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The Free Amino Groups of Insulin

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That the free amino groups in proteins may be the ϵ-amino group of lysine was first suggested by Skraup & Kaas (1906), who failed to isolate lysine from deaminized proteins. Van Slyke & Birchard (1913) later found an apparent equality between the free amino-N of proteins and one-half of their total lysine-N, which suggested that the free amino groups were due exclusively to such a source. Methods were then developed to confirm the presence of the free ϵ-amino group of lysine in proteins; these consisted in treating a protein with a reagent that would react with free amino groups to give derivatives that were stable to acid hydrolysis, so that on hydrolysis of the substituted protein the derivative of lysine could be isolated. The most satisfactory was that of Gurin & Clarke (1934), who treated gelatin with benzenesulphonyl chloride, hydrolyzed the resulting benzenesulphonyl gelatin and isolated ϵ-benzenesulphonyllysine from the hydrolysate.

In some proteins, and particularly in insulin, the number of free amino groups (Van Slyke) is far in excess of that which can now be ascribed to lysine, which suggests that the protein must contain residues of certain amino-acids which are condensed in such a way that their α-amino groups remain free. Jensen & Evans (1935) have, in fact, been able to isolate the phenylhydantoin of phenylalanine from a hydrolysate of insulin that had been treated with phenylisocyanate, thus demonstrating that some free amino groups of insulin are present on phenylalanyl residues. Chibnall (1942) has suggested that the free amino groups of proteins over and above the ϵ-amino groups of lysine are due to terminal

residues of polypeptide chains, and that the number of these groups must therefore give a measure of the number of polypeptide chains in the protein.

We found that methanesulphonyl amino-acids were stable to acid hydrolysis and that they could be fractionated by partition chromatography (Gordon, Martin & Synge, 1943) with an indicator in the aqueous phase of the column. The rates at which the bands moved on the columns were similar to those of the acetamino-acids. The method was satisfactory when applied to synthetic peptides, but with insulin the number of terminal groups estimated by titration of the methanesulphonyl amino-acids was far less than that suggested by the method of Van Slyke. The procedure was accordingly abandoned.

Abderhalden & Stix (1923) attempted to use 2:4-dinitrochlorobenzene (DNCB) for the identification of the terminal groups of a partial hydrolysate of silk fibroin. They did not meet with much success, chiefly owing to the presence of anhydrides in the hydrolysate and the difficulties of separating the products. It seemed, nevertheless, worth while to investigate this reagent, especially as all the 2:4-dinitrophenyl-amino-acids (referred to henceforth as DNP-amino-acids) produced are bright yellow, thereby facilitating chromatographic separation. DNCB will not react with amino-acids in $NaHCO_3$ solution unless heat is applied, and this brings about a certain amount of hydrolysis of the protein. Fortunately, however, the corresponding fluoro-compound, 2:4-dinitrofluorobenzene (DNFB) was found to react readily at room temperature, and the use of this has met with considerable success, for the DNP-amino-acids produced can be estimated

colorimetrically and separated almost completely from one another by partition chromatography. The solvent systems normally used for separating the acetyl-derivatives were not entirely satisfactory for the DNP-monoamino-acids, and several new systems had to be introduced; nevertheless, the method finally adopted embraced all amino-acids, though this was not possible with the methanesulphonyl derivatives.

The method as applied to insulin consisted of three stages. In the first the protein was treated with DNFB, hydrolyzed and the resulting coloured compounds separated chromatographically. The identification of these was based on band rates, and was confirmed by mixed chromatograms. Secondly, knowing which DNP-derivatives were present, one could assess the amount of each with reasonable accuracy by separating the fraction quantitatively and estimating the material present colorimetrically, using the pure DNP-amino-acid as a standard. Thirdly, the whole operation was carried out on a larger scale, so that the DNP-amino-acids could be isolated and satisfactorily characterized. The procedure should be applicable to the identification and estimation of the terminal residues in all peptides and proteins.

EXPERIMENTAL

Preparation of DNP-amino-acids

In order to work out a comprehensive chromatographic method for the identification of any DNP-amino-acid by its behaviour on a partition column, it was necessary to prepare the derivatives of all the naturally occurring amino-acids. Not all of these were obtained in pure form or completely characterized, as this was unnecessary for a study of their chromatographic behaviour; nevertheless, all the derivatives corresponding to those obtained from treated insulin were carefully purified, so that they could be used as standards of reference.

The derivatives of glycine, dl-alanine, dl-valine, dl-leucine and l-asparagine were prepared by the method of Abderhalden & Blumberg (1910). By a similar method, the derivatives of dl-methionine, l-proline, l-tryptophan, dl-aspartic acid, l-cystine and l-lysine were prepared but not completely characterized. The methods of purification and characterization of these compounds will be published later.

N-2:4-*Dinitrophenyl*-l-*phenylalanine*. 0·2 g. l-Phenylalanine and 0·4 g. NaHCO$_3$ were dissolved in 5 ml. water and to this was added a solution of 0·4 g. (0·28 ml.) DNFB in 10 ml. ethanol. The mixture was shaken for 2 hr. at room temperature, concentrated to remove ethanol, dissolved in water and extracted with ether to remove excess DNFB. The aqueous solution was then acidified, which caused the separation of an oil that immediately solidified in an amorphous form. The N-2:4-*dinitrophenyl*-l-*phenylalanine* was recrystallized twice from aqueous methanol. Yield, 0·27 g.; m.p. 186°. (Found: C, 54·2; H, 4·2; N, 12·6 %. C$_{15}$H$_{13}$O$_6$N$_3$ requires C, 54·4; H, 3·9; N, 12·7 %.)

The derivatives of dl-serine, dl-threonine, l-hydroxyproline, l-glutamic acid and l-arginine were prepared in a

similar way using DNFB. Their purification and characterization will be published later. The acid equiv. wt. (277) of the serine derivative showed that it was mono-DNP-serine (271), and that DNFB does not react with aliphatic hydroxyl groups.

ε-N-2:4-*Dinitrophenyl*-l-*lysine*. Several attempts to prepare this compound by the action of DNCB or DNFB on the copper complex of lysine proved unsuccessful. It was eventually prepared from the α-acetyl-compound as follows. 0·48 g. α-acetyl-l-lysine (Neuberger & Sanger, 1943) and 0·75 g. NaHCO$_3$ were dissolved in 3 ml. water. To this was added a solution of 0·5 g. DNCB in 10 ml. ethanol. The mixture was then heated under reflux on a water-bath for 4 hr. The ethanol was removed by evaporation *in vacuo* and the residue dissolved in water, filtered to remove excess DNCB and acidified with HCl while hot. An oil separated, which was extracted into chloroform. The chloroform solution, after drying with anhyd. Na$_2$SO$_4$, was taken to dryness, leaving an oil. On rubbing this with dry ether the α-*acetyl*-ε-*DNP*-l-*lysine* crystallized and could be recrystallized from a mixture of ethanol and ether: m.p. 110°; yield 0·7 g. (75 % theoretical). 0·2 g. of the compound was boiled under reflux for 3 hr. with 5 ml. 20 % HCl. On cooling, the ε-*DNP*-l-*lysine hydrochloride* separated as crystals containing 1 mol. of water of crystallization. After standing overnight it was filtered off and washed with very little water and ethanol and then thoroughly with ether. It was recrystallized from dilute HCl; m.p. 186°. (Found: C, 39·0; H, 5·5; N, 15·6; Cl, 9·5 %. C$_{12}$H$_{16}$O$_6$N$_4$.HCl.H$_2$O requires C, 39·5; H, 5·2; N, 15·4; Cl, 9·8 %.)

α-N-2:4-*Dinitrophenyl*-l-*lysine*. It is generally believed that the lysine residues in proteins are combined through the α-amino groups while the ε-amino groups are free. One would thus expect to isolate only ε-DNP-lysine from a hydrolysate of a DNP-protein. It is not impossible, however, that some, at least, of the lysine residues in proteins are combined through the ε-amino group, in which case α-*DNP*-lysine would be formed on hydrolysis of a DNP-protein. This second derivative was accordingly synthesized as follows. 1·25 g. ε-benzoyl-l-lysine were treated for 4 hr. with 1·0 g. DNCB as described above. On acidification an oil separated which solidified on standing. It weighed 2·0 g. 0·5 g. of this α-*DNP*-ε-*benzoyl*-l-*lysine* was boiled under reflux for 3 days with a mixture of 5 ml. acetic acid and 5 ml. conc. HCl. The mixture was then evaporated to dryness, taken up in water and filtered to remove unchanged substance. On neutralization with pyridine the α-DNP-l-lysine crystallized out in 60 % yield; m.p. 260° (decomp.). (Found: C, 45·6; H, 5·3; N, 17·5 %. C$_{12}$H$_{16}$O$_6$N$_4$ requires C, 46·2; H, 5·1; N, 17·9 %.)

Tyrosine derivatives. When l-tyrosine was treated with DNCB in excess two coloured compounds were produced, which moved down a 1 % butanol-chloroform column at R = 0·5 and 0·2 respectively. With DNFB only the faster-moving compound was produced. The acid equiv. wt. (519) indicated that this was O:N-diDNP-l-tyrosine (513), which had been prepared by Abderhalden & Stix (1923). The slower-moving compound was presumably N-DNP-l-tyrosine, since O-*DNP*-l-tyrosine is colourless. The monoDNP-tyrosine prepared by Abderhalden & Stix (1923) was presumably the O-DNP-derivative in an impure form.

O-2:4-*Dinitrophenyl*-l-*tyrosine*. Since DNFB reacts with the hydroxyl group of tyrosine, a hydrolysate of a DNP-protein would be expected to yield O-DNP-l-tyrosine. It

was accordingly necessary to synthesize this latter compound. 0·55 g. N-acetyl-l-tyrosine (du Vigneaud & Meyer, 1932) was treated for 4 hr. with 2·0 g. DNCB as described above. The solution was only faintly coloured at the end of the reaction, in contrast to the bright colours obtained when DNCB reacts with amino groups. On acidification an oil was produced which partly crystallized. From aqueous ethanol the N-*acetyl*-O-*DNP*-l-*tyrosine* crystallized in white needles; m.p. 194°; yield 0·1 g. The product was boiled for 3 hr. under reflux with 20 % HCl; on cooling and evaporating to a small volume, a precipitate was obtained which was filtered off, dissolved in warm dilute HNO_3 and neutralized with pyridine while hot. O-DNP-l-tyrosine crystallized in white needles containing 1 mol. of water of crystallization; m.p. 202° (decomp.). (Found: C, 49·9; H, 4·2; N, 11·9 %. $C_{15}H_{13}O_7N_3.H_2O$ requires C, 49·4; H, 4·1; N, 11·6 %.)

Reaction of DNFB with histidine. By the action of DNCB on histidine Abderhalden & Blumberg (1910) obtained two compounds containing one and two DNP-groups respectively. I find that on treatment with excess DNFB only the diDNP-histidine is obtained, indicating that DNFB reacts with the imidazole ring. As this is free in proteins it seemed probable that a monoDNP-histidine would be formed on hydrolysis of a DNP-protein. In an attempt to prepare this compound, α-acetylhistidine (Bergmann & Zervas, 1928) was treated with DNFB, but there was very little reaction and only a small amount of a yellow oil was obtained. A portion of this was hydrolyzed with 20 % HCl and the products studied chromatographically. Two compounds were obtained. The first of these was not extracted from acid solution into ether and moved fast on a 17 % butanol-chloroform column; it was not observed when the hydrolysis products of DNP-insulin were treated. The second was ether-soluble and moved fast on a chloroform column. It seemed probable that this latter might be 2:4-dinitroaniline, derived by breakdown of the substituted histidine, and it was indeed found to have the same R values on ethanol-ligroin and acetone-cyclohexane columns as the aniline derivative itself. This same band was obtained on hydrolysis of both diDNP-histidine and DNP-insulin. These results indicate that DNFB reacts only slowly with α-acetylhistidine, in contrast to the almost quantitative reaction with histidine itself. It is interesting to note that benzoylation of the imidazole group of histidine is affected by the presence of other polar groups in the molecule (Gerngross, 1919) and that the same appears to be true on reaction with DNFB. To ascertain how histidine would react if present in a polypeptide chain with its carboxyl as well as its amino group in peptide linkage, the action of DNFB on α-benzoylhistidine methyl ester (Gerngross, 1919) was studied; the reaction was very slow and the same products were given on hydrolysis as with α-acetylhistidine. Finally, to ascertain how histidine

would react if present as a terminal residue in a peptide chain with only the amino group free, the reaction with histidine methyl ester (Pauly, 1904) was also studied. In this case the product obtained on hydrolysis consisted entirely of the diDNP-derivative, indicating easy reaction with the imidazole group. To summarize, it may be inferred that when histidine is present in the body of a polypeptide chain of a protein or as a terminal residue with its carboxyl group free, the imidazole group will react slowly with DNFB so that on hydrolysis some 2:4-dinitroaniline will be obtained together with a possible unknown breakdown product which is insoluble in ether and moves fast on a 17 % butanol-chloroform column. If histidine is present as a terminal residue with its amino group free the diDNP-derivative will be obtained.

Reaction of DNFB with —SH groups. Saunders (1934) showed that DNCB reacts more readily with —SH groups than with amino groups, so that one is led to infer that when a protein containing —SH groups is treated with DNFB and hydrolyzed, S-DNP-cysteine would be produced. Saunders (1934) prepared a derivative by the action of DNCB on cysteine, which he stated crystallized with difficulty. I have repeated the preparation and find that the product does not give a positive ninhydrin reaction and is extracted from dilute HCl into ether. On a 3 % butanol-chloroform column it forms two bands having R = 1·2 and 0·3 respectively. It would appear, therefore, that the condensation does not run smoothly, and the product does not exhibit the expected properties.

To obtain information as to the compound to be expected from DNP-proteins, a small amount of the S-DNP-derivative of reduced glutathione was prepared and hydrolyzed for 6 hr. with 20 % HCl. S-DNP-glutathione is a well-characterized product of known structure (Saunders, 1934). The hydrolysate appeared to contain one substance which moved fast down a 66 % methyl ethyl ketone (M.E.K.)-ether column and at R = 0·08 in 17 % butanol-chloroform. It was not extracted from dilute HCl into ether. It is suggested that this band represents S-DNP-cysteine, and that the substances prepared by the action of DNCB on cysteine are formed from S-DNP-cysteine by the action of alkali. More experimentation is necessary to verify this, but it appears that the product obtained from glutathione would also be obtained from a protein.

Chromatographic separation of DNP-derivatives

In general the partition chromatographic method of Gordon et al. (1943) was employed to separate the DNP-derivatives, using a stationary aqueous phase adsorbed on the silica gel and a moving organic phase. It was, of course, unnecessary to use an indicator in the aqueous phase as the derivatives

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themselves are coloured. To describe such solvent systems the terminology of the above-mentioned workers is followed, i.e. 1 % butanol-chloroform refers to a solution in chloroform of butanol, 1 % (by vol.) subsequently saturated with water. In some cases glycol or an aqueous organic solvent such as ethanol or acetone was employed as the stationary phase, and a non-polar solvent in equilibrium with it as the moving phase. Such systems do no more than indicate the relative rates of the different derivatives on the columns, and R values alone cannot be used for identification unless a parallel experiment with a sample of the authentic derivative itself is run.

The data given in Table 1 show that all the amino-acid derivatives listed can be separated by one or other of the solvent systems employed, the sole exception being leucine and *iso*leucine. The method

Table 1. *Band rates of ether-soluble DNP-amino-acids*

(Figures are values of R—Gordon *et al.* 1943.)

Stationary phase ...	Water		Water	Water	Water	Ethanol	Methanol	Acetone	Glycol
Mobile phase ...	Butanol-chloroform		Chloroform	5% propanol-cyclohexane	33% ether-ligroin	Ligroin	Carbon tetrachloride	Cyclohexane	Benzene
	3%	1%							
2:4-Dinitroaniline	—	—	Fast	—	—	0·35	—	0·8	—
DNP-leucine	—	Fast	0·45	Fast	Fast	0·75	0·6	0·5	0·65
DNP-*iso*leucine	—	Fast	0·45	Fast	Fast	0·8	0·6	0·55	0·6
DNP-valine	—	Fast	0·4	Fast	Fast	0·55	0·5	0·4	—
DNP-phenylalanine	—	Fast	0·2	Fast	Fast	0·5	0·4	0·25	—
DNP-methionine	—	Fast	0·25	Fast	1·0	0·3	0·4	—	—
DNP-proline	Fast	0·7	0·2	1·0	0·9	0·2	0·3	0·4	0·3
DNP-alanine	Fast	0·7	0·15	1·0	1·0	0·25	0·25	0·4	0·2
DNP-tryptophan	—	—	0·06	0·9	0·8	0·1	—	—	—
DNP-glycine	—	0·5	0·08	0·6	0·6	0·1	—	—	0·07
diDNP-lysine	—	0·5	0·06	Insol.	0·25	Insol.	—	—	0·3
diDNP-tyrosine	—	0·5	0·08	Insol.	0·25	—	—	—	0·5
DNP-hydroxyproline	—	0·13	0·02	—	—	—	—	—	—
DNP-threonine	—	0·07	—	0·2	0·2	—	—	—	—
DNP-serine	—	0·05	—	Insol.	0·1	—	—	—	—
DNP-glutamic acid	0·4	0·03	—	0·35	0·3	—	—	—	—
DNP-aspartic acid	0·4	0·02	—	0·2	0·2	—	—	—	—
diDNP-cystine	0·35	—	—	Insol.	0·0	—	—	—	—

are especially useful for the separation of the mono-amino-acid derivatives. To prepare an ethanol-ligroin column, 1 vol. water, 1 vol. ethanol and 10 vol. ligroin (b.p. 80–100°) are shaken together until the two phases are in equilibrium. The lower aqueous phase is used as the stationary phase of the column, 1 ml. being added to each 2 g. dry silica, and the upper ethanol-ligroin phase is used as the moving phase of the column. A methanol-carbon tetrachloride column is prepared as above from 1 vol. water, 1 vol. methanol and 15 vol. carbon tetrachloride; and an acetone-cyclohexane column from 1 vol. water, 1 vol. acetone and 10 vol. cyclohexane. To prepare a glycol-benzene column, glycol and benzene are shaken together; 1 ml. of the glycol layer is added to each 1 g. dry silica, and the benzene layer used as the moving phase.

In Table 1 are shown the R values for the various ether-soluble DNP-amino-acids using various solvent systems. These values have been found to vary considerably with such factors as the particular batch of silica or solvent and with the distance travelled down the column. It is probable that there is a certain amount of adsorption of the compounds on the silica. The figures in Table 1, therefore,

may thus be capable of estimating the component amino-acids given on hydrolysis of a protein, but the more quantitative aspects of such a procedure have not yet been fully explored. All the derivatives

Table 2. *Band rates of acid-soluble DNP-amino-acids*

(Figures are values of R—Gordon *et al.* 1943.)

	Developing solvents		
	Methyl ethyl ketone-ether	Butanol-chloroform	
DNP-amino-acids	66%	17%	30%
diDNP-histidine	Fast	Fast	—
S-DNP-cysteine	Fast	0·08	Fast
α-DNP-arginine	0·35	—	0·2
ε-DNP-lysine	0·20	—	0·15
α-DNP-lysine	0·11	—	—

shown in Table 1 are extracted from acid solution into ether, and move fast on an ether column. This fact is made use of to separate them from the acid-soluble derivatives, the R values for which on various solvents are given in Table 2.

Attempts to separate the DNP-derivatives by adsorption chromatography have not been very successful. When magnesium oxide or alumina were used as adsorbents decomposition took place. It was found possible, nevertheless, to separate the glycine and leucine derivatives in acid solution on a column of talc and in ether solution on a column of calcium carbonate, in neither case, however, so effectively as in the above-mentioned partition chromatography. Likewise the use of partition chromatography using filter paper (Consden, Gordon & Martin, 1944) or starch (Synge, 1944) was not very satisfactory, due to 'tailing' of the spots or bands.

Estimation of DNP-amino-acids

The DNP-amino-acids, dissolved in N-HCl (about 0·1–0·3 mg. amino-acid-N/100 ml. N-HCl), were estimated colorimetrically with a photoelectric absorptiometer. In the case of DNP-phenylalanine the solubility in this solvent was too low, and 1 % NaHCO₃ had to be used. Standard curves were plotted for the derivatives of the naturally occurring amino-acids. These varied considerably from one to another, and differences were noted between the curves representing the *l* and *dl* samples of the same amino-acid. In all cases the colour intensities do not obey Beer's law.

Stability of DNP-derivatives to acid hydrolysis

Since the liberation of DNP-amino-acids from a DNP-protein requires acid hydrolysis, it was necessary to investigate the stability of the former products during such treatment. To effect this, a standard solution of the derivative was made up
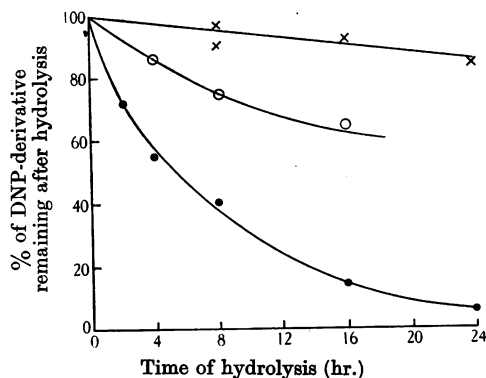

Fig. 1. Rate of hydrolysis of DNP-amino-acids with boiling 20 % (w/v) HCl. ● DNP-glycine; ⊙ DNP-phenylalanine; × ε-DNP-lysine.

and samples were hydrolyzed with 20 % HCl for varying periods. These were then taken to dryness, purified on a suitable column and the amount of unchanged derivative estimated. The results of such experiments with DNP-phenylalanine, DNP-glycine and ε-DNP-lysine are shown in Fig. 1.

Approximate experiments with the DNP-derivatives of alanine, valine, arginine, serine, tryptophan, aspartic acid and diDNP-lysine indicated less than 20 % breakdown in 8 hr. It would thus appear that, with the single exception of DNP-glycine, all the derivatives exhibit satisfactory stability for the purpose intended.

Preparation of DNP-insulin

0·5 g. crystalline insulin and 0·5 g. NaHCO₃ were dissolved in 5 ml. water; 10 ml. ethanol and 0·5 ml. DNFB were then added and the mixture mechanically shaken for 2 hr. The DNP-insulin, which had precipitated as an insoluble yellow powder, was centrifuged down and washed with water, ethanol and ether, and air dried.

For the experiments which follow it was necessary to know the insulin content of a given sample of DNP-insulin. This could not be ascertained directly by a determination of N, as the nitro-groups would interfere, and it was therefore computed indirectly from an estimation of the amide-N. To do this a sample of the DNP-insulin was boiled for 3 hr. with 2N-HCl and, after neutralization, the liberated NH₃ estimated in the micro-Kjeldahl apparatus using a borate buffer of pH 8·5. A control experiment with asparagine showed that treatment with DNFB did not alter the amide-N content as determined in this way. In one particular experiment, where all the values quoted below are calculated on a moisture-free basis, the sample of DNP-insulin contained 1·07 % amide-N and 0·04 % amino-N (Van Slyke). Since insulin itself contains 1·38 % amide-N (Chibnall, 1942; Rees, unpublished), it follows that 100 mg. of the DNP-insulin correspond to 77·2 mg. of insulin. If the amino-N (Van Slyke) value for insulin be taken as 0·85 % (Chibnall, 1942) it would appear that 93 % of the free amino groups of the insulin have been substituted.

Identification of the terminal residues in insulin

100 mg. DNP-insulin were boiled under reflux for 8 hr. with 10 ml. 20 % (w/w) HCl. After cooling it was extracted three times with ether. This separated the hydrolysate into two fractions; the ether extract, which contained the DNP-derivatives listed in Table 1, and the aqueous solution which contained free amino-acids and any of the DNP-derivatives listed in Table 2.

The ether extract was taken to dryness, and the material passed through an ether column to remove HCl, which would cause adsorption of the derivatives at the top of the subsequent column. The material was then fractionated on a chloroform column. There was a faint band that moved fast

and so could not be an amino-acid derivative; it seems probable that it was 2:4-dinitroaniline, derived from the breakdown of a substituted histidine derivative. It could not be separated from 2:4-dinitroaniline by the use of an ethanol-ligroin or an acetone-cyclohexane column. The rest of the colour was in two bands having $R = 0.2$ and 0.1 respectively. Each was 'run out' of the column separately, taken to dryness and samples used for the following experiments.

Fraction 1 (R in chloroform = 0.2) formed one band having $R = 0.5$ on an ethanol-ligroin column, and one band having $R = 0.3$ on an acetone-cyclohexane column. These rates suggested that the material was DNP-phenylalanine, which was confirmed by the following mixed chromatograms. A sample mixed with DNP-leucine formed two bands having $R = 0.6$ and 0.4 respectively on an ethanol-ligroin column. Another sample mixed with DNP-alanine formed two bands on an ethanol-ligroin column having $R = 0.4$ and 0.2 respectively. A third sample mixed with DNP-valine formed two bands having $R = 0.5$ and 0.3 respectively on an acetone-cyclohexane column. On mixing with authentic DNP-phenylalanine only one band was obtained on each of the above columns. Since the original hydrolysis was carried out for only 8 hr. it was possible that either band could represent a DNP-derivative of a peptide. In order to show that fraction 1 was not a peptide, a sample was hydrolyzed for a further 8 hr. with 20 % HCl, extracted with ether and analyzed on the various columns given above. Only one band was obtained and the R values were unchanged. If it had been a peptide one would have expected to find evidence for a new band, representing the terminal group. It is, of course, possible that fraction 1 contained a DNP-phenylalanyl-peptide that is inseparable from DNP-phenylalanine on the columns used, but this seems unlikely and does not alter the fact that DNP-phenylalanine is present.

A sample of fraction 2 (R in chloroform = 0.1) on a 5 % propanol-cyclohexane column gave one band having $R = 0.6$, and on a 33 % ether-ligroin column one band having $R = 0.55$. These rates suggested that the material was DNP-glycine and this was confirmed by the following mixed chromatograms. A sample mixed with DNP-alanine gave two bands having $R = 0.3$ and 0.2 respectively on a chloroform column. Mixed with diDNP-lysine, two bands having $R = 0.6$ and 0.2 respectively were formed on a 33 % ether-ligroin column. Mixed with DNP-tryptophan two bands were formed which moved at $R = 0.8$ and 0.5 respectively in 5 % propanol-cyclohexane. When mixed with DNP-glycine only one band was obtained on each of the above columns. Further hydrolysis of the material brought about no change, showing that it was not a DNP-peptide.

After thorough ether extraction the original aqueous solution was still brightly coloured, indicative of substances listed in Table 2. The presence of ϵ-DNP-lysine was to be expected, and in order to ascertain what others might be there, a sample of the acid solution was taken to dryness and its behaviour on various columns studied. On a 66 % M.E.K.-ether column one band was formed, having $R = 0.2$. In 30 % butanol-chloroform it moved at $R = 0.2$. When mixed with α-DNP-arginine, two bands having $R = 0.35$ and 0.2 respectively were formed on a 66 % M.E.K.-ether column. These facts indicated that ϵ-DNP-lysine was the only coloured DNP-derivative in the aqueous solution.

The above experiments showed that the hydrolysate of DNP-insulin contained DNP-phenylalanine, DNP-glycine, ϵ-DNP-lysine, and no other coloured DNP-derivative.

Estimation of the amino-acids representing terminal residues in insulin

After the identity of the DNP-amino-acids produced on hydrolysis of DNP-insulin had been established, the next step was to estimate them quantitatively. It can be seen from Fig. 1 that DNP-glycine is relatively unstable to acid hydrolysis, so that if a time period of as long as 8 hr. is used a rather large correction for breakdown must be applied. Estimations were accordingly made after shorter periods of hydrolysis. In each case it was necessary to test the various fractions for the presence of peptides as described in the previous section. No evidence for these was obtained after hydrolysis for 4 hr. but after only 2 hr. a phenylalanyl-peptide was detected in fraction 2. This was inseparable from DNP-glycine on a chloroform column, but could be separated on a special acetone-cyclohexane column made from 1 vol. water, 3 vol. acetone and 10 vol. cyclohexane. The peptide moved at $R = 0.7$ and DNP-glycine at $R = 0.45$. To estimate the DNP-phenylalanine contained in the peptide, it was subjected to a further 4 hr. hydrolysis.

The experimental details were as follows. Three samples of about 100 mg. DNP-insulin were boiled under reflux with 10 ml. 20 % HCl for 8, 4 or 2 hr. respectively. In each case the acid solution was extracted five times with ether, the ether extracts being washed with water and the washings returned to the original aqueous solution. The collected ether extracts were reduced to dryness, passed through an ether column prepared from 2 g. silica, evaporated to dryness and fractionated on a 6 g. chloroform column. The two fractions representing DNP-phenylalanine and DNP-glycine were separately collected and taken to dryness. In the 2 hr. hydrolysate the DNP-glycine fraction was purified on an acetone-cyclohexane column (containing 3 vol. acetone). The DNP-phenylalanine

fraction was dissolved in 1 % $NaHCO_3$, made up to 50 ml. and estimated colorimetrically. The DNP-glycine fraction was made up to 25 ml. with N-HCl. As standards the pure derivatives of the naturally occurring amino-acids were used.

The aqueous solution, after extraction with ether, was taken to dryness and subjected to a further 8 hr. hydrolysis with 20 % HCl to break down any lysine peptides; this should not greatly affect the lysine estimation as the ε-DNP-derivative is relatively stable (Fig. 1). The acid solution was then made up to 100 ml., 10 ml. samples taken, reduced to dryness and passed through a 66 % M.E.K.-ether column. The band was 'run out', taken to dryness and made up with N-HCl to 25 ml. for estimation.

Table 3. *Estimation of the free amino groups of insulin*

Time of hydrolysis (hr.)	Fraction	Amino-N as % insulin-N	Amino-N corrected for hydrolysis	No. of groups per insulin submolecule (mol. wt. 12,000)
8	Glycine	0·67	1·65	2·2
	Phenylalanine	1·00	1·35	1·8
	Lysine	1·16	1·3	1·7
4	Glycine	0·86	1·5	2·0
	Phenylalanine	1·05	1·2	1·6
	Lysine	1·32	1·4	1·9
2	Glycine	1·13	1·55	2·1
	Phenylalanine	1·07	—	—
	Phenylalanine from peptide	0·32	—	—
	Total phenylalanine	1·39	1·5	2·0
	Lysine	1·43	1·55	2·1
Mean	Glycine	—	—	2·1
	Phenylalanine	—	—	1·8
	Lysine	—	—	1·9

The results of the three experiments are set out in Table 3. The values given in column 3 represent the free amino-N of the amino-acid as a percentage of the original insulin-N. In column 4 these values are corrected by means of the data given in Fig. 1 for the breakdown which the derivatives are as-sumed to have undergone as a result of the hydro-lysis; the corrections may not be strictly valid as the stability of the derivatives while still condensed in the protein or in peptide split-products may not be the same as that found for the derivatives them-selves. In column 5 the corrected values are pre-sented in terms of amino-acid residues per insulin submolecule of mol. wt. 12,000 (Chibnall, unpub-lished). It can be seen that within the limits of experimental error there are two residues of each amino-acid per insulin submolecule.

Isolation of DNP-amino-acids representing the terminal residues in insulin

To confirm the identity of the three DNP-deri-vatives obtained from insulin it was necessary to carry out an experiment on a larger scale and isolate the compounds concerned in a pure form. A 4 hr. period of hydrolysis was chosen for reasons given above.

1·03 g. air-dried DNP-insulin (corresponding to 0·755 g. moisture-free insulin) were boiled under reflux with 100 ml. 20 % HCl for 4 hr. and the cooled hydrolysate extracted with ether as described above. The ether extract. was taken to dryness and the material first passed through an ether column and then fractionated on a chloroform column prepared from 40 g. silica. The DNP-phenyl-alanine and DNP-glycine fractions were collected and worked up as follows.

N-2:4-Dinitrophenyl-l-phenylalanine. The chloro-form solution was taken to dryness and the material passed through a 20 g. acetone-cyclohexane column to ensure purification. The coloured solution was taken to dryness and dissolved in a minimum of warm dilute $NaHCO_3$ solution. This solution was acidified and after standing for a few days crystals of DNP-phenylalanine had separated. The crude product weighed 14·0 mg. (38 % of the material present as estimated colorimetrically). It was re-crystallized from aqueous methanol and then from a mixture of acetone and cyclohexane, yielding 10·1 mg. of material which melted at 186° alone or when mixed with authentic DNP-phenylalanine. (Found: C, 55·2; H, 4·1 %. Calc. for $C_{15}H_{13}O_6N_3$: C, 54·4; H, 3·9 %.)

N-2:4-Dinitrophenylglycine. After evaporation of the chloroform solution, the whole fraction was purified on an acetone-cyclohexane column (con-taining 3 vol. acetone). The DNP-glycine fraction was taken to dryness, and crystallized from hot water; yield 10·5 mg. Another 5·3 mg. were ob-tained by working over the mother liquors, making a total yield of 15·8 mg. (90 % of the amount esti-mated). Abderhalden & Blumberg (1910) report a m.p. of 205° for DNP-glycine. The purest samples I have synthesized usually decompose between 195 and 202° according to the rate of heating. To make the necessary comparison three melting-point tubes containing synthetic DNP-glycine, the above crys-talline material and a mixture of the two respec-tively were heated side by side in the melting-point apparatus. They all decomposed simultaneously at 195°. (Found: C, 40·0; H, 2·9 %. Calc. for $C_8H_7O_6N_3$: C, 39·8; H, 2·9 %.)

ε-N-2:4-Dinitrophenyl-l-lysine. The aqueous solu-tion containing the ε-DNP-lysine was taken to dryness and hydrolyzed for a further 20 hr. with 20 % HCl. It was then evaporated to a small

volume and allowed to stand overnight. An amorphous yellowish grey precipitate had formed, which was filtered off. The nature of this precipitate, which weighed 100 mg., is not yet clear. The filtrate was then taken to dryness. A column was next prepared, using 40 g. silica, on which were adsorbed 20 ml. N-HCl, 66 % M.E.K.-ether being used as the mobile phase. The ε-DNP-lysine fraction was then dissolved in 2 ml. water, which were mixed with 4 g. dry silica, suspended in the solvent, poured on to the previously prepared column and developed with 66 % M.E.K.-ether. This procedure was found more satisfactory than the normal one in the presence of large amounts of free amino-acids. As it passed out of the column the yellow material was collected in three fractions. Each of these was separately evaporated to dryness and dissolved in warm dilute HCl, filtered and allowed to evaporate slowly in a desiccator. The first fraction produced well-formed crystals of the hydrochloride, the second rather impure crystals and the third fraction would not crystallize. This suggested the presence of some colourless impurity that moves on the column at a rate somewhat slower than ε-DNP-lysine; it may have been O-DNP-tyrosine. On working over the various fractions, and applying chromatography to those known to be somewhat impure, there were finally obtained 10·2 mg. of pure crystals and another 14 mg. of cruder crystals, making a total yield of 24 mg. (55 % of the amount estimated). The pure material melted at 186°, alone and when mixed with authentic ε-DNP-lysine hydrochloride. (Found: C, 39·6; H, 5·4 %. Calc. for $C_{12}H_{16}O_6N_4$. HCl.H_2O: C, 39·5; H, 5·2 %.)

DISCUSSION

Under the conditions used in the above-mentioned experiments DNFB reacts with amino, phenolic-hydroxyl, thiol and possibly imidazole groups. The O-DNP-tyrosine formed by the reaction with phenolic-hydroxyl groups is colourless and thus does not interfere with the estimation of the coloured amino-derivatives. The nature of the reaction with —SH groups is not quite clear, but it appears that S-DNP-cysteine should be distinguishable from other DNP-derivatives chromatographically. It was not present in the insulin experiment. The reaction with the histidine imidazole group appears to be very slow, unless histidine is present as a terminal residue with its amino group free.

The results with insulin show that in every insulin submolecule of mol. wt. 12,000, two of the free amino groups are located on glycine residues, two on phenylalanine residues and two represent the ε-amino groups of lysine residues. The presence of free amino groups of phenylalanine has already been demonstrated by Jensen & Evans (1935), though

not quantitatively, but terminal glycine residues have not been previously reported, nor has it been shown before that the ε-amino groups of lysine are free. The present results indicate that there are six free amino groups per submolecule of insulin. From amino-N determinations by the Van Slyke method, Chibnall (1942) suggested the presence of 21 free amino groups per molecule of mol. wt. 36,000 or seven per submolecule of 12,000. This rather higher value can probably be accounted for by the presence of glycine as a terminal residue, since glycyl-peptides have been shown to give abnormally high amino-N values by the Van Slyke method (Abderhalden & Van Slyke, 1911; Schmidt, 1929). According to Schmidt, glycylglycine gives about 135 % of the theoretical value in 10 min. so that two terminal glycine residues would react as 2·7 amino groups. Using these enhanced values for glycyl-peptides, and those of Greenstein (1933) for lysyl-peptides, it is possible to compute the course of the reaction of insulin under the Van Slyke conditions. As shown in Table 4, the agreement between the computed and experimental values is reasonable.

Table 4. *Van Slyke amino-N of insulin*

Reaction time (min.)	Apparent no. of amino groups per submolecule of mol. wt. 12,000				Found by Van Slyke method
	2 glycyl-residues	2 phenyl-analyl-residues	2 lysyl-residues	Total	
6	2·5	2·0	1·9	6·4	5·9
11	2·7	2·0	2·0	6·7	6·7
30	2·7	2·0	2·0	6·7	7·1

The presence of four free α-amino groups suggests that the submolecule is built up of four open polypeptide chains bound together by cross-linkages, presumably chiefly —S—S— linkages. It is, of course, possible that other chains may be present in the form of a ring structure with no free amino groups.

SUMMARY

1. A new method is described for the identification and estimation of the free amino groups of proteins and peptides by the formation of derivatives with 2:4-dinitrofluorobenzene.

2. The method has been applied to insulin. Assuming a minimum mol. wt. of 12,000, it is shown that six free amino groups are present; two of these are located on glycine residues, two on phenylalanine residues and two represent the ε-amino groups of lysine.

3. The results suggest that the insulin submolecule of mol. wt. 12,000 is made up of four open peptide chains, two of these having terminal glycyl-

residues and the other two terminal phenylalanyl-residues respectively.

I wish to express my thanks to Dr B. C. Saunders for

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Growth and Diabetes in Normal Animals Treated with Pituitary (Anterior Lobe) Diabetogenic Extract

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When a dog receives daily injections of a diabetogenic extract of anterior pituitary tissue his body weight may rise during the course of the treatment despite the induction of an intensely diabetic condition (Young, 1937, 1938). This phenomenon, which is curiously in contrast with the behaviour of the dog rendered diabetic by pancreatectomy, may well be relevant to the fact (Young, 1939) that the diabetogenic agent in anterior pituitary extracts, effective in the partially depancreatized rat (Shipley & Long, 1938) or in the intact adult dog* (Young, 1939), has not, as yet, been clearly separated from the pituitary substance which induces growth and nitrogen retention in the rat—an animal which is not sensitive to the diabetogenic action of anterior pituitary extract unless the major part of the pancreas is first removed. Moreover, to the daily injection of a pituitary extract which is highly diabetogenic in the adult dog, the puppy responds with an accelerated rate of growth unaccompanied by symptoms of diabetes (Young, 1941). Daily treatment for 4 months or more, i.e. until the animal has become a dog rather than a puppy, ultimately may

bring about a condition in which the diabetogenic action of the extract becomes manifest and the body weight ceases to rise, though a further increase in weight can then be brought about by simultaneous treatment with pituitary extract and insulin (Young, 1944).

Preliminary communications (Young, 1942) have briefly recorded the observation that when an adult dog, consuming a limited and constant daily amount of meat just sufficient to maintain a steady body weight under normal conditions, is given daily injections of diabetogenic pituitary extract, a rise in body weight and the appearance of symptoms of diabetes are observed. The investigations described in the present paper were undertaken in order to ascertain the nature of the metabolic changes which, in the intact dog under pituitary influence, bring about a rise of body weight and excretion of sugar even in the absence of extra food. In this connexion, investigations concerning the influence of treatment with diabetogenic pituitary extract on cats and on rats have also been carried out.

METHODS

Chemical

The N content of urine, faeces, tissues and food was determined by the Kjeldahl method.

* In the present paper the term 'diabetogenic' connotes ability to induce hyperglycaemia and glycosuria (with or without ketonuria) in an intact normal animal (adult dog or cat).