Diabetologia 6, 15–20 (1970)

The Antigenicity of Pig Insulin*

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Received: April 5, 1969

Summary. Treatment of human subjects with a neutral solution of pure pig insulin crystals does not lead to the formation of insulin antibodies. Thirty-six non-diabetics with psychiatric diseases were treated with a neutral solution of crystalline pig insulin for a maximum period of 104 days, using a 24-h dosage of up to 208 i.u. In patients who had previously not received insulin treatment (24 patients), insulin antibodies could not be demonstrated after the termination of the insulin treatment. However, in patients who had previously received treatment with acid solutions of insulin consisting of pig and ox insulin (12 patients), it was possible in almost all cases to demonstrate insulin antibodies after the termination of the insulin treatment. In addition, 10 pigs received treatment with solutions of pure pig insulin. Insulin antibodies could be demonstrated in only 2 pigs, both treated with acid solutions of recrystallized pig insulin, whereas antibodies could not be demonstrated in pigs treated with neutral solutions of recrystallized pig insulin. The reason why pure preparations of pig insulin are in most cases also antigenic to man, is presumably that the pig insulin preparations are injected as suspensions (zinc insulins, NPH insulin) or as is the case with acid solutions of insulin, converted to suspensions after injection, since insulin precipitates when the acid insulin solution is neutralized by tissue fluid.

L'antigénicité de l'insuline de porc

Résumé. Le traitement de sujets humains avec une solution neutre de cristaux d'insuline pure de porc ne provoque pas la formation d'anticorps insuliniques. Trente-six sujets non-diabétiques, atteints de maladies psychiatriques, ont été traités avec une solution neutre d'insuline cristallisée de porc, pendant 104 jours au maximum, en utilisant une dose quotidienne allant jusqu'à 208 U. Chez les patients qui n'avaient pas été traités auparavant par l'insuline (24 patients), on ne pouvait pas démontrer la présence d'anticorps insuliniques après la fin du traitement à l'insuline. Par contre, chez les patients (12) qui avaient été traités auparavant par des solutions acides d'insuline (insuline de porc et insuline de boeuf), il était possible dans presque tous les cas de démontrer la présence d'anticorps insuliniques après la fin du traitement par l'insuline. — En outre, 10 porcs ont reçu un raitement avec des solutions d'insuline pure de porc. On a pu trouver des anticorps insuliniques seulement chez 2 porcs, traités avec des solutions acides d'insuline de porc recristallisée, tandis qu'on n'a pas pu trouver d'anticorps chez les porcs traités avec des solutions neutres d'insuline de porc recristallisée. La raison pour laquelle les préparations pures d'insuline de porc sont dans la plupart des cas également antigéniques chez l'homme, est probablement que les préparations d'insuline de porc sont injectées en suspensions (insuline-zinc, NPH-insuline), ou que, comme c'est le cas pour les solutions acides d'insuline, elles se transforment en suspensions après l'injection, puisque l'insuline précipite quand la solution acide d'insuline est neutralisée par les liquides tissulaires.

Die Antigen-Wirkung von Schweine-Insulin

Zusammenfassung. Behandlung von Menschen mit neutralen Lösungen von reinen Schweineinsulinkristallen führte nicht zur Bildung von Insulinantikörpern. 36 Patienten mit psychiatrischen Krankheiten ohne Diabetes wurden maximal 104 Tage lang täglich mit einer neutralen Lösung von reinen Schweineinsulinkristallen gespritzt. Die höchste Tagesdosis war 208 E. Bei Patienten, die früher niemals eine Insulinkur durchgemacht hatten (24 Patienten), konnten nach der Kur mit neutralem Schweineinsulin keine Insulinantikörper nachgewiesen werden. Bei Patienten, die früher einmal Insulinkuren mit Schweine-Rinderinsulinkristallen, gelöst in verdünnter HCl, durchgemacht hatten (12 Patienten), konnten jedoch in beinahe allen Fällen nach der Kur mit neutralem Schweineinsulin, Insulinantikörper nachgewiesen werden. — Von 10 Schweinen, die mit Schweineinsulin gespritzt wurden, konnten Insulinantikörper nur bei Schweinen nachgewiesen werden, die mit Schweineinsulin in saurer Lösung gespritzt waren. Es wird angenommen, daß die Ursache zur Antigenizität vieler Schweineinsulinpräparate Menschen gegenüber daran liegt, daß sie als Suspensionen gespritzt werden (Zink-Insuline, NPH-Insulin) oder — wie es mit den sauren Insulin-Lösungen der Fall ist — nach der Injektion zu Suspensionen umgewandelt werden, indem Insulin präcipitiert, wenn die sauren Lösungen von der Gewebeflüssigkeit neutralisiert werden.

Key-words: Insulin antibodies, pig insulin, isoimmunization, insulin dose.

Humoral insulin antibodies can be demonstrated in almost all subjects who have been treated with insulin (Deckert, 1967). In man, however, the antigenicity of preparations of pig insulin seems to be less pronounced than the antigenicity of preparations of pure ox insulin or mixtures of pig and ox insulin (Devlin and O'Donovan, 1966). This is presumably because ox insulin differs more in structure from human insulin than pig insulin does.

The sole structural difference between pig insulin and human insulin is the C-terminal amino acid in the B-chain, which is alanine in pig insulin and threonine in human insulin (Table 1). It has, nevertheless, been found that antibody formation in diabetic subjects cannot be avoided, even though these patients are treated with long-acting insulin preparations pre-

* Delivered as a lecture in a slightly altered form, at the III Congress of the Deutschen Diab. Gesellschaft, Göttingen 1968.

pared exclusively from pig insulin (Berson and Yalow, 1963; Deckert, 1964; Lockwood and Prout, 1965).

The reason for the antigenicity of pig insulin in man has not been clarified. It can hardly be due to the structural difference existing between pig insulin and

Table 1. *The characteristic differences in the primary structure of insulin from various animal species*

	A_8	A_9	A_{10}	B_{30}
ox insulin	ala	ser	val	ala
sei-whale insulin	ala	ser	thr	ala
sheep insulin	ala	gly	val	ala
horse insulin	thr	gly	ileu	ala
sperm-whale insulin	thr	ser	ileu	ala
pig insulin	thr	ser	ileu	ala
rabbit insulin	thr	ser	ileu	ser
human insulin	thr	ser	ileu	thr

human insulin, since it has not been possible to demonstrate any immunological difference between pig insulin and human insulin (Deckert and Jørgensen, 1966), nor between pig insulin and desalanine-pig insulin (Berson and Yalow, 1963), a preparation of pig insulin from which the C-terminal amino acid, alanine, has been split off.

Further, the supposition that the structure of circulating endogenous human insulin is different from that of crystallized pig insulin or human insulin after preparatory purification, must be regarded as unlikely, since it can be shown that plasma insulin from normal subjects as well as overweight subjects and diabetics does not differ immunologically from crystalline pig insulin or human insulin (Deckert and Jørgensen, 1966).

The following investigations were made in an attempt to determine the reason for the antigenicity of long-acting pig insulin preparations.

Material and Methods

Twenty-four patients with psychiatric diseases who were non-diabetics and who had not previously received treatment with insulin, were given an intramuscular injection of a neutral solution of recrystallized pig insulin nearly every day for periods up to 104 days.

The size of the insulin dose varied from patient to patient and from day to day, depending on the aim and effect of the treatment. Table 2 shows the duration of the treatment and the maximum dose of insulin per 24 h. A similar treatment was given to 12 patients with psychiatric diseases who were non-diabetic, but who had previously, some months to several years before, been treated with acid solutions of recrystallized pig-ox insulin for a shorter period (Table 3). Blood samples were taken from the cubital vein on the day prior to the commencement of the insulin treatment, and 2 days after the termination of the insulin treatment. Serum for insulin antibody investigation was stored at −20° C.

For isoimmunization tests, 12 pigs were used weighing between 22 and 28 kg, 11 of them from one litter (Brunfeldt and Deckert, 1964a). Six pigs were treated with recrystallized pig insulin dissolved in phosphate buffer (pH 7.3), free from antiseptics. Four

Table 2. *Patients treated with a neutral solution of pig insulin. None of these patients had ever been treated with insulin. n.e. = not examined*

Name	Age (years)	Duration of treatment (days)	Maximum insulin dose/day (units)	Insulin antibody concentration (per cent)	
				before	after
A.B.H.	20	13	48	1.0	0.5
B.M.C.	50	44	40	0.4	0.6
H.M.	47	23	40	0.1	0.2
I.S.	47	27	16	0.8	0.1
J.S.	33	44	8	0.3	1.1
G.N.	33	45	40	0.4	0.7
L.P.	48	44	32	1.1	0.6
J.J.H.	32	44	56	n.e.	0.3
G.L.J.	45	51	24	n.e.	0.5
E.P.	41	32	32	0.2	0.7
A.M.	30	99	40	0.3	0.6
L.R.	31	55	32	n.e.	0.3
K.B.J.	33	44	4	n.e.	1.2
B.P.	35	44	32	n.e.	0.5
O.J.	46	48	48	0.8	1.3
I.B.M.	25	86	208	0.4	0.3
A.H.B.	51	54	128	0.0	0.5
I.N.	36	52	128	0.7	0.5
E.R.J.	20	62	128	0.0	0.3
L.N.	50	54	80	n.e.	1.1
E.T.	37	53	144	n.e.	0.0
B.H.	44	104	144	n.e.	0.9
J.Ø.	41	64	96	0.6	0.6
J.B.	31	80	80	0.3	0.2

pigs were treated with recrystallized pig insulin dissolved in diluted hydrochloric acid (pH 3.2) to which glycerol and an antiseptic had been added. Two pigs were used as controls (Table 4). The biological strength

Table 3. *Patients treated with a neutral solution of pig insulin. All these patients had been treated with acid solutions of pig-ox-insulin in earlier periods. n.e. = not examined*

Name	Age (years)	Duration of treatment (days)	Maximum insulin dose/day (units)	Insulin antibody concentration (per cent)	
				before	after
V.J.	46	42	40	0.1	4.5
K.I.N.	47	45	32	0.6	11.0
E.M.J.	30	45	56	n.e.	0.3
T.L.	58	50	24	0.7	12.7
E.L.H.	30	46	8	0.3	0.1
E.N.	33	49	32	0.2	7.4
V.A.	56	49	20	0.2	2.8
K.H.P.	38	69	40	0.9	8.2
N.E.M.	44	46	144	n.e.	6.6
E.T.	58	51	64	0.4	17.2
A.H.	33	103	64	0.5	4.8
B.N.	35	104	48	0.1	7.5

of the insulin preparations was 24—26 i.u./mg. Insulin without the addition of adjuvants was injected subcutaneously daily for 87—90 days. Nine of the animals

received a total of 2250 i.u. insulin each, beginning with 15 i.u. daily, later with increasing doses. One animal (Brunfeldt and Deckert, 1964a) received a total of 1050 i.u. insulin. The day prior to and two days after the termination of the insulin treatment, blood samples

Table 4. *Pigs treated with pig insulin. Pigs 1—6 were injected with recrystallized pig insulin dissolved in neutral solution (pH 7.3). Pigs 7—10 were injected with recrystallized pig insulin dissolved in acid solution (pH 3.2)*

No.	Insulin preparation	Duration of treatment (days)	Total insulin dose (units)	Insulin antibodies before	Insulin antibodies after
1	neutral pig	90	2250	0	0
2	neutral pig	90	2250	0	0
3	neutral pig	90	2250	0	0
4	neutral pig	90	2250	0	0
5	neutral pig	90	2250	0	0
6	neutral pig	90	2250	0	0
7	acid pig	87	1050	0	++
8	acid pig	90	2250	0	0
9	acid pig	90	2250	0	+++
10	acid pig	90	2250	0	0
11	control	—	—	0	0
12	control	—	—	0	0

were taken and serum for antibody investigation was stored at −20°C.

The human sera were examined for insulin antibodies by a modification of the double antibody technique described by Skom and Talmage (1958). 100 µl of serum to which 2.5 µl heparin LEO was added (5000 units/ml) and 100 µl of blind (0.04 M phosphate buffer, pH 7.4 with 5% human albumin) were both incubated for 4 days at 4°C with 100 µl of an 0.04 M phosphate buffer, pH 7.4, containing 0.5% human albumin and 0.9% NaCl together with not quite 0.2 µg ^{125}I-pig insulin/ml. The specific radioactivity of the iodinated insulin was about 60 mCi/mg. After standing for 4 days, 50 µl of the incubation mixture was diluted with 5000 µl 0.04 M phosphate buffer, pH 7.4, containing 0.25% human albumin. Two hundred µl of this dilution was incubated for 24 h with 100 µl of a dilution of serum from rabbits immunized with human gammaglobulin. The dilution of the rabbit antihuman gammaglobulin was made with 0.04 M phosphate buffer, pH 7.4, and was so adjusted that 100 µl of the dilution could completely precipitate the amount of gamma globulin present in the reaction mixture. After the incubation, the precipitate formed was filtered off on oxoid filters. The precipitate was rinsed twice with 0.04 M phosphate buffer containing 0.5% human albumin, and the radioactivity then measured in a Wellcounter (Philips).

The "insulin-antibody concentration" was expressed as the amount of radioactive insulin bound to the immunoglobulins, as a percentage of the total radioactivity, *i.e.* the radioactivity of the sample on the filter minus the radioactivity of the blind sample on the filter, as a percentage of the total activity. The total activity corresponded to the overall radioactivity in the second incubation mixture (corresponding to approximately 2 mµg ^{125}I-insulin). All determinations were performed as double tests. SEM (double determinations) = 0.19%.

The ^{125}I-pig insulin was supplied by Nordisk Insulinlaboratorium. The rabbit anti-human gamma-globulin was supplied by Dansk Svovlsyre and Superphosphat Fabrik A/S, and the freeze-dried human albumin was supplied by Statens Seruminstitut.

Using the same method, the insulin antibodies were examined for their ability to discriminate between pig insulin and ox insulin, after varying amounts of recrystallized pig insulin and recrystallized ox insulin, respectively, had previously been added to the serum. The pig and ox insulin crystalline powders were supplied by Nordisk Insulinlaboratorium.

After the addition of ^{131}I-pig insulin, the sera from the pigs were examined by means of immunoelectrophoresis and autoradiography as previously described (Brunfeldt and Deckert, 1964a). The specific activity of the isotope-labelled insulin was 140—180 mCi/mg. It was used in a concentration of approximately 0.04 µg ^{131}I-insulin/ml serum.

Results

Patients who had not previously been treated with insulin had an "insulin-antibody concentration" of < 1.2%, namely 0.46 ± 0.34.

Elevated insulin-antibody concentration could not be demonstrated in any of the patients or the animals, prior to the commencement of the insulin treatment.

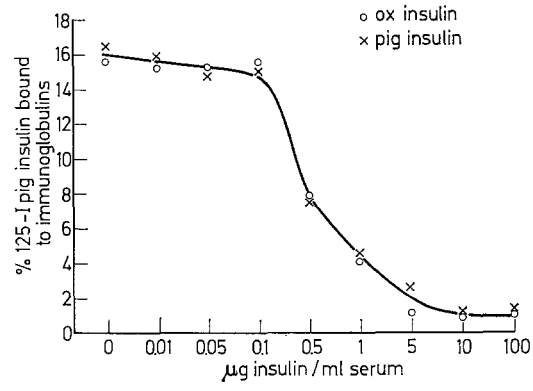

Fig. 1. Serum containing insulin antibodies from a patient after re-immunization with a neutral solution of pig insulin. For some years, the patient had been treated with an acid solution of pig-ox insulin. Various amounts of pig and ox insulin, respectively, were added to the serum (abscissa). The insulin antibodies (ordinate) could not discriminate between pig and ox insulin

In only one (O.J.) of the 24 patients who had not previously been treated with insulin, could insulin antibodies be demonstrated after the termination of the insulin treatment (Table 2). However, the concen-

tration of insulin antibody was very slight, being only significantly higher than in non-insulin-treated patients at the 5% level, but not at the 1% level.

In contrast to the absence of antibody formation in the patients not previously treated with insulin, 10 out of the 12 patients who had previously received insulin treatment with mixed pig-ox insulin preparations showed insulin antibodies following the renewed insulin treatment (Table 4).

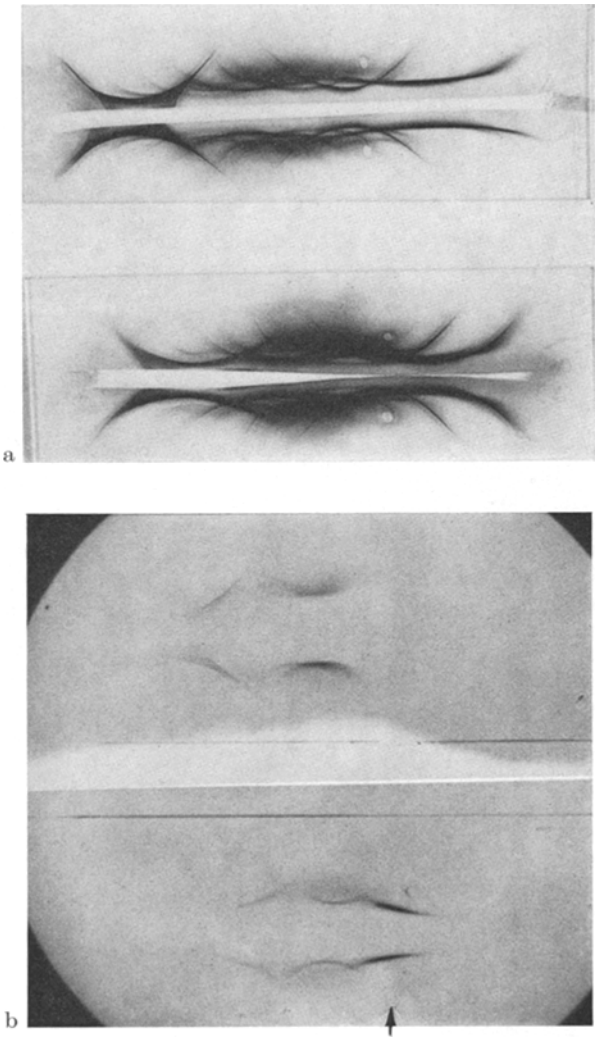

Fig. 2. Immunoelectrophoresis (a) and autoradiography (b) of serum from pig No. 9, to which ^{131}I-pig insulin was added, before and after immunization with pig insulin. The arrow indicates the position of the most rapidly migrating complex consisting of IgG and ^{131}I-pig insulin. Radioactivity, arising from radioactive degradation products is bound to albumin and alpha-2-globulins

In one case, the insulin antibodies formed were examined for their ability to discriminate between pig and ox insulin. No discrimination was found (Fig. 1).

None of the 6 pigs treated with recrystallized pig insulin in neutral solution showed insulin antibodies after the termination of the insulin treatment. However, 2 of the 4 pigs that had been treated with recrystallized pig insulin in acid solution showed insulin antibodies after the termination of the insulin treatment (Fig. 2, Table 4).

Discussion

The absence of insulin antibody formation after treatment with solutions of neutral pig insulin in patients not previously treated with insulin, is not due to the treatment being too low in intensity, since a corresponding intensity of treatment with acid pig-ox insulin solutions resulted in the formation of insulin antibodies (Table 5) in almost all cases — as previously demonstrated (Deckert, 1964).

Table 5. *Patients treated with different insulin preparations. (1^x) = insulin antibody demonstration in this one case was not significant at the 1% level (see page)*

Number of patients	Treated with	Duration of of treatment	Insulin antibodies found in
9	acid solution of recrystallized pig-ox-insulin	40—70 days	8 cases
12	NPH-pig insulin	6—48 months	11 cases
24	neutral solution of recrystallized pig insulin	13—104 days (see Table 1)	0 (1^x) cases

It is remarkable that the treatment of human subjects with large doses of pig insulin in neutral solution did not lead to a demonstrable formation of antibodies. This is in contrast to the findings in patients treated with longacting pig insulin preparations, since it had already been reported (Deckert, 1964) that circulating insulin antibodies could be demonstrated in 11 out of 12 patients after treatment with NPH-pig insulin (Table 5). It thus appears as if the very slight antigenicity of pig insulin in human subjects is potentiated when the insulin is injected subcutaneously as a suspension of crystals with a long-acting effect. There are a number of factors which suggest that a suspension of insulin particles constitutes a greater stimulus to the reticuloendothelial system of the organism around the site of injection than a neutral solution, which is rapidly reabsorbed. Thus, experiments with pigs show that the injection of acid insulin preparations, which following injection are transformed into a suspension as a result of isoelectric precipitation at the site of injection,can likewise lead to the formation of insulin antibodies, whereas this is not the case when the insulin is injected as a solution whose pH corresponds to the pH of the tissue fluid. Frankhauser and Morell (1968) were only rarely able to demonstrate insulin antibody formation in patients treated with neutral solutions of pig insulin, whereas in patients treated exclusively with semilente

suspensions, which are almost pure pig insulin, Devlin and Duggan (1968) found insulin antibodies in all cases. The antigenicity of pig insulin would thus appear to depend on whether the pig insulin is injected as a suspension or whether it becomes precipitated at the site of injection. It is well known in experimental immunology that an antigen is more immunogenic as a suspension than as a solution, since phagocytosis of the particles of antigen by macrophages potentiates the antigenicity of protein (Frei *et al.*, 1965).

It cannot be overlooked that factors other than the state of the insulin may be of significance for potentiating the antigenicity of pig insulin. Thus, it is possible that contamination of pig insulin by small amounts of ox insulin, proinsulin (Steiner *et al.*, 1967) or proteins alien to insulin (Brunfeldt and Deckert, 1964b) may be of significance for the formation of insulin antibodies in patients treated with suspensions of pig insulin. Nor can the possibility be excluded that a small part of the pig insulin is so modified during preparation that it may be significant for the antigenicity of the pig insulin. For example, the splitting-off of amide groups might well be significant for the antigenic characteristics of pig insulin.

It is not clear why patients who had previously produced insulin antibodies following treatment with mixed pig-ox insulin preparations in acid solution could be re-immunized following treatment with neutral solutions of pig insulin. The result must presumably mean that neutral solutions of pig insulin represent such a weak antigenic stimulus that antibody formation does not occur unless the immune apparatus of the organism is already sensitized. The fact that the insulin antibodies formed are unable to discriminate between pig insulin and ox insulin agrees well with this.

The present investigations suggest that the formation of insulin antibodies in human subjects can be avoided by using rapidly-acting neutral solutions of pig insulin crystals. However, the problem of insulin antibodies has already been reduced to a clinically insignificant phenomenon even with the use of long-acting insulin preparations. This is seen from the experience obtained in the Scandinavian countries, where insulin resistance is a practically unknown situation. During the last 15 years, several thousand diabetics with insulin requirements have undergone treatment at Niels Steensen's Hospital, Gentofte. During this period, not one single case of insulin resistance has been observed. Likewise, the insulin requirements of patients in Scandinavia appear to be lower than elsewhere. At Niels Steensen's Hospital, the 24-hour insulin dosage in diabetics was examined over 2 periods, namely 1942—1952 and 1963—1967. The patients, practically all with diabetes starting before the age of 30, had all received insulin suspensions containing mainly pig insulin for a period of more than 1 year, all patients were over the age of 18 years, had normal renal function, no infectious disease, and were not overweight or pregnant. All patients had recently terminated a hospital stay for the control of their diabetes, were well controlled and ambulant. The 24-hour insulin dosage the day prior to returning home from hospital was noted. The distribution of the 24-hour insulin dosage during the 2 periods mentioned is seen in Fig. 3. This shows that only 1 out of 1006 patients had an insulin requirement which was greater than 100 i.u./24 h. For comparison, it may be mentioned that out of 1089 patients examined at the university diabetes clinic in Frankfurt am Main, 36 patients were found with an insulin requirement greater

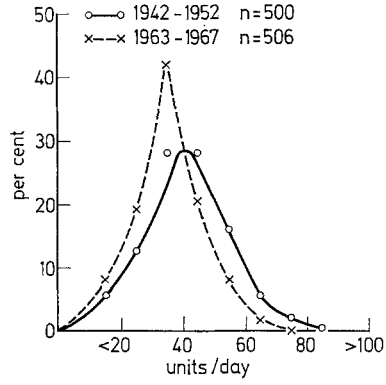

Fig. 3. The distribution of the size of the dose of insulin in 1006 diabetic subjects treated with insulin during the periods 1942—1952 and 1963—1967. n = number of patients examined

than 100 i.u. per 24 hours (Ditschuneit and Federlin, 1965). Insulin resistance is almost always due to the presence of large amounts of insulin antibodies, and as there is in addition a relationship between insulin requirement and insulin antibody titer, the extremely rare occurrence of insulin resistance and the low insulin requirement might suggest that the formation of insulin antibodies is less pronounced using the insulin preparations employed in Scandinavia.

It is not clear why there should be a shift in the distribution curve for 1963—1967 towards a lower 24-h dosage (*cf.* Fig. 3), but this is probably related to the fact that in 1963—1967 the insulin preparations had a greater degree of purity.

Acknowledgements. The author wishes to thank Physician-in-chief Jacob E. Poulsen, M.D., K. Brunfeldt, M. Sc., and Physician-in-chief Otto Jacobsen, for their helpful criticism.

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