ELECTROPHORETIC BEHAVIOUR IN FILTER PAPER AND MOLECULAR WEIGHT OF INSULIN*

by

L. A. Æ. SLUYTERMAN

Philips Research Laboratories, N.V. Philips' Gloeilampenfabrieken, Eindhoven (Netherlands)

PAPER ELECTROPHORESIS OF INSULIN

Electrophoresis in strips of filter paper has been applied with success to many proteins, veronal solution pH 8.6 being most frequently used as a buffer[1]. For paper electrophoresis of insulin KUNKEL recommends a buffer of pH 3.0[2], without specifying the composition of the buffer. However, the present author has found by experience that insulin travels as a poorly-defined diffuse band with heavy tailing (Fig. 1), at both pH 8.0 and pH 3.0 This is probably due to some irreversible adsorption to the paper.

KALLEE[3] found even stronger adsorption of radioactive iodinated insulin. The adsorption could be diminished by addition of serum to the buffer. This method, however, has obviously only limited applicability.

It has now been found that excellent electropherograms of insulin can be obtained if a mixture of one volume of glacial acetic acid and two volumes of water is used as a buffer (pH 1.7). The protein travels as a well-defined narrow band without any tailing or residue at the point of application (Fig. 1 C).

Insulin is sufficiently stable in 33% acetic acid as judged by its crystallization ability.

In 1 ml 33% acetic acid 20 mg insulin was dissolved. After standing at room temperature (20–23° C) for one week 10 ml water (containing 1 mg zinc acetate) and sufficient ammonia to bring the pH to 5.4 were added. The resulting preparation was almost completely crystallized after three days at room temperature.

The application of the method (for details see METHODS AND MATERIALS) is illustrated in the following examples.

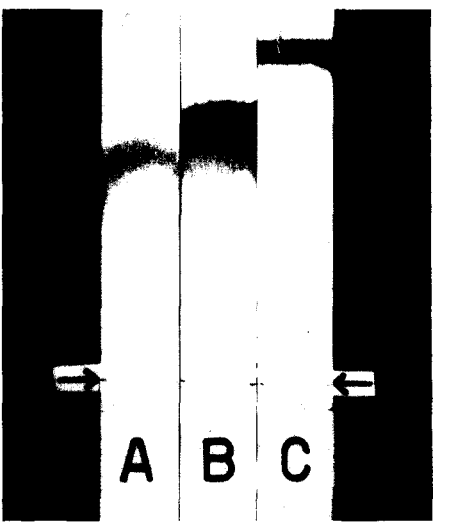

Fig. 1. Electropherograms of insulin (0.5 mg per strip) A. Veronal buffer pH 8.6, μ = 0.04, 16 h, 150 V, 0.8–1.0 mA. B. Glycine buffer pH 3.0, μ = 0.04 (without NaCl) 16 h, 150 V, 0.85–1.0 mA. A similar result was obtained in citrate buffer pH 3.0. C. 33% acetic acid, pH 1.7, 16 h, 150 V, 0.4 mA. Arrows: point of application.

* Presented in part before a joint meeting of the "Belgische Biochemische Vereniging" and the "Nederlandse Vereniging voor Biochemie", Ghent, Nov. 1954.

References p. 176.

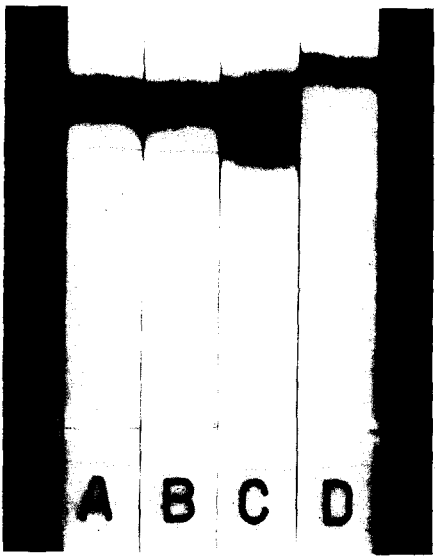

Fig. 2. A. Insulin of Philips-Roxane (batch 058/666), 16 h, 230 V. B. Old sample of insulin (war-time preparation), 16 h, 230 V. C. Preparation of low potency (6 IU/mg), 16 h, 140 V. D. On the right-hand half of the paper I insulin, on the left-hand half normal insulin was applied. 16 h, 200 V. Buffer 33 % acetic acid.

1. Crystalline insulin.

On careful examination of electropherograms containing 1 mg insulin, it appeared that all samples analysed up to the present contained a small amount of material of only slightly lower mobility than insulin and which was therefore not completely separated from the main band (Fig. 2A and B). A similar pattern was observed by TIMASHEFF[4] by free electrophoresis at pH 3.0 in the rising branch of the Tiselius cell.

Sometimes traces of material of higher mobility than insulin were found also. Only an old insulin sample contained more than negligible amounts of this material (Fig. 2B).

2. An insulin preparation of low potency (6 IU/mg). The narrow insulin band can be easily distinguished from the impurities of higher mobility (Fig. 2C).

3. Iodinated insulin of low I content (2.1 %)[5*]. It cannot be distinguished from normal insulin by electrophoresis under the present conditions (Fig. 2D).

The application of electrophoresis to the determination of the molecular weight of insulin will be discussed in the next section.

THE MOLECULAR WEIGHT OF INSULIN

At the present time there is a certain lack of agreement about the molecular weight of insulin. According to analysis of the chemical structure the molecular weight must be 6,000 (more accurately 5,732) or multiples thereof[6]. Several authors calculated a value of 12,000 from measurements of osmotic pressure[7], sedimentation velocity[8,9] and light scattering[9,10]. CRAIG, however, derived a molecular weight of 6,000 by application of the method of partial substitution combined with counter-current distribution[11]. The same value was calculated from the surface pressure of insulin monolayers[12], from sedimentation experiments[13] and from osmotic presure data in guanidinium chloride solution[14]. For the present investigation a variation of the method of partial substitution has been used, which is partially similar to the procedure applied by BATTERSBY[15] to the evaluation of the molecular weight of gramicidine-S and tyrocidine. The present method is based upon the following line of reasoning.

According to structural analysis insulin contains three amino groups (two amino end groups and one ε amino group of lysine) and three other basic groups (two imidazole groups of histidine and one guanidyl group of arginine) per unit of molecular weight 6,000. The charge of an insulin molecule at pH < 2 is therefore $6+$ if the molecular weight is 6,000 or $12+$ if the molecular weight is 12,000.

If the insulin is treated with a specific amino group reagent, which is capable of

* A sample was kindly supplied to us by Dr. O. A. DE BRUIN, Philips-Roxane, Weesp.

References p. 176.

abolishing the basicity of these groups, the charge will be diminished by a number of units equal to the number of amino groups that have reacted. Conversion of insulin in all its amino groups occurs in as many stages as there are amino groups in the molecule, yielding an equal number of compounds of successively lower charge and hence lower electrophoretic mobility. Therefore these various insulin derivatives may be detected experimentally by making electropherograms of several preparations of insulin, whose amino groups have been blocked to various degrees by variation of the reaction conditions. The total number of new compounds, of lower mobility than normal insulin, is then equal to the number of amino groups in the insulin molecule. Therefore, if the proposed experiments reveal three compounds besides insulin itself, the molecular weight of insulin is 6,000. If six such compounds are found, the molecular weight is 12,000.

Concerning the electrophoretic mobility of the modified insulins the following predictions may be made. The charge of the three insulin derivatives, to be found if the molecular weight is 6,000, is 5/6, 4/6 and 3/6 times the charge of untreated insulin. Assuming proportionality between charge and mobility, the mobilities of the three bands relative to the band of normal insulin will show the same ratio. Likewise the mobilities of the six bands to be found if the molecular weight is 12,000, will be 11/12, 10/12.... 6/12 times the mobility of normal insulin.

Generally, however, the increase in electrophoretic mobility of highly charged particles is a little less than the increase in charge[16]. Therefore the mobilities of the insulin derivatives relative to normal insulin, which carries the highest charge, may be expected to be somewhat higher than the calculated values given above.

For the actual application of the method two amino-group reagents, *viz.* dinitrofluorobenzene and acetic anhydride, have been used.

1. *Electrophoresis of insulin preparations treated with 2,4-dinitrofluorobenzene (DNFB)*

Although this reagent may act also upon the imidazole group of histidine, it does so at a much slower rate than it converts amino groups into dinitrophenyl (DNP) amino groups[17]. For the present purpose it may therefore be regarded as a specific amino-group reagent.

Insulin was treated with DNFB for 15 minutes, according to the directions of HARFENIST[11] and the resulting yellow compound subjected to electrophoresis. The results are given in Fig. 3. An unstained electropherogram (Fig. 3B) revealed two narrow yellow bands which will be due to DNP insulin species.* A duplicate strip was stained with bromophenol blue and then showed three narrow bands (Fig. 3C), two bands coinciding

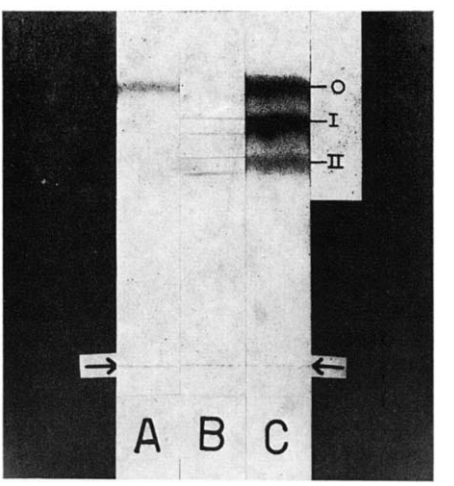

Fig. 3. A. Normal insulin. B. DNP insulin, unstained strip. C. DNP insulin, stained strip. Buffer 33% acetic acid, 16 h, 200 V.

* It will be recalled that DNP amino-acids and DNP peptides are yellow.

On the point of application a diffuse yellow band was observed, probably due to dinitrophenol (formed by hydrolysis of the excess DNFB). This band was removed during the staining procedure, as is to be expected for dinitrophenol.

References p. 176.

with the two previously yellow bands and one new band with a mobility equal to that of normal insulin (Fig. 3A).

These strips demonstrate that introduction of DNP groups indeed results in a decrease in electrophoretic mobility of insulin. Since the reaction mixture apparently still contained normal insulin, the two yellow bands may represent the first two stages of conversion ($(DNP)_1$ insulin and $(DNP)_2$ insulin). The mobilities of these bands (Table I) appear to be approximately 5/6 and 4/6 times the mobility of normal insulin. This corresponds to what has been predicted for the alternative that the molecular weight of insulin is 6,000.

TABLE I

ELECTROPHORETIC MOBILITY OF INSULIN DERIVATIVES

Derivative	*Band No.*	*Assumed identity of component*	*Relative mobility**		
			calc. (a)	*found (b)*	*found (c)*
DNP insulins	I	$(DNP)_1$ insulin	0.83	0.87	
	II	$(DNP)_2$ insulin	0.67	0.73	
Acetyl insulins	I	Ac_1 insulin	0.83	0.87	0.86
	II	Ac_2 insulin	0.67	0.72	0.72
	III	Ac_3 insulin	0.50	0.54	0.55

* Given as a fraction of the mobility of normal insulin.
(a) Calculated on the assumption of proportionality between charge and mobility.
(b) Electrophoresis in free hanging strips.
(c) Electrophoresis in strips between glass plates.

An attempt has been made to show the presence of a third yellow band, with 3/6 of the mobility of normal insulin, in an insulin preparation treated with DNFB for a longer period. However, such a preparation, resulting from a reaction period of 30 minutes, appeared to be insufficiently soluble in 33% acetic acid to allow electrophoresis. Since this

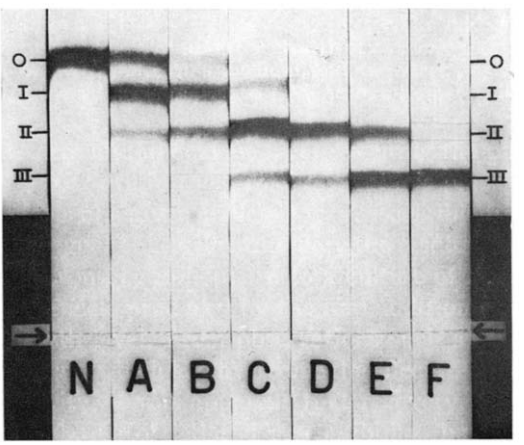

Fig. 4. Acetyl insulin. N. Normal insulin. A–F Acetylated insulin preparations (a–f). Buffer 33% acetic acid, 16 h, 230 V.

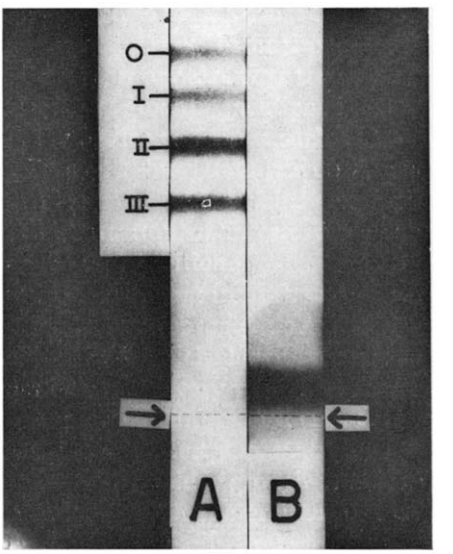

Fig. 5. Acetyl insulin. A. Mixture of preparations analysed in Fig. 4. B. Glucose (reference point). Buffer 33% acetic acid, 16 h, 230 V.

References p. 176.

lack of solubility might be due to the hydrophobic properties of the DNP group, the introduction of a more favourable group was attempted. The acetyl group appeared to be suitable, as will be described below.

2. Electrophoresis of insulin preparations treated with acetic anhydride

It has been shown by OLCOTT that acetic anhydride is a specific acetylating agent of the amino groups in proteins[18]. Insulin was one of the proteins studied by these authors.

For the present investigation insulin preparations of various degrees of acetylation were prepared by varying the amount of acetic anhydride and the reaction time. In Table II the mean degrees of acetylation, calculated from the content of remaining free amino groups, are presented. The corresponding electropherograms of the preparations (Fig. 4) clearly reflect the progress of the reaction. With increasing acetylation bands of lower mobility appear, those of higher mobility disappear. When virtually all amino groups have been converted, the band of lowest mobility (Fig. 4F, band III) is the principal one, the preceding band (Fig. 4F, band II) being only very faint.

Fig. 5 shows the electropherogram of a mixture of all preparations.

Apparently a total number of four bands can be observed, one due to normal insulin, three to acetylated insulin species. The mobilities of the latter three compounds (Table I), are approximately 5/6, 4/6 and 3/6 times the mobility of normal insulin. Hence both the number of bands and their relative mobilities are in accord with the assumption that the molecular weight of insulin is 6,000.

TABLE II

APPARENT AMINO-NITROGEN CONTENT OF INSULIN PREPARATIONS

Preparation	*Reaction conditions**	N_{NH_2} %	*% acetylation*
insulin	—	0.73 %**	0%
prep. (a)	½ drop	0.49%	33%
(b)	¾ drop	0.42%	42%
(c)	1 drop	0.30%	59%
(d)	2 drop	0.18%	75%
(e)	180 mg	0.10%	86 %
(f)	twice 180 mg	(0.05%)	(93 %)

* Amount of acetic anhydride per 200 mg insulin.
** Calculated value 0.76 % (see experimental part).

In order to be certain that the insulin molecule is not broken down into units of molecular weight 6,000 during the acetylation procedure, a sample of insulin was subjected to the treatment of preparation (e), using acetic acid instead of acetic anhydride. The resulting preparation could be crystallized in the normal manner. Therefore the acetylation conditions are sufficiently mild not to lead to denaturation.

It can be learned from Table I that the mobilities of the two yellow bands of the DNFB-treated insulin are equal to the mobilities of the first two bands of acetylated insulin. This confirms the assumed identity of the yellow bands as given in Table I.

DISCUSSION

If the actual molecular weight of insulin were 12,000, the observed facts could be explained only by assuming that the reaction rate of the second, fourth and sixth acetylation steps is considerably higher than the rate of the first, third and fifth steps.

References p. 176.

This, however, seems very unlikely. The present experiments therefore strongly support the assumption that the molecular weight of insulin is 6,000.

Up to the present, separation of the components in the method of partial substitution was achieved by counter current distribution. The present experiments demonstrate that it is possible to use electrophoresis instead. This may widen the field of application since most proteins can be subjected to electrophoresis but not to counter current distribution.

Finally, it may be worth pointing out that partially acetylated proteins can be used to study the electrophoretic mobility as a function of the charge of the molecule. This procedure has the advantage that molecules of different charge can be obtained at the same time in one medium, since there is no need to use a variety of buffers, as must be done when the charge is varied by shifting the pH. As far as insulin is concerned, the present method apparently demonstrates an approximately linear relationship between charge and mobility in 33% acetic acid, pH 1.7. This may be due to a low maximum charge (6+) and a sufficiently high ionic strength (0.02).

METHODS AND MATERIALS

Preparation of DNP insulins

Two preparations were made according to the directions of HARFENIST[11] for reaction periods of 15 minutes (preparation (a)) and 30 minutes (preparation (b)). Most of a sample of 90 mg preparation (a) could be dissolved in 2 ml 33% acetic acid and dissolved completely on addition of 0.5 ml 96% acetic acid to the mixture. This solution was used for making electropherograms (Fig. 3), using 33% acetic acid as a buffer.

Preparation (b) did not dissolve very well in 33% acetic acid but was taken up in 50% acetic acid. Electropherograms made with 33% acetic acid as a buffer, revealed the three bands found in preparation (a) and a band at the point of application, which may have contained $(DNP)_3$ insulin. The solution of preparation (b) appeared to be rather unstable, since some precipitate was formed after standing at room temperature for 4 days.

Preparation of insulin of low zinc content

Samples of insulin were made virtually zinc free in order to make them more readily soluble in weakly basic medium.

In 100 ml 2% acetic acid 1.0 g crystalline insulin (Philips-Roxane, batches OWO, 06050 H3 and 658/666, zinc content 0.4%) was suspended. On addition of one drop of concentrated HCl the insulin readily dissolved. Some disodium salt of ethylenediaminetetraacetic-acid (75 mg) was added and the solution brought to pH 5.4. After standing at room temperature overnight the precipitate was centrifuged off, washed twice with distilled water, with alcohol and ether and dried *in vacuo*. Yield 919 mg, zinc content 0.03% (determined by spectrochemical analysis).

Preparation of acetyl insulins

Preparation (a). In 12 ml water 200 mg zinc-free insulin was suspended and cooled in icewater. On addition of 4 drops of 2 *N* KOH and stirring, the insulin dissolved slowly. The pH of the solution, measured with universal indicator paper, was about pH 8.5. Sodium acetate 3 aq. (3 g) was added and dissolved. The solution became turbid. While stirring, one drop of a mixture of equal volumes of acetic anhydride and acetic acid was added. The stirring was continued for a few minutes. The reaction mixture was left standing at 0° C for 5 more minutes. The pH was then about 6.5. One drop of acetic acid was added to make the pH 5–6.

The reaction mixture was dialysed for 10 hours against several changes of distilled water at room temperature and lyophilized. Recovery 195 mg. The sample contained 2.7% sodium ion (determined as Na_2SO_4 after destruction with H_2SO_4-HNO_3).

The dialysis was executed in cellulose dialysing tubing of 2 cm width. Since this membrane is not absolutely impermeable to insulin, the dialysis could not be prolonged in order to obtain a salt-free preparation.

Preparation (b). In 12 ml water 200 mg insulin was dissolved at 0° C and sodium acetate added as described for preparation (a).

In 2 ml dry carbon tetrachloride 1 drop of acetic anhydride was dissolved. Of this solution 1.5 ml was added to the insulin-acetate solution. The reaction mixture was stirred during three minutes and left standing for 10 minutes. The carbon tetrachloride was separated. All operations were carried out at 0° C.

References p. 176.

The insulin was separated from the reaction mixture as described for preparation (a). Recovery 185 mg. Sodium content 2.0%.

Preparation (c). One drop of pure acetic anhydride was used. The remaining parts of the procedure were similar to the preparation of (a). Recovery 195 mg. Sodium content 3.2%.

Preparation (d). The main difference in the procedure compared with the above preparations is the addition of 2 drops of acetic anhydride. The second drop was added 5 minutes after the first. The pH after the completion of the reaction was about 5. Therefore no acetic acid was added. Recovery 184 mg, sodium content 2.3%.

Preparation (e) was made similarly. An amount of 180 mg acetic anhydride was added dropwise over a period of 40 minutes. Recovery 191 mg, sodium content 1.4%.

Preparation (f). A sample of preparation (e) was subjected to the same treatment for a second time. Recovery 89%.

Amino-nitrogen determinations

These have been made in a VAN SLYKE-NEILL chamber[19], from which the constrictions for measurement of the gas volume had been omitted. After completion of the reaction the gases were transferred into the buret and hempel pipet of a micro volumetric VAN SLYKE apparatus and treated in a manner conventional with the latter apparatus.

For each determination 1.8–2 ml protein solution in 30% acetic acid, 0.2 ml caprylalcohol, 0.35 ml acetic acid and 1 ml water were brought into the reaction chamber in the order given. After evacuation of the chamber and expulsion of the bubble of air[19], 2 ml $NaNO_2$ solution (80 g/100 ml water) and 2 ml mercuric-acetate solution (0.6 g/10 ml) were mixed and brought into the reaction chamber. The reaction mixture was shaken during a few seconds at the start, after 1, 3, 6 and 10 minutes and during the whole of the last minute. The total reaction time was 15 minutes.

Although insulin contains 3 amino groups per unit of molecular weight 5,732 an apparent number of 3.35 groups has been found[6]. The discrepancy is known to be due to the terminal glycyl-residue, since peptides with a terminal glycyl-residue yield more than the theoretical amount of nitrogen[20]. Glycylglycine, for instance, gives 135% nitrogen.

Addition of mercuric salts to the reaction mixture has a favourable effect. Glycyl-peptides then give almost theoretical values in a *volumetric* apparatus[21]. Under the conditions described above, glycylglycine appeared to yield 109% nitrogen even in the presence of mercuric salts. If this is taken into account insulin may be expected to have an apparent number of 3.1 groups, or 0.76% amino nitrogen. Actually a value of 0.73 ± 0.02% was found, which is sufficiently accurate for the present purpose.

The values presented in Table II are corrected for the sodium content of the preparations. This sodium was assumed to be present in the form of anhydrous sodium acetate.

Paper electrophoresis

Mainly the method of free hanging strips was used, following the procedure of FLYNN[22]. The electrophoretic tank was made of polymethylmethacrylate. The buffer was contained in glass troughs. The space of the tank above the troughs was divided into 8 compartments in order to ensure equal conditions for all paper strips. Each compartment contained one strip.

The walls and cover of the tank were lined with filter paper moistened with buffer in order to saturate the atmosphere with vapour. Although all evaporation from the paper strips during the runs can not be prevented, this is no serious disadvantage, since a simple calculation shows that the relative mobilities are not affected if the evaporation is uniform along the strip[23]. This could be confirmed by a few experiments with strips sandwiched between glass plates[2]. The relative mobilities found by the former and the latter procedures were only slightly different (Table I).

Strips of filter paper Whatman No. 3 mm, 4 × 45 cm, were immersed in the buffer and blotted between sheets of filter paper in order to remove the excess of liquid. Samples of 0.05 ml 1–2%, protein solution were applied to the strips by means of a comb as proposed by TOENNIES[24]. A large drop of the solution to be analysed is brought on a surface made water-repellent with silicone grease. A comb, whose teeth have been ground down until the appropriate volume could be taken up in the spaces between the teeth within a length of 4 cm, is passed through the top of this drop. Thus the spaces between the teeth are filled with solution. The comb is then placed upon the paper strip along the starting line. The strip sucks up the solution. This procedure has three advantages:

1. In a simple manner the same volume is always applied.
2. A well-defined uniform band is obtained.
3. One half of the comb can be filled with one solution, the other half with another solution. Thus two different solutions can be applied very close to each other on one strip. This procedure has been used for instance for the strip presented in Fig. 2 D.

It is essential for successful application that the comb be kept in water or in a dilute soap solution. The comb is dried only immediately before use.

The protein bands were stained by immersing the strips for 16 hours in a dye bath containing 0.2 g bromophenol blue, 50 g $HgCl_2$ and 50 ml acetic acid in 1 l water[25]. The excess dye was removed

References p. 176.

by washing for half an hour in three changes of 2% acetic acid. The strips were dried and held in ammonia vapour in order to produce the blue form of bromophenol blue.

ACKNOWLEDGEMENTS

Grateful thanks are due to Dr J. DE JONGE and Dr S. A. TROELSTRA for various discussions and to Mr H. A. HAUMERSEN for his technical assistance.

SUMMARY

Insulin travels as a well defined band in electropherograms if acetic acid-water 1:2 (v/v) is used as a buffer. Preparations of partially acetylated insulin were analysed by this method. From the results it could be derived that the molecular weight of insulin is 6,000. An improvement in the amino-nitrogen determination of insulin is described in the experimental part.

RÉSUMÉ

L'insuline se déplace sous forme d'une bande bien définie sur des électrophérogrammes lorsqu'on utilise comme tampon un mélange acide acétique-eau 1:2 (v/v). Des préparations d'insuline partiellement acétylée ont été analysées par cette méthode. Les résultats permettent d'attribuer à l'insuline un poids moléculaire de 6,000. Dans la partie expérimentale, on décrit une amélioration du dosage de l'amino-N de l'insuline.

ZUSAMMENFASSUNG

Insulin wanderte als ein genau begrenzter Streifen in einem Elektrophoregramm, in dem eine Mischung von Essigsäure-Wasser 1:2 (v/v) als Puffer verwendet wurde. Präparate, die aus teilweise acetyliertem Insulin bestanden, wurden mit Hilfe dieser Methode analysiert. Aus den Ergebnissen wurde gefolgert, dass das Molekulargewicht des Insulins 6,000 ist. Im experimentellen Teil wird eine verbesserte Bestimmungsmethode für Aminostickstoff im Insulin beschrieben.

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Received December 8th, 1954