydroxyproline were found to deactivate the diazonium compound, from which it was concluded that reaction occurs with aliphatic amino and imino groups in proteins but not with peptide linkages, guanidino or amide groupings. No attempt was made to prepare derivatives of compounds containing the groupings mentioned above: the destruction of the diazonium compound was followed by the addition of alkaline β -naphthol.

Derivatives of glycine have been isolated by treating it with diazotized aniline and nitroaniline (Busch, Patrascanu & Weber, 1934). Subsequently derivatives have been isolated from the products of

interaction of diazotized 5-chloro-2-methylaniline and (a) proline, (b) cysteine respectively (Zahn, Wollemann & Waschka, 1954). Zahn *et al.* also state that with aliphatic amino acids a diazoaminobenzene is formed.

In the present paper the reaction of diazonium compounds with amino acids and compounds containing groupings present in proteins is described. Derivatives have been prepared and isolated, and the influence of the position of the amino group on the nature of the product of reaction has been studied. The results of these experiments have been compared with analytical data on the products obtained by coupling proteins with diazotized *p*-arsanilic acid.

RESULTS

Reaction with amino groups

Glycine was found to react with diazotized aniline in alkaline solution to form bisbenzenediazoglycine, $(C_6H_5.N:N)_2N.CH_2.CO_2H$. A small amount of diazoaminobenzene also was produced. It might be expected that α -amino acids other than glycine would react similarly, but our experiments have shown that this is not so. Three α -amino acids, namely α -alanine, phenylalanine and leucine, gave diazoaminobenzene as the main product and no triazen was formed. The conversion of a diazonium compound into a diazoamino compound by α -amino acids could be explained by the following series of reactions:

Support for this mechanism is provided by the isolation of DL-phenyllactic acid in the experiments with DL-phenylalanine. The hydroxy acid was prepared from the amino acid and pure crystalline diazobenzene hydrogen sulphate to prevent its formation from excess of nitrous acid used in diazotization. Further, the percentage yield of DL-phenyllactic acid was comparable with that of diazoaminobenzene. No evidence was obtained that the diazoaminobenzene and α -hydroxy acid are formed by the spontaneous decomposition of the aliphatic triazen, for bis(benzenediazo)glycine was quite stable in cold sodium carbonate solution, as were the other aliphatic triazens which were prepared.

An investigation was made of the influence of the position of the amino group in the acid chain on the nature of the products. In experiments with very dilute solutions (about 1%) of methylamine and ethylamine in M sodium carbonate solution, the bis(benzenediazoamino)amine was formed almost

quantitatively. Amino acids in which the amine grouping is terminal but not in the α -position, such as β -alanine, γ -aminobutyric acid, ϵ -aminocaproic acid and glycylglycine, all gave high yields of the bis(benzenediazoamino) compound, with no detectable formation of diazoaminobenzene. It is concluded, therefore, that compounds containing the $-CH_2.NH_2$ grouping all react to form aliphatic triazens.

An amino acid in which the amino group was neither terminal nor in the α -position, such as β -aminobutyric acid, gave diazoaminobenzene as the chief product, and in contrast to the above-mentioned amino acids gave no evidence of triazen formation. A similar result was obtained with *iso*-propylamine, and it is concluded that compounds containing the group $CH_3.CH(NH_2).CH_2-$ do not form bis(benzenediazo)amino compounds.

It was noted that the rate of formation of diazoaminobenzene was slow, several hours being necessary for complete reaction. It is suggested, therefore, that diazoaminobenzene is produced only when the amino compound does not form a triazen or forms one slowly as does glycine. The groupings adjacent to the amino group affect the rate of reaction with diazonium salts, and the following combinations cause a decrease in reactivity:

Our experiments with diazotized *p*-arsanilic acid have not been as extensive or as detailed as those described with aniline. The diazonium compound is difficult to work with and the products of reaction are insoluble in non-polar and unstable in polar solvents. Two derivatives, however, were prepared as acids from methylamine and *n*-butylamine, and three more from amino acids were isolated as their sodium salts. A complete analysis was not possible on the last three compounds, owing to the coprecipitation of small amounts of inorganic salts, but the As/N quotients indicated formation of products analogous to those obtained with diazotized aniline: further, DL-phenyllactic acid was isolated from the reaction with DL-phenylalanine in good yield.

Reaction with guanidino groupings

Three equivalents of diazotized aniline were added to methylguanidine, and benzenediazomethylguanidine was isolated. The structure of this compound is probably represented by

analogous to the derivative prepared from dicyanodiamide by Walther & Grieshammer (1915).

Although a large excess of the phenyldiazonium salt was used there was no evidence that further reaction could take place.

Reaction with sulphhydryl and aliphatic hydroxyl groups

In an experiment with *p*-diphenylamine and ethanethiol, a compound was isolated which contained no nitrogen, but had a sulphur content corresponding to that of ethyl *p*-diphenyl thio ether. This compound was presumably formed from the extremely unstable *S*-azo derivative.

Reaction with indolyl groups

Experiments with 3-indolylacetic acid showed that reaction with indoles substituted in the 3-position takes place to give an unstable product. It seems probable that the diazonium salt reacts with 3-indolylacetic acid at the imino group to form a triazen, and that the triazen can undergo rearrangement to form a coloured *C*-azo compound.

Reaction with proteins

Effect of pH on the reaction. Solutions obtained by diazotizing 0.25 g. or 0.5 g. of *p*-arsanilic acid were added to 0.25 g. of casein dissolved in 12.5 ml. or 25 ml. respectively of the following reagents: *m*-NaHCO₃, 0.5 *m*-Na₂CO₃, *m*-Na₂CO₃, *m*-Na₂CO₃ and *N*-NaOH (1:1, v/v). The pH was determined with a glass electrode and the products were treated as described in the Experimental section. The results (Table 1) show that a product with the highest As/N quotient was obtained when the reaction was carried out at pH 9.8.

Maximum reaction at pH 9.8. *p*-Arsanilic acid (0.25 g.) was diazotized and added to a cold soln. of 0.25 g. of protein in 12.5 ml. of *m*-Na₂CO₃. Multiples of this amount of *p*-arsanilic acid were used with the same weight of protein dissolved in correspondingly larger volumes of *m*-Na₂CO₃. A series of compounds was thus prepared for each of four proteins, with the quotient *p*-arsanilic acid/protein (w/w) ranging from 1 to 6. The analytical results are shown in Table 2. The As/N quotients in the products

Table 1. Variation of arsenic/nitrogen quotient of casein conjugated with *p*-arsanilic acid at various pH values

Alkaline solution	pH	<i>p</i> -Arsanilic acid (g./g. of protein)	Analysis of product		
			As (%)	N (%)	As/N
<i>m</i> -NaHCO ₃	7.5	1	7.1	11.6	0.612
0.5 <i>m</i> -Na ₂ CO ₃	9.3	1	8.9	11.1	0.802
0.5 <i>m</i> -Na ₂ CO ₃	9.1	2	9.5	11.6	0.819
<i>m</i> -Na ₂ CO ₃	9.8	1	10.2	12.3	0.848
<i>m</i> -Na ₂ CO ₃	9.8	2	11.1	11.7	0.949
<i>m</i> -Na ₂ CO ₃ - <i>N</i> -NaOH	11.5	1	4.6	11.8	0.390

Table 2. Arsenic/nitrogen quotients of proteins conjugated with diazotized *p*-arsanilic acid at pH 9.8, and comparison with values theoretically possible

Protein	<i>p</i> -Arsanilic acid (g./g. of protein)	Analysis of product			Theoretical values for As/N*		
		As (%)	N (%)	As/N	1	2	3
Casein	4	11.5	11.7	0.983	0.939	0.837	0.440
	3	11.7	11.9	0.983			
	2	11.1	11.7	0.949			
	1	10.2	12.3	0.848			
Ovalbumin	4	10.8	11.8	0.915	0.883	0.721	0.300
	3	11.1	12.0	0.925			
	2	10.1	12.0	0.855			
Edestin	6	12.1	12.1	1.000	1.034	0.678	0.289
	4	12.1	12.2	0.992			
	3	11.6	12.4	0.936			
	2	11.2	12.3	0.911			
Bovine-plasma albumin	4	12.7	11.6	1.095	1.088	0.968	0.424
	3	12.7	11.6	1.095			
	2	11.8	12.1	0.975			
	1	9.6	12.4	0.774			

* Column 1. Theoretical values of As/N assuming that 2 moles of diazotized *p*-arsanilic acid react with 1 mole of phenolic, imidazole, α -amino groups of terminal glycine residues, ϵ -amino groups; 3 moles with 1 mole of guanidino group and 1 mole with indolyl and sulphhydryl groups, also assuming deamination of α -amino groups of terminal amino acids other than glycine. Column 2. As above, except 1 mole of diazotized *p*-arsanilic acid assumed to react with 1 guanidino group. Column 3. Assuming 2 moles of diazotized *p*-arsanilic react with 1 mole of phenolic and imidazole groups only.

obtained are compared with those theoretically possible from the amino acid compositions of the proteins given by Tristram (1953).

Stability of the azoproteins. The ppt. (0.5 g.) from casein coupled with 1 g. of diazotized *p*-arsanilic acid/g. of protein and having a value for the As/N quotient of 0.848 was dissolved in 25 ml. of 0.5M-NaHCO₃. This solution was divided into two parts; one part, left at 5° for 24 hr. and then treated in the normal manner, gave a product with an unchanged As/N quotient of 0.842, but the other, kept at 37° for the same time, gave a product with a diminished As/N quotient of 0.765. A product obtained from bovine-plasma albumin, having an initial As/N quotient of 0.774, was also dissolved in NaHCO₃. After 24 hr. at 5° the value was also unchanged, but after the same time at 56° it had changed to 0.536. These azoproteins therefore contain linkages unstable to heat.

Homogeneity of the azoproteins. The azoproteins (10 mg.), obtained from diazotized *p*-arsanilic acid and casein, ovalbumin, edestin, and bovine-plasma albumin, were dissolved in 0.5M-NaHCO₃ (about 5 ml.). A sample (0.01 ml.) of each solution was applied to Whatman no. 1 paper, and electrophoresis was carried out in a veronal buffer (pH 8.6, *I* = 0.05) for 7 hr. with a current of 2 mA/5 cm. strip and potential gradient of 5 v/cm. Typical products from each of the purified azoproteins were thus developed and in none was more than one band, orange-coloured, observed. Migration occurred towards the anode compartment and for proteins coupled with 4 g. of *p*-arsanilic acid/g. of protein the distances moved were as follows: casein, 5.5 cm.; ovalbumin, 6.1 cm.; bovine-plasma albumin, 4.9 cm.; edestin 5.8 cm.

DISCUSSION

Diazonium compounds have been used extensively to produce artificial antigens, Landsteiner & Lampl (1917, 1918) being the pioneers in this field. A more recent use of the method is demonstrated in papers in which the present authors collaborated (Coombs, Mynors & Wild, 1951; Britton, Coombs, Mynors & Wild, 1951; Coombs, Howard & Wild, 1952; Coombs, Howard & Mynors, 1953). The reactions of diazonium compounds with histidine and tyrosine were the first reactions to be investigated and derivatives were isolated by Pauly (1915) from the highly coloured reaction mixtures. It has not been realized previously that coupling reactions with other amino acids are possible to give colourless products, and a recent review (Herriott, 1947) has emphasized the incomplete state of present knowledge of this reaction. It was thought that further work was necessary in the investigation of the reactions of diazonium compounds with individual amino acids and the correlation of these

results with the possible reactions with proteins. The results published in the early literature are considered in this paper and explained in a general reaction mechanism.

The isolation of an aliphatic triazen by the reaction of an amino acid with a diazonium compound was first reported by Busch *et al.* (1934), who isolated bis(benzenediazo)glycine from the reaction of diazotized aniline and glycine in neutral solution. They reported in addition that no product was formed when excess of sodium hydroxide was present. We have confirmed these results but have noted that in weak alkali, such as sodium carbonate, the yield of triazen is similar to that obtained in neutral solution. Like Busch *et al.* (1934), we have also isolated diazoaminobenzene from the products of the reaction.

Glycylglycine was found to react under the same conditions as glycine to form a triazen exclusively, no diazoaminobenzene being formed. As the reaction for proteins is usually carried out in weak alkali, these results indicate that proteins containing *N*-terminal glycine residues could react to form bis-(diazo)amino linkages.

Not all amino acids were found to behave like glycine in their reaction with diazonium compounds. In experiments with diazotized aniline and diazotized *p*-arsanilic acid, only those compounds containing a $-\text{CH}_2\text{NH}_2$ group were found to form triazens. Amino compounds containing the group $\text{R.CH}(\text{NH}_2)-$ gave a diazoaminobenzene as the chief product. The latter type of reaction is not peculiar to amino acids, since Bucherer & Wolf (1904) found that ammonia and ammonium acetate convert diazotized *p*-nitroaniline quantitatively into *pp'*-dinitrodiazoaminobenzene. These workers suggested that *p*-nitroaniline was formed in the reaction and reacted spontaneously with unchanged diazonium compound. The application of this hypothesis to the reaction of certain amino acids with diazonium compounds entails the conversion of the amino acid into a hydroxy acid. Our experiments support the view that this change occurs, since we isolated phenyllactic acid from the products of the reaction with phenylalanine.

Zahn *et al.* (1954) have also found that valine, glycine and leucine give a diazoaminobenzene when allowed to react with diazonium compounds. Our results are in agreement with their findings, but not all amino acids give diazoaminobenzenes with diazonium compounds. Apart from glycine none of the compounds they used contained a $-\text{CH}_2\text{NH}_2$ group, which we find gives a triazen and not (except with glycine) a diazoaminobenzene.

ϵ -Aminocaproic acid was among the compounds which were found to give a triazen. This result would indicate that in the diazo reaction with proteins reaction could occur to form bis(diazo)amino links with $\epsilon\text{-NH}_2$ contributed by lysine.

α -Amino acids other than glycine have been found not to form triazens. In proteins which contain amino groups contributed by α -amino acids other than glycine, attack by the diazonium compound would be expected to proceed by the proposed 'deamination' mechanism. This would involve the deamination of the amino group.

Our results are in complete disagreement with those of Kapeller-Adler & Boxer (1936), who described the preparation of a derivative of phenylalanine in which two moles of diazotized *p*-arsanilic acid combined with one mole of the amino acid. The proposed structure of the highly coloured material was that of a *C*-azo compound, analogous to that of the tyrosine derivative previously isolated by Pauly (1915). We have found that the chief products of the above reaction are phenyllactic acid and sodium *pp'*-diazaminobenzenearsonate.

Diazonium compounds can also react with the guanidino group. In experiments with methylguanidine and diazotized aniline, a new derivative, benzenediazomethylguanidine, was isolated. This confirms the early work of Walther & Grieshammer (1915), who showed that the guanidino group of dicyanodiamide, $\text{H}_2\text{N} \cdot \text{C}(\text{NH}) \cdot \text{NH} \cdot \text{CN}$, could form a derivative with diazonium compounds. Similar compounds prepared from guanidino-taurine have been reported in the patent literature (Lecher & Orem, 1939). Although we have found that at least one diazonium group reacts with one guanidino group, it is our opinion that further reaction involving a total of three diazonium groups and one guanidino group could occur since the latter contains three active hydrogen atoms. Failure to obtain further reaction with methylguanidine was probably due to the extreme insolubility of the product, benzenediazomethylguanidine, in the aqueous media.

The isolation of a new derivative from diazotized *p*-diphenylamine and ethanethiol confirms the work of Stadler (1884), who showed that sulphhydryl groups give unstable *S*-azo derivatives with diazotized *p*-sulphanilic acid and diazotized aniline. Similar *S*-azo compounds have been obtained by Friedländer & Chwala (1907). Sulphydryl groups in proteins would be expected to react in a similar manner.

The reaction of aliphatic hydroxyl groupings was not examined, as it has been fully investigated already (Hantzsch, 1898). It is possible that hydroxyl groups in proteins may be oxidized slowly to aldehydic groupings by diazonium compounds when present in excess.

Aliphatic imino compounds have been shown by Jacobs & Heidelberger (1921) to form diazoimino linkages with diazotized *p*-arsanilic acid. Furthermore, derivatives of proline with diazonium compounds involving the same linkage has been re-

ported in the patent literature (Markush, 1933). In proteins, however, the free imino groups of proline and hydroxyproline occur only if these amino acids are present as end groups. The results of these workers do not support the claim of Kapeller-Adler & Boxer (1936) that highly coloured *C*-azo compounds can be formed from proline and hydroxyproline.

It has been suggested by Pauly & Gundermann (1908) that tryptophan and other 3-substituted indoles do not react with alkaline solutions of diazonium compounds, as the colour which is produced by tyrosine and histidine is not given by tryptophan. Our experiments with 3-indolylacetic acid have shown that reaction with 3-substituted indoles such as tryptophan can occur. Although the product was too unstable in acid to isolate, it is suggested that the tryptophyl group in proteins would react with at least one diazonium group.

All other groupings known to be present in proteins, including the amide linkage, have been considered and no evidence that they react with diazonium compounds has been obtained. We believe that the only reactions possible are those considered above.

The capacity of proteins to couple with diazotized *p*-arsanilic acid has been closely investigated by several workers (Hooker & Boyd, 1934, Boyd & Mover, 1935, Kapeller-Adler & Boxer, 1936), but we believe the results as yet reported are inaccurate and that the explanation of the reactions is incomplete. The extent of coupling has been calculated from the arsenic/nitrogen quotient of the azo-protein, but as an excess of the diazo compound is used to ensure complete reaction with the protein it is essential to carry out the analyses on a derivative which is not contaminated with decomposition products of diazotized arsanilic acid and to use methods of analysis which are accurate. The method of purification, therefore, is of great importance. Kapeller-Adler & Boxer (1936) precipitated the azo-proteins by acidifying the aqueous alkaline reaction mixture. It is certain, therefore, that the derivatives used in their analyses were impure, for we have found that if *p*-arsanilic acid is diazotized, left to stand in alkaline solution for a number of hours and then acidified, a copious brown precipitate is formed. The precipitate will dissolve in alkali but is reprecipitated on acidification. Hooker & Boyd (1934) acidified an 80% alcoholic solution, in which the decomposition products of the diazotized *p*-arsanilic acid are soluble, and so prevented coprecipitation. The percentage of nitrogen in the azoprotein was determined by the Kjeldahl method, and it is accepted that this method of estimation gives a low value when the azo linkage is present (Kirk, 1950). We have repeated the preparations and estimations of the previous workers and confirm

that Kjeldahl values for nitrogen in the azoproteins containing a large number of azo linkages may be 20% lower than those found by the micro-Dumas estimation.

In our experiments diazotized *p*-arsanilic acid was coupled with casein, ovalbumin, edestin and bovine-plasma albumin, under the conditions described previously by Hooker & Boyd (1934). In addition, the solution of azoprotein was dialysed, which removed a further amount of impurity.

The precipitated azoproteins were not dried under vacuum, as the possibility existed of loss of nitrogen by this procedure. Analyses were done on the material dried in air, in contrast to the technique of analysis of the solution (Hooker & Boyd, 1934).

Kapeller-Adler & Boxer (1936) have reported that analysis for nitrogen by the micro-Dumas estimation gives a lower value than that obtained by Friedrich's method. We have not confirmed these findings. In our experiments only a small difference was noted and the micro-Dumas method gave the higher value. The estimation of the percentage of nitrogen, therefore, has been done by the micro-Dumas method.

Preliminary experiments were carried out to find the optimum pH for the reaction. It was found that the maximum coupling takes place at about pH 10. The main experiments were carried out at pH 9.8, so that it was possible to use the smallest amount of the diazotized *p*-arsanilic acid and obtain the greatest amount of coupling. We found that for complete reaction, 1 g. of casein, ovalbumin, or bovine-plasma albumin needed 3 g. of the diazonium compound, whereas edestin required 4 g. The derivatives so prepared were homogeneous and stable in the solid state. They could be stored in solution at 5°, but when kept at either 37° or 56° for some time the arsenic/nitrogen quotient fell considerably, showing that some of the linkages were labile to heat.

From the evidence considered above, it seemed probable that the reaction of proteins with diazonium compounds would occur through groupings other than the phenolic and imidazole groups which have been assumed to be involved since the work of Pauly (1904). The experimental values for the arsenic/nitrogen quotients were compared with the theoretical values on the assumption that reaction can take place with other groups present. On the basis of these experiments, we now propose that the phenolic group of tyrosine, the imidazole group of histidine and the ϵ -amino group of lysine react with 2 moles of a diazonium compound; that the sulphhydryl group of cysteine and the indolyl group of tryptophan react with 1 mole; and that the guanidino group of arginine reacts with at least 1 and probably 3 moles. The α -amino group of glycine reacts with 2 moles of the diazonium salt when this

amino acid is present as a terminal residue, but the α -amino grouping of other amino acids present are deaminated. Our experiments show that the $-\text{CH}_2\text{NH}_2$ group must be present to allow the formation of a triazen. In the proteins we have used, the imino groupings of proline and hydroxyproline do not occur as end groups, but it is probable that if proteins containing such groupings were used reaction would take place between the *N*-terminal residue and 1 mole of the diazo reagent.

The experimental and theoretical arsenic/nitrogen quotients are in close agreement, for the proteins used, on the assumption that coupling takes place in this manner and that the guanidino group reacts with a maximum of 3 moles of the diazo reagent. In particular, this is true for edestin with its very high arginine content.

EXPERIMENTAL

Unless otherwise stated, 'light petroleum' refers to solvent boiling within the range 60–80°.

Reactions with diazotized aniline

An aqueous solution of benzenediazonium chloride was prepared by dissolving 1.2 g. of aniline hydrochloride in 25 ml. of water and 2 ml. of conc. HCl. To the ice-cold solution was added 11.3 ml. of *m*-NaNO₂ slowly and the mixture kept at 5° for 15 min. The diazo solution was added to the following compounds, in the quantities indicated, dissolved in 50 ml. of cold *m*-Na₂CO₃. The reaction mixtures were left overnight at 4°.

(a) *Primary amino compounds which react with diazotized aniline to form triazens.* (i) Methylamine (0.64 g. of methylamine hydrochloride). A yellow oil separated rapidly and on standing solidified to a red solid. Light-yellow crystals of bis(benzenediazo)methylamine were obtained after recrystallization from light petroleum, the solution being decolorized with charcoal; yield 90%; m.p. 113–114°. (Found: N, 29.2. Calc. for C₁₃H₁₃N₅: N, 29.3%.) Goldschmidt & Badl (1889) give m.p. 112–113°.

(ii) Ethylamine (0.77 g. of ethylamine hydrochloride). A yellow oil separated immediately and solidified. Light-yellow crystals of bis(benzenediazo)ethylamine were obtained after recrystallization from aqueous ethanol, the solution being decolorized with charcoal; yield, 90%; m.p. 69.5°. (Found: N, 27.6. Calc. for C₁₄H₁₅N₅: N, 27.7%.) Goldschmidt & Badl (1889) give m.p. 70–71°.

(iii) Glycine (0.345 g.). A red-orange solution with some brown solid was obtained. The filtered solution was acidified with 2.5 *N*-HCl and the yellow precipitate formed filtered, washed with water and dried. Light-yellow crystals of bis(benzenediazo)glycine were obtained by crystallization from benzene; yield 35%; explodes 109°. (Found: N, 24.5. Calc. for C₁₄H₁₃O₂N₅: N, 24.7%.) Busch *et al.* (1934) give decomp. 124°. The brown solid from the reaction mixture was dried; light-yellow crystals of diazoaminobenzene were obtained after recrystallization from light petroleum and decolorizing with charcoal; yield 10%; m.p. 99° (unchanged by admixture with an authentic sample, m.p. 99°).

(iv) β -Alanine (0.5 g.). A yellow solid separated after 2 min. This solid was completely soluble in a large excess of

water and was presumably the sodium salt of the compound formed. The reaction mixture was acidified with 2.5 N-HCl and the resulting solid filtered, washed with water and dried, giving a light-brown powder. Light-yellow crystals of bis(benzenediazo)- β -alanine were obtained after recrystallization from benzene-light petroleum; yield 65%; decomp. 101° (Found: N, 23.4. $C_{15}H_{15}O_2N_4$ requires N, 23.6%).

The reaction of γ -aminobutyric acid, ϵ -aminocaproic acid and glycylglycine was similar to that described above for β -alanine. The treatment was in every respect identical except for the solvents used in recrystallization.

(v) γ -Aminobutyric acid (0.474 g.). Yellow crystals of bis(benzenediazo)- γ -aminobutyric acid were obtained after recrystallization from light petroleum; yield 60%; m.p. 103° (decomp.). (Found: N, 22.4. $C_{16}H_{17}O_2N_4$ requires N, 22.5%.)

(vi) ϵ -Aminocaproic acid (0.603 g.). Yellow crystals of bis(benzenediazo)- ϵ -aminocaproic acid were obtained after recrystallization from light petroleum, yield 60%; m.p. 93°. (Found: N, 20.5. $C_{18}H_{21}O_2N_4$ requires N, 20.7%.)

(vii) Glycylglycine (0.625 g.). Light-yellow crystals of bis(benzenediazo)glycylglycine were obtained after recrystallization from benzene-light petroleum; yield 70%; decomp. 105°. (Found: N, 24.6. $C_{18}H_{16}O_2N_4$ requires N, 24.7%.)

(b) *Amino compounds which yield diazoaminobenzene as the chief product.* (i) α -Alanine (0.42 g.). After the addition of the diazotized aniline, the reaction mixture became opalescent and within 10 min. a ppt. formed which gradually increased over several hours. A continuous evolution of N_2 occurred throughout the reaction. (A Na_2CO_3 soln. without α -alanine gave no visible evolution of N_2 gas or opalescence until after 1 hr.) The yellow-brown ppt. was filtered off and dried. The light-yellow filtrate gave no ppt. on acidifying with 2.5 N-HCl. Since recrystallization proved ineffective, the brown product was purified in the following way. Alumina slurry with light petroleum was used to prepare a column 30 cm. diam., 150 cm. in height. The product (300 mg.) was dissolved in 20 ml. of light petroleum and added to the column, which was developed with light petroleum. A yellow band separated from a stationary black band at the top of the column. Both bands were eluted with ethanol after the column had been cut into sections, and the products obtained by evaporation of the solvent. The black band could not be recrystallized and was probably a mixture of decomposition products of the diazonium compound. The yellow band yielded a yellow solid from which light-yellow crystals of diazoaminobenzene were obtained after recrystallization from light petroleum; yield 45%; m.p. 99° (not depressed by an authentic sample of diazoaminobenzene, m.p. 99°). (Found: N, 21.4. Calc. for $C_{12}H_{11}N_3$: N, 21.3%.)

The behaviour of the following compounds was found to be similar to that described for α -alanine. Diazoaminobenzene was isolated in each case by means of the chromatographic method, and identified by means of a mixed m.p. determination. The presence of an aliphatic triazen could not be demonstrated by acidification of the alkaline filtrate.

(ii) *iso*Propylamine (0.34 ml.); yield of diazoaminobenzene 40%.

(iii) β -Aminobutyric acid (0.474 g.); yield 45%.

(iv) DL-Leucine (0.60 g.); yield 25%.

(v) DL-Phenylalanine (0.76 g.); yield 40%. This compound was also allowed to react with benzenediazonium

hydrogen sulphate, which was prepared in solid form (Mann & Saunders, 1952) and washed well with ethanol and then with ether to remove impurities. Benzenediazonium hydrogen sulphate (2.5 g.) was dissolved in 10 ml. of ice-cold water and a small quantity of green precipitate removed by filtration. The filtrate was added to 1 g. of phenylalanine dissolved in 40 ml. of cold M- Na_2CO_3 (reaction mixture at pH 10). The mixture was left overnight at 4°. Evolution of N_2 occurred throughout the reaction. The reaction mixture was filtered and the precipitate purified by chromatography on alumina as described above. Diazoaminobenzene was obtained with m.p. 99° (not depressed by an authentic sample, m.p. 99°); yield 45%. The filtrate from the reaction mixture was acidified with 2.5 N-HCl and gave no ppt. The soln. was then extracted with ether several times (total vol. 500 ml.) and the ethereal layer dried with anhydrous $CaSO_4$. Evaporation of the ether yielded a brown syrup which solidified in the presence of light petroleum, b.p. 40–60°. Colourless crystals of DL-phenyllactic acid were obtained after recrystallization from benzene; yield, 43%; m.p. 97° (not depressed by an authentic sample, prepared by a new and simpler method described below). (Found: C, 65.0; H, 6.1. Calc. for $C_9H_{10}O_3$: C, 65.1; H, 6.0%.)

Authentic DL-phenyllactic acid was prepared by dissolving 1 g. of DL-phenylalanine in 50 ml. of water and 4 ml. of conc. HCl; 1.3 g. of NaNO₂ in 25 ml. of water was added to the cold soln. and the mixture left overnight at 5°. Extraction of the soln. with ether (200 ml.) and its subsequent distillation yielded a colourless syrup which solidified on rubbing. Colourless crystals of DL-phenyllactic acid were obtained by recrystallization from benzene; yield, 60%; m.p. 97°. (Found: C, 65.1; H, 6.2. Calc. for $C_9H_{10}O_3$: C, 65.1; H, 6.0%.) Erlenmeyer (1880) gives m.p. 97–98°.

(c) *Reactions with miscellaneous compounds.* (i) Methylguanidine (0.483 g. of methylguanidine sulphate). A ppt. was visible after 5 min. and gradually increased during several hours. The product was filtered off, washed with water and dried. Light-yellow crystals of benzenediazo-methylguanidine were obtained by recrystallization from benzene; yield 80%; m.p. 89–90° (decomp.). (Found: N, 38.8. $C_8H_{11}N_3$ requires N, 39.5%.)

(ii) 3-Indolylacetic acid (0.826 g.). A yellow precipitate was formed in the initially orange solution after 2 min. Decomposition of this precipitate appeared to take place as a gas was evolved and the yellow precipitate turned brown after 15 min. After standing, the mixture was acidified and the precipitate separated and stirred with 500 ml. of 2.5 N-NaOH, about one-half of the precipitate dissolving. The red solution was filtered and the filtrate acidified with 2.5 N-HCl. A dark-red gelatinous precipitate was obtained. This precipitate was found to decompose on drying to yield a product insoluble in 2.5 N-NaOH. Hence the gelatinous precipitate was dissolved in $CHCl_3$ and reprecipitated from the $CHCl_3$ layer by light petroleum. The product obtained was unstable in hot $CHCl_3$ and benzene, and gave a black syrup. The substance was soluble in 2.5 N-NaOH and dil. aqueous NH_3 .

Reaction with diazotized p-diphenylamine

An aqueous soln. of *p*-diphenyldiazonium chloride was prepared by dissolving 1.5 g. of *p*-diphenylamine in 50 ml. of hot water and 6 ml. of conc. HCl; the hot solution was filtered and cooled in ice, when *p*-diphenylamine hydrochloride separated as a white ppt. M- $NaNO_2$ (15 ml.) was

added to the mixture with stirring and the opalescent soln. filtered after standing for 15 min.

Ethanethiol. The above soln. was added to 1.43 ml. of ethanethiol in 50 ml. of cold $m\text{-Na}_2\text{CO}_3$. A yellow oil separated immediately and the mixture effervesced vigorously. The oil solidified on stirring to form a yellow solid, which was filtered off, washed with water and dried. Yellow crystals of *p*-diphenyl ethyl thio ether were obtained by recrystallization from light petroleum, b.p. 40–60°; yield 95%; m.p. 71–72°. (Found: S, 14.9; N, nil. $\text{C}_{14}\text{H}_{14}\text{S}$ requires S, 14.9%.) A sample of the product obtained before recrystallization was dissolved in acetone, filtered and precipitated by the addition of water in the cold. Analysis by the micro-Dumas method showed the absence of nitrogen. Hence the formation of the thio ether was not due to decomposition of the *S*-azo compound during recrystallization from a hot solvent.

Reactions with diazotized *p*-arsanilic acid

An aqueous solution of diazotized *p*-arsanilic acid was prepared by dissolving 1 g. of *p*-arsanilic in 50 ml. of water and 1.5 ml. of conc. HCl; to the ice-cold soln. 23 ml. of 0.2M- NaNO_2 was added slowly and the mixture allowed to stand for 15 min.

(a) *Reactions in which the product was isolated as an acid.* The following compounds were dissolved in 50 ml. of cold $m\text{-Na}_2\text{CO}_3$, the diazo reagent was added and the mixture set aside overnight at 4°.

(i) *n*-Butylamine (0.45 ml.). The yellow-orange soln. was acidified with dil. HCl to yield a yellow ppt. This product was filtered off and purified by dissolving in 2.5N-NaOH and reprecipitating with 2.5N-HCl several times. Yellow crystals of bis-(*p*-benzenearsonic acid diazo)-*n*-butylamine were obtained after drying over conc. H_2SO_4 ; yield, 60%; decomp. 148°. (Found: N, 13.1; As, 28.2. $\text{C}_{18}\text{H}_{21}\text{O}_6\text{N}_4\text{As}_2$ requires N, 13.1; As 28.2%.)

(ii) Methylamine (0.318 g. of methylamine hydrochloride). Light-yellow crystals of bis-(*p*-benzenearsonic acid diazo)-methylamine were obtained in a similar manner to that described above; yield 50%; decomp. 137°. (Found: As, 30.4. $\text{C}_{13}\text{H}_{15}\text{O}_6\text{N}_4\text{As}_2$ requires As, 30.8%.)

(b) *Reactions in which the product was isolated as the sodium salt.* The compounds indicated were dissolved in 50 ml. of $m\text{-Na}_2\text{CO}_3$, the diazo solution was added and the mixture set aside overnight at 4°. The yellow-orange solutions were acidified with 2.5N-HCl in the presence of *n*-butanol, and the orange butanol layer was separated and shaken with water several times. By adding excess of dil. NaOH (50 ml.; 5%, w/v) the compound was extracted from the butanol in the form of an aqueous solution of the sodium salt. The compound was precipitated by the addition of ethanol (to 80%, v/v) as light-yellow crystals. Some purification was effected by dissolving the crystals in water and reprecipitating with ethanol. The compounds were found to be contaminated with small quantities of Na_2CO_3 ; hence an indication of the formula of the compounds formed was obtained only by means of the quotient As/N. Approximately 50% yields were obtained.

(i) DL- α -Alanine (0.410 g.). The product corresponds to sodium *pp'*-diazaminobenzenearsonate. (Found: As/N, 3.61; $\text{C}_{12}\text{H}_{10}\text{O}_6\text{N}_4\text{As}_2$ requires As/N, 3.59.)

(ii) γ -Aminobutyric acid (0.576 g.). The product corresponded to the sodium salt of bis-(*p*-benzenearsonic acid diazo)- γ -aminobutyric acid. (Found: As/N, 2.16. $\text{C}_{16}\text{H}_{18}\text{O}_6\text{N}_4\text{As}_2$ requires As/N, 2.14.)

(iii) DL-Phenylalanine (0.76 g.). The product corresponded to sodium *pp'*-diazaminobenzenearsonate. (Found: As/N, 3.64. $\text{C}_{12}\text{H}_{10}\text{O}_6\text{N}_4\text{As}_2$ requires As/N, 3.59.)

The isolation of DL-phenyllactic acid from the reaction of excess of diazo reagent and the amino acid was effected as follows. *p*-Arsanilic acid (1 g.) was dissolved in 15 ml. of $n\text{-HCl}$, the solution cooled and 4.6 ml. of $m\text{-NaNO}_2$ added. After standing for 15 min. the soln. was added to 0.25 g. of DL-phenylalanine in 10 ml. of 10% (w/v) Na_2CO_3 and stood for 24 hr. at 4°. The mixture was acidified in the presence of ether (50 ml.) and then extracted a further four times. The ethereal layer yielded a reddish solid which was recrystallized from benzene to give colourless crystals of DL-phenyllactic acid; yield, 90%; m.p. 97° (not depressed by an authentic sample, m.p. 97°).

(c) *Reactions with proteins.* Casein (whole) was prepared by the method of Warner (1944). Crystalline bovine-plasma albumin fraction V was obtained from the Armour Laboratories. Edestin was obtained from Roche Products Ltd. Ovalbumin was prepared by the method of Kekwick & Cannon (1936) in the form of a 7.5% (w/v) solution in 0.9% saline and frozen at -20° till required.

The diazo reagent was added to a cold alkaline solution of the protein (see Table 1) in the quantities indicated in the Results section, and the mixture left at 4° overnight. Ethanol was added to an 80% (v/v) concentration and the conjugated protein precipitated by addition of dil. HCl until the pH of the solution was 4.0 as indicated by bromocresol green paper. The ppt. was spun down, dissolved in 25 ml. of 0.5M- NaHCO_3 , and reprecipitated by addition of 100 ml. of ethanol and 5 ml. of 2.5N-HCl. The final ppt. was dissolved in 25 ml. of 0.5M- NaHCO_3 and dialysed for 3 days against 0.5M- NaHCO_3 . The latter was frequently changed and no coloured materials passed through the cellophan membrane after 24 hr. The recovered solution was precipitated from 80% ethanol as before and the precipitate washed with ethanol, twice with water, twice further with ethanol and finally with ether. The orange-red product was not dried in a desiccator but allowed to equilibrate with the atmosphere for 1 day. The average yield was 50%.

Methods of analysis

Arsenic was determined by the method of Wintersteiner (1926). Nitrogen was determined by a micro-Dumas method by Weiler and Strauss, Oxford. These analyses were accurate to $\pm 1\%$. Hence the As/N quotient was accurate to $\pm 2\%$. The Dumas method was used in preference to the Kjeldahl method or that of Friedrich (1933). A typical azo protein gave N=11.8% by Friedrich's method and N=10.4% by the Kjeldahl method, compared with N=12.2% by the Dumas method. Electrophoresis of the conjugated proteins on paper was carried out in an apparatus supplied by Evans Electroselenium Ltd.

Calculation of theoretical As/N quotients

The method adopted for the calculation of the As/N quotients which would be expected on the assumption that reaction occurred with various chemical groupings in the protein is an extension of that devised by Hooker & Boyd (1934).

Let the protein contain *n* different amino acids whose wt./100 g. of protein are $a_1, a_2, \dots, a_n$. Let the molecular weights of the amino acids be $m_1, m_2, \dots, m_n$ respectively. Finally, let the free groups of the amino acids combine with

$b_1, b_2, \dots, b_n$ moles of diazotized *p*-arsanilic acid/mole of amino acid. Then the wt. (g.) of As combined with 100 g. of protein is

$$75(b_1 a_1/m_1 + b_2 a_2/m_2 + \dots + b_n a_n/m_n).$$

If this last expression is designated by 75α the N introduced into the protein is $(28/75)75\alpha$ or 28α . If the wt. of N/100 g. of the untreated protein is M , then the As/N quotient (R) is given by the equation

$$R = \frac{75\alpha}{M + 28\alpha}. \quad (1)$$

Deamination of α -amino groups may possibly occur, in which case the original N content of the protein is reduced from M to M' , where

$$M' = M - 14(a_p/m_p + a_q/m_q \dots a_z/m_z), \quad (2)$$

$a_p, a_q, \dots, a_z$ being the weights of amino acids present in the form of terminal amino acid residues and $m_p, m_q, \dots, m_z$ being their respective molecular weights.

The values for the amino acid content of the proteins used were taken from Tristram (1953). Additional information about the terminal amino acid residues of bovine-plasma albumin was obtained from Vunakis (1951), and of whole casein from Mellon, Korn & Hoover (1953) and Gordon, Semmett, Cable & Morris (1949).

SUMMARY

1. Pauly (1915) has already shown that the phenolic group of tyrosine and the imidazole group of histidine can react with diazo reagents.

2. In a continuation of this work, reaction with other groups present in proteins has been considered and derivatives were prepared with compounds containing certain amino groups and guanidino, sulphydryl and indolyl groups.

3. From the evidence of other authors it is also concluded that imino and aliphatic hydroxyl groups react, whereas all other groups are inert.

4. Of the aliphatic primary amino compounds investigated, only those containing the grouping $-\text{CH}_2\text{NH}_2$ have been found to yield triazens. Other types of amino group were deaminated, an aromatic diazoamino compound being produced as the by-product.

5. It is inferred that for amino acids found in proteins, only the ϵ -amino group of lysine and the α -amino group of glycine are capable of forming bis(diazo)amino links.

6. The maximum amount of diazotized *p*-arsanilic acid which can react with four different proteins has been determined and the values of the arsenic/nitrogen quotients were obtained by analysis.

7. A comparison of these results with theoretical values calculated on the basis of their known amino acid content has given an indication of the extent of the reaction.

8. It is concluded that reaction occurs between one diazonium radical and each sulphydryl and indolyl group; between two diazonium radicals and each phenolic, imidazole, ϵ -amino group and α -

amino group of *N*-terminal glycine residues; and between three diazonium radicals and each guanidino group. It is also concluded that α -amino groups of *N*-terminal residues other than glycine are deaminated.

We wish to acknowledge the co-operation of Mr M. J. Newlands in the electrophoresis experiments.

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