SULPHATION OF INSULIN AND ELECTROPHORESIS OF THE PRODUCTS OBTAINED*

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SUMMARY

The SO_3 complexes of a few tertiary amines were tested for their ability to introduce SO_3 groups into insulin. Pyridinium sulphonic acid was found to be the most suitable one. This reagent is not hydrolyzed immediately and therefore is able to exert a sulphating action for some time after being dissolved in the reaction medium.

"Sulphate" groups** are introduced into amino groups and, at a slower rate into tyrosine and histidine residues. By variation of the reaction conditions, insulin preparations of various sulphate content were prepared and subjected to paper electrophoresis at pH 1.7 (Fig. 3). A total number of 13 well defined, approximately equidistant bands could be observed, corresponding to insulin molecules carrying different electrical charges and covering a range from + 6 units (native insulin) to — 6 units (completely sulphated insulin). The intensities of the bands in the electropherogram of a partly sulphated insulin could be related to the different reactivities of the groups concerned.

The biological activity of the preparations decreased with increasing sulphate content.

INTRODUCTION

The attachment of sulphate or sulphonate groups to protein molecules can be an effective method of obtaining protein derivatives of low isoelectric point. This has been accomplished by treating proteins with concentrated sulphuric acid at low temperatures[1,2], or with the reaction product of chlorosulphonic acid and pyridine under anhydrous conditions at 70–80° [3]. The former reaction converts aliphatic and aromatic hydroxyl groups to acid sulphate esters. The latter reaction involves the same groups and, in addition, the amino groups and sulphhydryl groups.

Far milder conditions for sulphation than those mentioned above have been described by BAUMGARTEN[4]. Pyridinium sulphonic acid (the addition complex of pyridine and sulphur trioxide) in aqueous potassium carbonate at 10° was found to

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** Although both sulphate monoester groups and N-sulphonic acid groups occur in the products, for the sake of brevity the terms "sulphation" and "sulphate groups" are generally used in this paper. This choice was made because both types of groups are converted into inorganic sulphate by acid hydrolysis.

sulphate aromatic hydroxyl groups, imidazole moieties, and amino groups of free amino acids and of a few simple peptides. No protein was investigated.

The present paper describes experiments designed to test a few tertiary amine–SO_3 complexes for their suitability to sulphate the protein insulin. The kinds of groups involved in the reaction and some properties of the insulin derivatives obtained will be reported and discussed.

SULPHATING ACTION OF A FEW REAGENTS

The sulphur trioxide complexes of four tertiary amines were tested for their ability to sulphate insulin. The preparative and analytical procedures were those described in the experimental part. A few representative data are shown in Table I.

TABLE I

REACTIVITY OF AMINE – SO_3 COMPLEXES AT pH 10, 0°

Amine	*Amount of reagent g/g insulin*	*Sulphate groups introduced per insulin molecule*
Pyridine	0.2	4.2
Pyridine	1.0	6.8
Dimethylaniline	0.3	3.3
Dimethylaniline	1.0	4.9
Dimethylaniline	2.5	5.6
Triethylamine	1.0*	$\leq$ 0.2
Trimethylamine	1.0*	0.7
Trimethylamine	1.0**	3.1

* Reaction period 3 h.
** Reaction period 24 h.

It may be concluded from Table I that, of the reagents tested, pyridinium sulphonic acid (PS)* is the most suitable one. The investigations we report on here were therefore carried out with this reagent.

The sulphonates of trimethylamine and triethylamine apparently react at a slow rate. This may be due to the stability of these complexes under the present conditions, judging from the very slow drop in pH observed after the addition of the reagents to the aqueous reaction mixture. This drop is caused mainly by the hydrolysis of the compounds. Even at the end of 24 hours only a small part of the reagent appeared to be hydrolyzed.

The hydrolysis of dimethylanilinium sulphonic acid, on the other hand, occurs in a few minutes; PS occupies an intermediate position. The different effectiveness of these two compounds as regards the sulphation reactions cannot be explained solely by the different stability, since it is the ratio of hydrolysis rate and sulphation rate that is the deciding factor. It may also be due to a difference in solubility of the suspended reagents. Naturally, the solubility of the complexes themselves cannot be measured very well, but those of the parent amines differ a great deal, since dimethylaniline is

* Abbreviations: PS, pyridinium sulphonic acid; DNFB, 2,4-dinitro fluoro benzene; DNP, 2,4-dinitrophenyl moiety.

only slightly soluble in water while pyridine can be mixed in all proportions. This difference may be reflected in the solubilities of the complexes. The following experiment indicates that it may be the dissolved part of the reagent that exerts the sulphating action.

In 10 ml borate buffer pH 10.0, 200 mg PS was suspended at room temperature. The reaction mixture was stirred and the pH kept at the same level for one hour. The small amount of reagent remaining was then filtered off. The filtrate was cooled to 0° and 200 mg insulin added. The reaction mixture was stirred and kept at pH 10.0 for one hour. The product was separated in the usual manner and found to contain an average number of two SO_3 groups per molecule.

Apparently PS is not hydrolysed immediately on being dissolved but is still reactive for some time.

THE GROUPS INVOLVED IN THE SULPHATION REACTION

Five samples of insulin were subjected to the action of different amounts of PS and the sulphate content of the products determined by the methods described in the experimental part of this paper. The more reagent was applied, the more sulphate groups were found to be introduced. The most sulphated product contained about 8 ½ sulphate groups per molecule (Table III, Fig. 1).

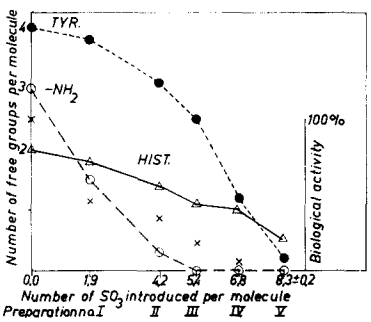


Fig. 1. Distribution of sulphate groups over tyrosine, histidine and amino groups in five sulphated insulin preparations.

(a) *Qualitative investigation*

In order to establish in a qualitative manner which groups are involved in the sulphation reaction, a preparation of high sulphate content (prep. V, Fig. 1) was subjected to the action of DNFB, the resulting product hydrolysed and the hydrolysate applied to a paper chromatogram. On the same strip, solutions of tyrosine, histidine, hydrolysates of native insulin, sulphated insulin (preparation V) and the DNP derivative of native insulin were applied. The strip was developed and stained as described in the experimental part. The result is shown in Fig. 2. The hydrolysate of insulin contains histidine and so does the hydrolysate of sulphated insulin (preparation V), because the N-sulphonate group, if present, is removed by hydrolysis. Virtually no histidine is found in dinitrophenyl insulin, because the imidazole nucleus is modified by the alkylation reaction. On the other hand, sulphated insulin, after reaction with dinitrofluoro benzene and hydrolysis, does contain histidine, apparently

because the imidazole group was shielded from the interaction with dinitrofluoro benzene by a sulphonate group. Hence *histidine* participates in the sulphation reaction.

In the same manner the participation of *tyrosine* is apparent. Finally, Fig. 2 demonstrates the complete sulphation of the *ε-amino group of lysine*. The hydrolysate of DNP insulin produced the yellow spot of ε-DNP lysine (Fig. 2, L); the hydrolysate

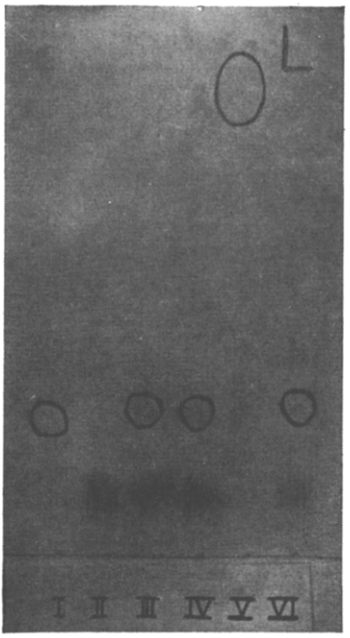


Fig. 2. Lower part of paper chromatogram of I tyrosine, II histidine, III hydrolysate of insulin, IV hydrolysate of sulphated insulin, V hydrolysate of DNP insulin, VI hydrolysate of DNP sulphated insulin.

of DNP insulin sulphate, however, did not contain any of this compound, apparently because the ε-NH_2 group was shielded by a SO_3 group against the interaction of DNFB. Even when 5 times as much hydrolysate was applied to the chromatogram no ε-DNP lysine could be detected*. This indicates that amino groups are completely sulphated by PS. Hence, it may be concluded that *tyrosine*, *histidine* and *amino groups* participate in the sulphation reaction.

(*b*) *Quantitative investigation*

The distribution of the sulphate groups over the three types of groups mentioned above was established by quantitative determination of free amino groups and free phenolic groups, the former groups by amino nitrogen determination according to Van Slyke, the latter groups by u.v. absorption in a strongly alkaline medium, as described in EXPERIMENTAL. The sulphate groups in excess of amino groups and phenolic groups were considered to be attached to histidine. The results are given in Fig. 1. The data concerning histidine are, of course, of an approximate nature only, but serve to show the trend.

* Small amounts of yellow compounds of unknown identity were present. These could be plainly distinguished from ε-DNP lysine.

The degree of sulphation of histidine in preparation V was also derived in a more direct manner from histidine determinations in the hydrolysates of Fig. 2, (for details see EXPERIMENTAL). It appeared that 0.6 out of the 2.0 histidine residues of preparation V had been free and reacted with DNFB. This compares favourably with the 0.5 free histidine residues recorded in Fig. 1. Hence all sulphate groups of preparation V are accounted for in terms of tyrosine, histidine and amino groups and no other groups of insulin are involved in the sulphation reaction.

It may be concluded that sulphation of insulin by the method described in this paper is not a specific reaction, since it acts upon three kinds of groups*. In insulin the same groups react as those of the free amino acids as found by BAUMGARTEN.

Before going on to the next paragraph one should note that the amino groups react at a higher rate than the other groups do, as shown in Fig. 1.

ELECTROPHORETIC PROPERTIES OF SULPHATED INSULIN PREPARATIONS

In acid medium, insulin is known to carry a net charge of + 6 units. It will be clear that the introduction of sulphate groups will affect this charge, making the molecules less positive or even negative, depending upon the number and site of the sulphate groups introduced.

The result of a sulphation experiment, in which, for example, on the average 2 sulphate groups are introduced per insulin molecule, will be a mixture of various molecular species, *viz.* insulin molecules carrying zero, one, two and more sulphate groups. It is therefore a mixture of molecules of almost the same molecular weight differing in electrical charge by steps of one electronic charge. These species, present in such a mixture, will manifest themselves in electrophoretic experiments on paper, giving rise to bands which, in a first approximation, will be equidistant. This has actually been found and it will be shown that the results of electrophoretic experiments are in perfect agreement with the analytical results described in the preceding paragraph.

The charge of + 6 units of insulin in acid medium is known to be due to 3 amino groups, 2 histidine residues and one arginine residue[5]. The introduction of SO_3 groups into the protein molecule causes a shift in the charge of the groups that participate in the reaction. These groups and their charge are tabulated in Table II, prior to and after sulphation.

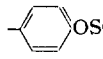
Monophenol sulphate and aminosulphonic acid are known to be strong acids. Therefore the corresponding groups in protein derivatives will carry a negative charge, as indicated in Table II.

Sulphonation of the imidazole nucleus was assumed to produce a compound with no net charge, since the nitrogen atom not involved in the reaction might retain its basic properties. This was verified in the following manner. Benzimidazole and benzimidazole N-sulphonic acid were subjected to paper electrophoresis in 33 % acetic acid. Glucose was run at the same time for the purpose of marking the point of zero charge. It was found that benzimidazole had travelled a certain distance from the glucose spot because of its positive charge; the sulphonic acid, on the other hand,

* Possibly upon four kinds of groups in SH-containing proteins, as was derived from sulphation experiments on glutathione in argon atmosphere, which indicated sulphation of the sulphhydryl group (see EXPERIMENTAL).

TABLE II

CHARGE OF INSULIN GROUPS AT pH 1,7

Native insulin				*Completely sulfated insulin*				
	n	*e*	*ne*		*n*	*e*	*ne*	*Δe*
$-NH_3^{\oplus}$	3	+1	+3	$-NH-SO_3^{\ominus}$	3	−1	−3	−2
	2	+1	+2		2	0	0	−1
	4	0	0		4	−1	−4	−1
	1	+1	+1		1	+1	+1	0
		Total charge	+6			Total charge	−6	

n number of groups per insulin molecule, *e* charge of each group, *Δe* shift in charge on sulphation of each group.

was scarcely displaced and therefore carried no overall charge. Hence, the assumption concerning the converted imidazole nucleus, as indicated in Table II, corresponds with actual fact.

These data may be applied to the preparations of Fig. 1. Preparation I probably consists of a mixture of some native insulin molecules and molecules having one, two or all three of its amino groups converted to sulphamate groups. Taking into account the shift of −2 charges for each sulphamate group, these four kinds of molecules will carry +6, +4, +2 and 0 charges. A few of each kind will carry a SO_3 group also on a tyrosine or histidine residue and therefore carry +5, +3, +1 and −1 charges, since for sulphation of a tyrosine or histidine residue a further shift of −1 unit occurs. Hence one may expect that an electropherogram of preparation I will show four relatively strong bands due to molecules carrying an even number of charges and four relatively weak bands due to molecules carrying an uneven number of charges. This has actually been found (Fig. 3, A). Clearly the even-numbered bands are relatively stronger than the uneven-numbered ones. The mobilities of the first four bands are similar to those that have previously been observed with partially acetylated insulin[6].

In the other preparations recorded in Fig. 1 most of the amino groups have reacted. The different charge species differ mainly in the degree of reaction of their tyrosine and histidine residues. Each reaction with one of these groups results in a shift of only one charge. Therefore no alternation of weak and strong bands can be expected, as was in fact observed (Fig. 3, B–E).

As indicated in Table II a completely sulphated insulin molecule may carry a net charge of −6 units. Band "−6" of Fig. 3 E corresponds to this compound. Hence all of the 13 bands corresponding to the charge species +6 to −6 have now been found.

It is apparent from the data of Fig. 1 that preparation V also contains some

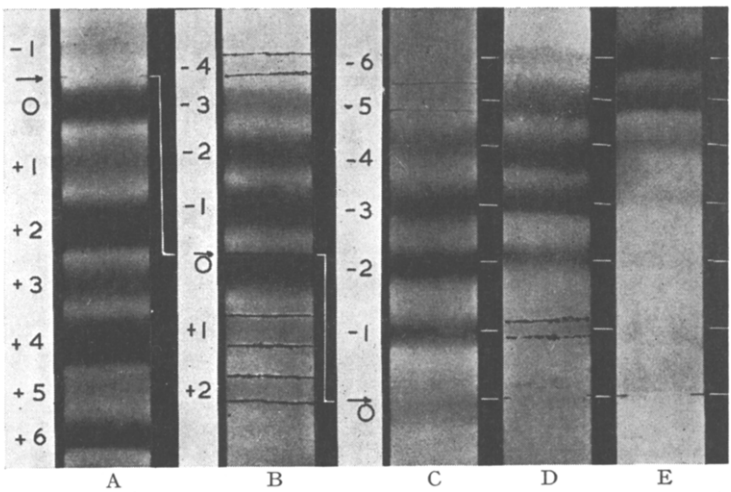


Fig. 3. Electropherograms of five insulin preparations of increasing sulphate content. A and B, 16 h, 4.4 V/cm. C, D and E, 16 h, 6.6 V/cm. Buffers: 33 % acetic acid pH 1.7. → point of application. A 50 μl 3 % protein solution, B–E 50 μl 1 % protein solution applied to the starting point.

insulin molecules having one or two free tyrosine and/or histidine residues. The bands "— 5" and "— 4" of Fig. 3 E correspond to these kinds of molecules*.

It may be concluded that the analytical data of Fig. 1 correspond very well with the electropherograms of Fig. 3.

The bands obtained are as sharp as those of native insulin at comparable concentrations. This may indicate that the electrophoretic mobility of a protein molecule is not greatly affected by whether a charged group is situated at one site or another on the molecule.

Clearly, sulphation is an effective method of obtaining protein derivatives of low isoelectric point. Even after limited sulphation of insulin (preparation II) the mean isoelectric point of the product is below pH 1.7, as shown by Fig. 3 B, since band "— 1", which is the strongest one, travels to the anode at pH 1.7.

Finally, a few remarks concerning the electrophoretic mobility of the compounds. One might expect that, in paper electrophoresis, completely sulphated insulin (charge of — 6 units) would travel to the anode at the same rate as native insulin (charge of +6 units) travels to the kathode. In fact, however, sulphated insulin moves at a lower rate than native insulin does, as can be observed by comparing strips A and E of Fig. 4. The bands "+ 6" of Fig. 3 A and "— 6" of Fig. 3 B travelled at approximately the same distance, but the former strip was run at 4.4 V/cm, the latter at 6.6 V/cm. Among other factors this lower mobility may be due to incomplete dissociation of the sulphate groups at pH 1.7.

Incomplete dissociation may also explain the electrophoretic behaviour of band "o". If glucose is run at the same time in order to mark the point of zero mobility, the glucose band does not coincide with band "o" but is found near the middle, between the bands "o" and "— 1". Thus band "o" actually carries a charge of about — ½ unit. The three sulphamate groups therefore seem to decrease the charge not by 6 units, as assumed in Table II, but by about 5 ½ units. If the groups were not

* A faint ill defined band of higher mobility than band "-6" is probably due to impurities.

dissociated at all, the decrease would have been 3 units. Consequently, the actual decrease of 5 ½ units could be explained by a dissociation of 80 % of the sulphamate groups at pH 1.7. This corresponds reasonably well with 80 % dissociation of sulphamic acid at pH 1.6, as calculated from dissociation data of solutions of the latter acid[7]. However, proton binding on other sites may explain the phenomena just as well.

BIOLOGICAL ACTIVITY

From the available data, some conclusions concerning the biological activity of insulin may be made. By increasing the degree of sulphation the biological activity of insulin decreases (Fig. 1). When most of the amino groups are converted (preparation II) a certain amount of activity still remains. This indicates that the amino groups are not essential for activity. The same conclusion has been drawn by FRAENKEL-CONRAT[3]. He found 80 % activity in an insulin preparation whose amino groups had been almost completely acetylated by means of acetic anhydride. This fact has been confirmed by us[9].

The data of preparations III and IV indicate that at least one tyrosine or histidine is not essential for activity. Preparation III has about 1/6 of the activity of native insulin (or 1/5 of the activity of acetyl insulin). The only fraction which may contain no sulphate groups on tyrosine or histidine, band "0" of Fig. 3 C, is certainly smaller than 1/6 of all fractions as judged from the electropherogram. Therefore at least one of the other fractions must have some activity.

The same line of reasoning may be followed for preparation IV, which has 1/16 of the activity of native insulin. Fig. 3 D shows that not a trace of band "0" was present. Even band "— 1" may be present in smaller amounts than 1/16 of the other fractions, indicating that even introduction of two sulphate groups on tyrosine and/or histidine residues may not lead to total loss of activity.

Non-essentiality of a part of the tyrosines is known already from iodination experiments[3] and sulphation with sulphuric acid in the cold[2]. Similar knowledge about histidine must await other experiments.

Finally, one should not overlook the possibility that an increase in negative charge, as demonstrated in the present preparations, may in itself, cause a decrease of activity.

EXPERIMENTAL

The crystalline insulin, supplied by Philips-Roxane, Netherlands, batch 04/05 A5-V, biological activity 24.5 I.U./mg, contained 8 % moisture. After drying, the analytical data were those presented in Table I.

Preparation of sulphur trioxide complexes. Pyridinium sulphonic acid was prepared from pyridine and chlorosulphonic acid[10]. The other complexes were obtained by adding a solution of sulphur trioxide in chloroform to a solution of the amine in chloroform cooled by ice–salt mixture[11].

Sulphation conditions. 200 mg crystalline insulin is dissolved in 10 ml 0.1 *M* borax at 0° and the solution adjusted to pH 10.0 with 1.5 *N* KOH from a syringe whilst vigorously stirring with a magnetic stirrer. In small portions 50–200 mg PS is suspended in the reaction mixture at intervals of about 15 min for a period of from one to three hours. The pH is maintained between 9.9 and 10.1 by slow addition of

KOH. The reaction is considered to be completed when all reagent is dissolved and the pH remains constant. The total reaction time was about 8 h for the largest amount of reagent applied. The reaction mixture is acidified to pH 5.5, and dialysed at room temperature against several changes of distilled water under stirring conditions for 65 h, and lyophylised. Yields were almost quantitative. A second 65 h period of dialysis did not decrease the total sulphur content of a few preparations.

Sulphation at pH 11.0 was carried out in 0.1 *M* soda buffer, the other conditions remaining the same.

Glutathione, 20 mg, was sulphated in 10 ml borax buffer pH 10.0 by 200 mg PS. In order to prevent oxidation a current of argon was passed through during the reaction. After the reaction was completed the mixture was acidified to pH 7 and diluted to 50 ml. Samples of 1 ml were used for amperometric titration in "tris" buffer using $AgNO_3$. No sulphhydryl groups remained.

A "blank sulphation" was carried out by adding pyridine plus sulphuric acid, instead of PS, the other reaction conditions remaining the same. In this way the sulphhydryl content was decreased by 15 %.

Dinitrophenylation. DNP insulin was prepared from zinc-free insulin according to SANGER[8]. Samples of sulphated insulin were treated in the following manner:

50 mg of preparation V (Fig. 1) and 100 mg sodium bicarbonate were dissolved in 1 ml water. 100 mg DNFB, dissolved in 2 ml alcohol, was added and the mixture shaken for 2 h at room temperature. Since no insoluble product separated after acidification with 2 drops of concentrated HCl, the reaction mixture was diluted with 3 ml water, three times extracted with 5 ml portions of ether, dialysed and lyophylised.

Hydrolysis. Samples of protein (50 mg) were hydrolysed in 5 ml twice-distilled 6 *M* HCl in evacuated vials at 110° for 32 h. The excess HCl was evaporated in a desiccator over KOH. The residue was twice taken up in water, filtered if necessary, again evaporated *in vacuo* and finally dissolved in 2.5 ml 10 % isopropanol.

Paper chromatography was carried out on Whatman paper No. 1. Histidine and tyrosine were identified by ascending–descending chromatography[13] (solvent butanol–2 *N* NH_3) and diazotized sulphanilic acid.

Paper electrophoresis of insulin was carried out on Whatman paper No. 3 MM as described previously[6].

Benzimidazole and benzimidazole N-sulphonic acid, prepared according to WEIDENHAGEN[14] were run on Whatman paper No. 1 at 4.5 V/cm for 5 h and located by their ability to absorb u.v. light[15]. Benzimidazole had travelled 85 mm to the cathode, the sulphonic acid 3 mm to the anode.

Staining of insulin derivatives. Electropherograms of preparation I were the only ones that could be stained by bromophenol blue-$HgCl_2$ or amido black. The chlorine–tolidine reaction as described by REINDEL[16] was therefore applied with one modification: after the stain had developed the strips were not washed in 2 % acetic acid, as recommended by REINDEL, but in distilled water. This improved the stability of the colour sufficiently to allow the strips to be photographed without haste.

Biological assays were carried out on groups of 6 rabbits. The injection levels were a factor of 2 to 3 apart. The values recorded in Fig. 1 are considered to be accurate to approx. 20 %. No protracted action was observed.

Amino nitrogen analyses were carried out in threefold on 10 mg samples, according to VAN SLYKE's manometric method[17]. A solution of 6 % mercuric acetate in 2 %

acetic acid was applied, in a manner and for the purpose described previously[6]. Reaction period was 15 min at 22–23°. Strong light on the reaction chamber was avoided by the use of black paper.

The results are presented in Table III. Preparations III, IV and V had low and equal values. It was assumed that all amino groups had been converted and that the low values were only seeming ones and due to unknown side reactions. A correction was applied accordingly in calculating the number of free amino groups as presented in Fig. 1.

TABLE III

ANALYTICAL DATA

Preparation no.	*Reagent g/g insulin*	*pH*	*N %*	*S %*	*S/65 N*	$\Delta E^{1\,cm}_{1\%}$	*% NH_2*	*Biological activity*
Native insulin*	—	—	15.5	3.30	6.0[5]**	15.6[5]	0.77***	26.5 I.U.
I	0.1	10.0	13.9	3.88	7.9	13.5	0.36[4]	11 I.U.
II	0.2	10.0	13.5[5]	4.80	10.2	10.3[5]	0.14	8 I.U.
III	0.5	10.0	13.4	5.35	11.3[5]	8.5	0.07[3]	4 I.U.
IV	1.0	10.0	13.3	6.01	12.8	3.9[6]	0.06[7]	1.5 I.U.
V	1.0	11.0	13.0	6.55	14.3 ± 0.2	0.6	0.07[6]	

* Dried 16 h *in vacuo* at 100°.
** Calculated 6.00.
*** Calculated 0.76 % [6].

The assumption of complete sulphation of the amino groups was confirmed by HARDINGS's[18] ninhydrin method. Preparation V yielded only 3½ % of the colour of native insulin after a heating period of 3 min. This short heating period was chosen in order to limit hydrolytic fission of N–S and other N-bonds and precipitation of native insulin in the standard.

Further confirmation was obtained from the DNP derivative of preparation V. After hydrolysis no yellow colour (from DNP glycine and DNP phenylalanine) was observed in the ether extract and no ε-DNP lysine in the water layer (see page 105).

Free phenolic groups of tyrosine were determined from the difference in u.v. absorption at 295 mμ in 0.2 *N* NaOH and in neutral or weakly acid medium. The values observed are presented in column 7 of Table III in terms of

$$\Delta E^{1\,cm}_{1\%} = \left(E^{1\,cm}_{1\%}\right) \text{alkaline} - \left(E^{1\,cm}_{1\%}\right) \text{neutral}$$

where $E^{1\,cm}_{1\%}$ denotes the extinction of a 1 % solution of the preparation concerned in a 1 cm cuvet at 295 mμ. Since the absorption in alkaline medium slightly increased with time, it was measured as a function of time and extrapolated to zero time.

The absorption of native insulin was determined in a moist preparation and corrected for the moisture content. The value given in Table III corresponds to 3.95 tyrosine residues per insulin molecule, if the molecular extinction coefficient of tyrosine is taken as 2330[19] and the insulin contains 65 N atoms per molecule[5].

Histidine was determined by a combination of paper chromatography and diazo reaction[20]. The results recorded in Table IV are based upon N determinations in the hydrolysates.

As can be seen from this table, histidine is effectively removed by dinitrophenylation, since only 4 % of the initial content remains.

TABLE IV

HISTIDINE CONTENT OF INSULIN PREPARATIONS

Preparation	*Histidine* %
Native insulin	5.3*
DNP insulin	0.2
Insulin sulphate V	4.4[5]
DNP insulin sulphate V	3.1

* Calculated 5.4 %

The sulphated preparations proved to be resistant to hydrolysis. Even after 32 hours at 110° a small part of the samples was not dissolved in 6 *N* HCl and removed by filtration. This may explain why the recovery of histidine in preparation V is only 84 %, since those molecules which were dissolved shortly before the termination of the hydrolysis were only partly hydrolysed.

The histidine content of the DNP derivative of preparation V is 70 % of that of preparation V itself, *i.e.* 1.4 out of the 2.0 histidine residues were protected against DNFB by sulphation. This compares favourably with the 1.5 groups recorded in Fig. 1.

Total sulphur was determined by combustion in oxygen and conductometric titration[21].

Total nitrogen was determined by McKENZIE's modification of the Kjeldahl method[22].

The data of Fig. 1, including the biological activities, are calculated from those of Table III, and are based upon the N content of the preparations, assuming 65 N atoms per insulin molecule (equal to 15.9 % N)[5].

The number of SO_3 groups introduced is equal to the total number of S atoms per molecule, minus 6.0. The data are accurate to $\pm$ 0.2 group. The accuracy of free amino and phenolic groups is about 0.1 group.

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THE DENATURATION OF PEPSIN

IV. THE EFFECTS OF TEMPERATURE

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SUMMARY

The temperature coefficient of pepsin denaturation has been measured as a function of pH in solutions of neutral salt (KNO_3), guanidinium chloride and $Pb(NO_3)_2$. In KNO_3 the heat of activation has been found to decline uniformly by almost 50 kcal between pH 6.0 and 6.7. This is precisely the pH region where a number (5 or 6) of carboxyl groups, which appear to be hydrogen bonded in the native protein, are cleaved by a first order process when the enzyme is inactivated. In $6.6 \cdot 10^{-4}$ *M* $Pb(NO_3)_2$ solutions the $\Delta H^{\ddagger}$ *vs.* pH curve is displaced by about 0.4 pH units in an acid direction. Guanidine also diminishes the heat of activation. In 1.18 *M* guanidine, the heat of activation shows a similar decline above pH $\sim$ 6.0 as observed in KNO_3.

INTRODUCTION

In earlier reports potentiometric titration[1] and inactivation rate data[2] have been presented from which it has been concluded that about 5 or 6 carboxyl groups are hydrogen bonded in pepsin. Moreover, these groups are thought of as forming an integral part of the network of secondary valence structures which are responsible for maintaining the enzyme in its active state. However, it was also shown that the environmental conditions which determine the pH stability of pepsin could be modified by either hydrogen bond-breaking reagents, such as urea or guanidine or by small amounts of ions possessing a high affinity for either the proton or the carboxylate anion of the hydrogen bonded carboxyl groups.

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