Insulin-Like Activity and Insulin-Inhibitory Effect of Human Albumin Fractions Studied with Two Biological Test Methods

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Summary. Human albumin prepared by two different procedures from the blood of seven normal persons and three diabetic subjects has been tested in two biological systems *in vitro*, namely the glucose uptake of the rat diaphragm and the distribution of α -amino-iso-butyric acid in the intact levator ani muscle of immature male rats. — Albumin fractions prepared by the trichloroacetic acid-ethanol procedure as it has been used by VALLANCE-OWEN et al. (1958) showed in some cases an insulin antagonistic activity whereas other fractions did not show any insulin antagonism. — Albumin prepared as described by MICHAEL (1962) constantly showed a high insulin-like activity but no antagonism. To some of these albumin fractions a specific anti-insulin serum was added, which partly reduced the insulin-like activity found in these preparations. — The two different assay systems used were found to give closely parallel results, which indicates that the source of conflicting findings probably resides in the steps of the procedure before the assay and not in the assay itself. — The results are discussed in connection to the theories put forward by VALLANCE-OWEN and co-workers.

Activité insulínique et effet inhibiteur vis-à-vis de l'insuline de fractions d'albumine humaine, étudiées à l'aide de deux méthodes tests biologiques.

Résumé. On a testé l'albumine humaine, obtenue à partir de deux procédés de préparation différents, de sept sujets normaux et de trois diabétiques, à l'aide de deux systèmes biologiques *in vitro*, à savoir la captation de glucose par le diaphragme de rat et la répartition de l'acide α -amino-iso-butérique dans le muscle releveur de l'anus intact de rats mâles impubères. — Les fractions d'albumine préparées par le procédé de l'acide trichloroacétique-éthanol, comme l'ont utilisé VALLANCE-OWEN et coll. (1958) ont montré dans certains cas une activité antagoniste de l'insuline tandis que d'autres fractions n'ont montré aucun antagonisme vis-à-vis de l'insuline. — L'albumine préparée selon la méthode de MICHAEL (1962) a constamment montré une activité insulínique (ILA)

élevée, mais aucun antagonisme. On a ajouté à certaines de ces fractions albuminiques un sérum anti-insuline spécifique, qui a réduit en partie l'ILA trouvée dans ces préparations. — On a trouvé que les deux systèmes de dosages différents utilisés donnaient des résultats tout à fait parallèles, ce qui montre que la cause des résultats contradictoires réside probablement dans les étapes qui précèdent le dosage et non dans le dosage lui-même. — Les résultats sont discutés en relation avec les théories de VALLANCE-OWEN et collaborateurs.

Untersuchungen der insulinähnlichen Aktivität und der insulin-antagonistischen Wirkung menschlicher Albuminfraktionen mit zwei biologischen Methoden.

Zusammenfassung. Von 7 Normalpersonen und 3 Diabetikern wurde menschliches Albumin, das nach zwei verschiedenen Präparationsmethoden gewonnen wurde, mit zwei biologischen *in vitro*-Systemen geprüft, nämlich mit der Glucoseaufnahme des Rattenzwerchfells und der α -Amino-Iso-Buttersäure-Verteilung am intakten Musculus levator ani junger Ratten. — Die Albuminfraktionen, die mit der Trichloressigsäure-Alkohol Methode gewonnen wurden, wie sie VALLANCE-OWEN et al. (1958) verwendet haben, zeigten in einigen Fällen eine insulinantagonistische Aktivität, während andere Fraktionen nicht den geringsten Insulinantagonismus aufwiesen. — Nach der von MICHAEL (1962) beschriebenen Methode hergestelltes Albumin zeigte immer eine hohe insulinähnliche Aktivität aber keinen Antagonismus. Einigen dieser Albuminfraktionen wurde ein spezifisches Antiinsulinserum zugesetzt, das die insulinähnliche Aktivität dieser Präparate teilweise herabsetzte. — Es zeigte sich, daß die beiden verschiedenen Bestimmungssysteme vollständig übereinstimmende Ergebnisse lieferten, was darauf hinweist, daß die Ursache für die widersprechenden Ergebnisse wahrscheinlich in den einzelnen Stufen der Präparation vor der biologischen Bestimmung nicht in dieser Bestimmungsmethode selbst zu suchen ist. — Die Ergebnisse werden in Verbindung mit den Theorien von VALLANCE-OWEN und Mitarbeitern diskutiert.

The physico-chemical form of insulin in blood is still not known and it is therefore not surprising that there are many difficulties in, and discrepancies between, the various methods which are used for the estimation of the insulin concentration in blood plasma. Thus, when a sample of whole plasma is tested in two of the most frequently used bioassay methods for insulin, the isolated rat diaphragm method and the epididymal fat pad method, this sample shows much higher insulin-like activity (ILA) in the epididymal fat method than in the diaphragm method.

One possibility among others to explain this difference is that several types of insulin-inhibitory

factors might be present in the whole plasma and that only some of these are active in the fat pad assay system whereas all of them are active in the diaphragm system.

The possibility that inhibitory or antagonistic factors in plasma can be of crucial importance for the level of ILA found in various bioassay systems has been supported during recent years by findings reported by VALLANCE-OWEN et al. (1958, 1961), LOWY, et al. (1961), ALP and RECAN (1965) and JERVELL (1965). These authors have reported an insulin-antagonistic effect of albumin fractions from human plasma in the isolated rat diaphragm system

and, in addition, no antagonistic effect in the epididymal fat pad system. They have also found that the amount of antagonistic activity is higher in albumin fractions from diabetics and prediabetics than from normal subjects. VALLANCE-OWEN and ASHTON (1963) have reported increased antagonistic activity in albumin fractions from many patients with cardiac infarction. There are, however, other groups of investigators who have not been able to confirm these reports. KEEN (1963) and CAMERON et al. (1964) did not find any insulin-antagonistic effect in their albumin preparations, even though these were extracted by the same trichloroacetic acid-ethanol procedure as that used by the workers cited above. Their albumin fractions gave instead a marked ILA not only on fatty tissue but also on the diaphragm.

The reason why some groups find an insulin-antagonistic activity in their plasma albumin fractions and other groups find an ILA is not clear, and further studies within this field are therefore very desirable.

In the present experiments, two types of human albumin fractions have been studied, one prepared by the trichloroacetic acid-ethanol procedure as it has been used by VALLANCE-OWEN et al. (1958), and the other prepared as described by MICHAEL (1962). The albumin fractions were tested for insulin-antagonistic effect or insulin-like activity in two systems: 1. the glucose uptake by the isolated "cut" diaphragm from the rat (GEMMILL, 1940); and 2. the amino acid transport in the isolated "intact" levator ani muscle from immature male rats (ARVILL and ÅHRÉN, 1965, 1966). Some pilot experiments have also been carried out with an anti-insulin serum in order to analyse the nature of insulin-like activity found in some of the albumin fractions.

Methods

Animals: Male rats of the Sprague-Dawley strain weighing approximately 50 g (25–30 days old) were used. They were fed a semisynthetic diet (ÅHRÉN, 1959) and tap water *ad libitum*. They were deprived of food 24 hours prior to the experiments, but had free access to water at all times.

Isotope: α -amino-iso-butyric acid- ^{14}C (AIB- ^{14}C) was obtained from the Radiochemical Centre, Amersham, England. The specific activity used was $3.0 \mu\text{C}/\mu\text{mole}$.

Incubation medium: Krebs-Ringer bicarbonate buffer containing 13.9 mM glucose, pH 7.4, was equilibrated with a gas mixture of 95% O_2 –5% CO_2 for one hour before the start of the experiment. The concentration of AIB in the medium was 0.1 mM.

The albumin fractions were added in the medium to make a final concentration of 1.25% and 4% (w/v).

Albumin preparations: Blood was drawn from 7 normal persons without known diabetes in their family history and from 3 diabetic persons who had never received insulin or other hormones. The diabetic patients were in good metabolic balance without

ketonuria and with only moderate glucosuria. All samples were taken after an overnight fast.

Two different procedures were used for preparing the albumin fractions. In the first the albumin was prepared from both heparinized plasma and serum according to the method of DEBRO et al. (1957) as slightly modified by VALLANCE-OWEN (1958). The final albumin fraction in 1% TCA: 95% ethanol was dialysed in the present experiments in "Visking" Seamless Cellulose Tubing No. 29/32 for one day against running tap water and for two days against distilled water and finally freeze-dried. The "Visking" tubing had been soaked in water but not boiled. Further references to this albumin fraction will be indicated as that obtained by the "TCA/ethanol procedure". The albumin prepared with this procedure was never quite soluble in the buffer solution used. Before the incubation the albumin solution in buffer was centrifuged for 5–10 min at 3000 rev./min and the insoluble part rejected.

Albumin was also prepared from serum by the method of MICHAEL (1962). The serum was mixed with ten volumes of concentrated methanol, acidified with concentrated hydrochloric acid, stirred for 30 min and centrifuged for 45 min at 8500 rev./min. The albumin in the supernatant was precipitated by neutralization to pH 6 with 2 N NaOH, and centrifuged at 6000 rev./min for 10 min. The precipitate was then washed twice with concentrated methanol and twice with ether. After the albumin had been air-dried, complete removal of ether was assured by dissolving it in water and freeze-drying. The albumin was again dissolved in water, the pH was adjusted to pH 6 using 0.1 N NaOH, and the slight precipitate often formed during this neutralization was discarded after centrifuging. The clear solution of albumin was finally freeze-dried. The resulting albumin preparation was a white powder, easily soluble in water and in the buffer used in the biological assay. Further references to this albumin fraction will be indicated as that obtained by the "acid methanol procedure".

Insulin: A ten-fold recrystallized preparation of pig insulin with a potency of 24.5 IU/mg was used (Novo S 1562). It was dissolved in 3.3 mM HCl to a concentration of 20 IU/ml and then diluted with medium to 10^{-3} IU/ml medium.

Anti-insulin serum: The anti-insulin serum was obtained from guinea-pigs injected with human insulin, and used by JONSSON (1965) for immuno-assay purposes with the technique of HALES and RANDLE (1963). The amount used was calculated to be able to inactivate 2×10^{-3} IU insulin in the immunological system. As control solution serum was prepared from normal, non-immunized guinea-pigs and added to the control groups in equal amounts.

The glucose uptake determinations: Hemidiaphragms were prepared as described by GEMMILL (1940) and incubated in 10 ml Erlenmeyer flasks with 1 ml medium, without any preincubation. The flasks were gassed for

about 30 sec prior to the incubation and sealed with tightly fitted rubber stoppers. The muscles were incubated in a shaking bath at 37°C for 2 hours. The original solutions were always incubated without muscle tissue to give the initial glucose concentration of the medium of each group. After the incubation the muscles were removed, blotted and weighed on a torsion balance. The protein was precipitated from the medium with 10% TCA and the glucose of the medium was determined by the glucose oxidase method (HUGGET and NIXON, 1957). The uptake was then calculated from each original solution and expressed as mg glucose per g muscle tissue (wet weight) per 2 hours. Samples were always analyzed in duplicate.

Determination of AIB-¹⁴C distribution: The intact levator ani muscle was extirpated and treated as described by ARVILL and AHRÉN (1965, 1966). In short, the levator ani muscle was incubated with its normal anatomical connection to the bulbocavernosus muscles in 1 ml of medium containing the non-utilizable amino acid, α -amino-iso-butyric acid (AIB-¹⁴C) for 2 hours in a shaking bath at 37°C. After the incubation the levator ani muscle was removed, blotted on filter paper, weighed and homogenized in 10% TCA. The amount of radioactivity of the protein-free TCA-extract and medium was determined in a liquid scintillation counter (Packard-tri-carb). The uptake of amino acid during the incubation time was calculated as the AIB-distribution ratio (CPM in 1 ml intracellular water divided by CPM in 1 ml medium). For this calculation the extracellular water content of the muscle was determined by measuring the sucrose-¹⁴C space and the total water was determined by drying.

groups of buffer and buffer + 10⁻³ IU/ml of insulin were compared. For the glucose uptake assay the mean difference between these groups was 5.66 ± 1.46 . The 95% confidence limits for the difference are 2.70 to 8.62. For the AIB distribution assay the mean difference between the control and insulin groups was 4.58 ± 0.47 . The 95% confidence limits for the difference are 3.63 to 5.53.

Statistical differences between the groups were tested using Student's *t*-test. A difference is considered significant when $p < 0.05$.

The dose-response of insulin on distribution of AIB in the levator ani muscle was examined. A statistical analysis was made of the regression line of this dose-response between 100 and 800 μ IU/ml of insulin. With four muscles for each point this curve was found to be linear with the equation: $\bar{Y}_x = 6.54 + 1.39 (x - 2.50)$ (to be published).

A similar analysis of the glucose uptake assay was not undertaken.

Results

I. Effects of albumin fractions prepared by the TCA/ethanol procedure

a) **Effects on glucose uptake in the diaphragm.** Four albumin fractions from four normal persons were examined in six experiments. The albumin was added to make a final concentration of 4%. Representative results are shown in Table 1, groups A and B. Albumin from two of the four persons showed an insulin antagonistic activity (Table 1, group A), whereas albumin from the other two persons did not show any insulin antagonism (Table 1, group B).

Table 1. Effect of albumin from normal subjects on glucose uptake by rat diaphragm

Group ¹	Numbers of ² fractions	Buffer ³	Buffer + albumin ³	Buffer + insulin ³	Buffer + albumin ³ + insulin
A	2	8.50 ± 0.63 (6) n.s.	7.55 ± 0.81 (6)	16.57 ± 1.30 (9) $p < 0.02$	9.24 ± 1.79 (6)
B	2	9.30 ± 1.37 (6) n.s.	12.67 ± 0.75 (6)	13.88 ± 1.60 (6) n.s.	15.57 ± 1.34 (6)
C	2	4.51 ± 0.53 (6) $p < 0.001$	8.39 ± 0.50 (6)	9.22 ± 0.21 (6) n.s.	10.28 ± 0.74 (6)

¹ In groups A and B the albumin was prepared by the TCA/ethanol procedure and in group C by the acid methanol procedure. In all groups the albumin was added at a concentration of 4% (w/v) and the insulin concentration was 10⁻³ IU/ml.

² Each of these fractions represents different persons and each fraction was tested in one experiment.

³ Glucose uptake in mg/g wet weight during the incubation period of 2 hours. Mean $\pm$ S.E. Number of rats in parenthesis. P-values based on Student's *t*-test; a difference is considered significant when $p < 0.05$.

Reliability of the methods: Mean values were calculated for both methods with at least three muscles for each point of buffer, buffer + albumin, buffer + insulin, and buffer + albumin + insulin determined in the same experiment.

Confidence limits of the assay procedures were determined on the basis of six assays in which triplicate

Albumin fractions from two diabetic subjects, which were tested at a concentration of 1.25% did not show any insulin antagonistic activity.

b) **Effects on AIB-¹⁴C distribution in the levator ani muscle.** Seven experiments were made on five albumin fractions from four normal persons and representative results from these experiments are shown in Table 2,

groups A and B. The albumin was tested at a concentration of 4%. In albumin fractions from three of the people an insulin antagonism was found (Table 2, group A) whereas the albumin of the remaining one did not show any antagonistic activity (Table 2, group B). The albumin alone was found to stimulate the AIB-¹⁴C distribution ($p < 0.001$) both in the antagonistic and the non-antagonistic groups.

Two albumin preparations from two diabetic subjects were examined at an albumin concentration of 1.25%. No insulin antagonism was found.

II. Effects of albumin fractions prepared by the acid methanol procedure

a) *Effects on glucose uptake in the diaphragm.* In these experiments three fractions from three normal persons and one fraction from a diabetic subject were

b) *Effects on AIB-¹⁴C distribution in the levator ani muscle.* Five fractions from four normal persons and two fractions from two diabetic subjects were tested in 14 experiments at an albumin concentration of 4%. In all these experiments a stimulation of the AIB distribution was consistently found but no insulin antagonism. In addition the AIB-¹⁴C distribution was stimulated more when both insulin and albumin were added to the incubation medium. Representative results are shown in Table 2, group C.

No difference was found between the effects of the albumin fractions obtained from diabetic and normal subjects.

c) *Effects of anti-insulin serum.* In some preliminary experiments an attempt was made to analyze the nature of the above mentioned insulin-like activity of the albumin prepared by the acid methanol procedure.

Table 2. Effects of albumin from normal subjects on AIB-¹⁴C distribution in the isolated levator ani preparation

Group ¹	Numbers of ² fractions	Buffer ³	Buffer + albumin ³	Buffer + insulin ³	Buffer + albumin ³ + insulin
A	3	1.71 ± 0.10 (11)	2.77 ± 0.23 (9)	6.40 ± 0.77 (12)	3.74 ± 0.35 (11)
			$p < 0.001$		$p < 0.01$
B	1	1.67 ± 0.07 (4)	5.35 ± 0.41 (4)	6.85 ± 0.90 (4)	6.60 ± 0.34 (4)
			$p < 0.001$		n.s.
C	2	1.42 ± 0.05 (6)	4.10 ± 0.26 (6)	5.35 ± 0.43 (6)	7.78 ± 0.44 (6)
			$p < 0.001$		$p < 0.005$

¹ In groups A and B the albumin was prepared by the TCA/ethanol procedure and in group C by the acid methanol procedure. In all groups the albumin was added at a concentration of 4% (w/v) and the insulin concentration was 10⁻³ IU/ml.

² Each of these fractions represents different persons and each fraction was tested in one experiment.

³ AIB-¹⁴C distribution as $\frac{\text{CPM/ml intracell. water}}{\text{CPM/ml medium}}$ during the incubation period of 2 hours. Mean ± S.E. Number of rats in parenthesis. P-values based on Student's *t*-test; a difference is considered significant when $p < 0.05$.

Table 3. Effects of albumin and anti-insulin serum on the AIB-¹⁴C distribution in the isolated levator ani preparation¹

Group ²	Buffer ³	Buffer + insulin ³	Buffer + insulin ³ + anti-insulin	Buffer + albumin ³	Buffer + albumin ³ + anti-insulin
A	3.41 ± 0.29 (4)	10.16 ± 0.92 (4)	2.74 ± 0.23 (4)	9.48 ± 0.60 (4)	8.92 ± 0.77 (4)
			$p < 0.001$	n.s.	
B	1.45 ± 0.32 (3)	4.03 ± 0.86 (3)	1.15 ± 0.19 (3)	5.27 ± 0.42 (3)	3.92 ± 0.39 (4)
			$p < 0.01$	n.s.	
C	1.85 ± 0.38 (4)	4.37 ± 0.69 (4)	1.41 ± 0.28 (4)	5.24 ± 1.03 (3)	3.06 ± 0.45 (4)
			$p < 0.01$	n.s.	

¹ The anti-insulin serum was prepared from guinea-pigs as described under "Methods".

² In groups A and B the albumin was prepared from two normal subjects, in group C from a diabetic subject. The albumin was prepared by the acid methanol procedure. In all groups the albumin was added at a concentration of 4% (w/v) and the insulin concentration was 10⁻³ IU/ml.

³ AIB-¹⁴C distribution as $\frac{\text{CPM/ml intracell. water}}{\text{CPM/ml medium}}$ during the incubation period of 2 hours. Mean ± S.E. Number of rats in parenthesis. P-values based on Student's *t*-test; a difference is considered significant when $p < 0.05$.

tested at a concentration of 4% albumin in the incubation medium. In all experiments a stimulation of the glucose uptake was found and no insulin antagonistic activity. Representative results are shown in Table 1, group C. No difference between albumin from the diabetic and the normal persons was found.

The results are shown in Table 3. As can be seen from this table the amount of anti-insulin serum used completely inactivated the test dose of insulin of 10⁻³ IU/ml. The same amount of anti-insulin serum did not significantly reduce the high insulin-like activity that was found when the albumin was added in a concen-

tration of 4%. This was true both with albumin of normal persons (Table 3, groups A and B) and with albumin of diabetic subjects (Table 3, group C).

In another experiment (not shown in the Table) albumin was tested at a concentration of 0.4% of the incubation medium. A significant ($p < 0.05$) stimulation of the AIB- ^{14}C distribution was still found in this experiment. This stimulating effect was reduced to the control level with the same amount of anti-insulin serum as in the experiments mentioned above.

Discussion

In previous studies concerning insulin-antagonistic effect and ILA of human plasma albumin fractions, glucose uptake by the isolated cut diaphragm has been used as the main test method. This test system has, however, many disadvantages which are discussed in detail in a paper by BERSON and YALOW (1965). It was concluded in that paper that the diaphragm system is "a very insensitive one and significant result can be obtained only with great difficulty and by diligent attention to the utmost precision in pipetting, titrating, etc.". We thought therefore that the conflicting results with plasma albumin fractions reported by various investigators might be attributed to difficulties with the test system used; and we decided to test the albumin fractions also in another test system, the isolated intact levator ani muscle prepared as described by ARVILL and ÅHRÉN (1965, 1966). In this system the uptake of the non-utilizable amino acid AIB was measured, and by this means we have obtained much more stable and reproducible effects of various insulin concentrations in the medium than with the glucose uptake method in the isolated cut diaphragm.

It can, however, be seen from the results that with both test methods only some of the albumin fractions prepared from normal subjects by the TCA/ethanol procedure and present in the incubation medium at a concentration of 4% (the same concentration as that used by VALLANCE-OWEN and co-workers) showed an insulin-antagonistic effect. Other fractions prepared by the same procedure did not show any insulin-antagonistic effect (Tables 1 and 2, groups A and B). Thus, there were in the present study some experiments that gave the same results as those reported by e.g. VALLANCE-OWEN et al. (1958) and JERVELL (1965), and, in addition, some experiments which gave the same results as those reported by e.g. KEEN (1963) and CAMERON et al. (1964). Since in the present study two different test methods were used and since the same results were found with both these methods, it seems very unlikely that the difference in results can be attributed to the test system(s). It seems more likely that the difference in results between VALLANCE-OWEN et al. (1958) and KEEN (1963), the same difference as found *within* our own series of experiments, must be attributed either to an inherent property of the blood, to the procedure for blood sampling or to the extraction technique.

In the present experiments both heparinized plasma and serum were used for the preparation of TCA/ethanol extracts with no differences in the results. However, since it has been shown that heparin might influence the conditions for ILA determination (see e.g. GUNDERSEN and BONIFACE, 1965), our acid methanol extracted albumins were made exclusively from serum.

The extraction technique of the albumin fractions in the TCA/ethanol method was in the present study the same as that employed by VALLANCE-OWEN et al. (1958). The same technique was also used by CAMERON et al. (1964). Minor differences in the equipment and/or in the performance of the various steps of the extraction procedure cannot be excluded. It has, for instance, been shown that various types of "Visking" tubing have different permeability characteristics for insulin (CRAIG et al., 1957).

Another unclear and disturbing point with the dialysis is that an insulin-antagonistic factor can, under certain conditions, be derived from the "Visking" tubing itself as described by LILLEY and VALLANCE-OWEN (1961), but the same authors claim in their paper that "under the ordinary conditions of dialysis", this factor does not contaminate the contents of the tubing, but only diffuses into the dialysis bath. It is in this connection of great interest that CAMERON et al. (1964) did find an insulin-antagonistic effect in one of their albumin extracts from a normal person. During the preparation of this particular extract, the dialysis bath had run dry for quite a long time. They prepared two further extracts of the plasma from the same normal person and these two extracts did not show any insulin-antagonistic effect but instead an ILA. It is our opinion that an extraction method that implies the use of a "Visking" tubing from which, at least under certain conditions, insulin-antagonistic factor(s) can be extracted is not suitable for this type of experiment.

There are in the present results two observations on the TCA/ethanol extracted albumin fractions from normal subjects that are of particular interest. Firstly, when tested in the levator ani system, all extracts showed a significant ILA (Table 2, groups A and B). Thus the four fractions that showed an insulin-antagonistic effect when tested together with insulin (Table 2, group A) showed a small but significant ILA when tested alone in the system. The 5th fraction, which did not show any insulin-antagonistic effect (Table 2, group B), showed a very marked ILA. In this respect, our results are very similar to those reported by CAMERON et al. (1964) and in complete contrast to those reported by VALLANCE-OWEN et al. (1958). Secondly, the TCA-ethanol extracted fractions did in some cases show a clear insulin-antagonistic effect not only on the glucose uptake of the isolated cut diaphragm but also on the amino acid transport in the isolated intact levator ani muscle. This finding is of interest in relation to the report of JERVELL (1963) that he failed to demonstrate an insulin-antagonistic effect on the penetration of D-xylose in the isolated intact

diaphragm with the same albumin fractions that had an insulin-antagonistic effect on the glucose uptake. JERVELL concluded therefore that the albumin-bound insulin antagonist probably acts on some intracellular aspect of glucose metabolism rather than on the membrane transport. From our results it is obvious that the insulin-antagonistic factor(s) which could be found now and then in our experiments in the TCA-ethanol extracts influenced both the glucose uptake in the diaphragm and the amino acid transport in the levator ani muscle.

Our results with the albumin fractions extracted with the method described by MICHAEL (1962) are stable and do not vary from extract to extract. All fractions from plasma of normal persons showed with both test methods a marked ILA and no insulin-antagonistic effect at all (Tables 1 and 2, group C). These results are consistent with the very interesting results of DAVIDSON et al. (1963) who found remarkably high ILA in acid alcoholic extracts of human serum. Our results are, however, in contrast to the findings of JERVELL (1965) who did not find ILA but marked insulin-antagonistic effect of albumin fractions prepared with the above mentioned method of MICHAEL. This method does not include dialysis and therefore "Visking" tubing is not used. Small differences in the extraction procedure can, however, not be excluded even with this methods. Thus, JERVELL (1965) used centrifugation and filtration to collect the albumin precipitate from the neutralized methanol solution, while we have used only centrifugation.

The properties of the ILA in the albumin prepared by the method of MICHAEL (1962) have not yet been analysed in detail. We have, however, started such an analysis and in some preliminary experiments the effect of an anti-insulin serum was studied. In these experiments (Table 3) addition of anti-insulin serum to the levator ani muscle in an amount that completely suppressed the effect of a test dose of 10^{-3} IU/ml of crystalline insulin, did not significantly reduce the ILA of the albumin fractions that were added at a concentration of 4%. It is therefore possible that at least a large proportion of the ILA either is not insulin at all or that this portion represents a form of non-suppressible or "atypical" insulin described by other workers (e.g. SAMAN et al., 1963) in other biological test systems. It is, however, also possible that the amount of anti-insulin serum used in the present experiments was too small in relation to the amount of insulin present in the albumin fractions. Our result in one experiment with a lower concentration of albumin in the test system might indicate that this last mentioned possibility is the right one, but further experiments are necessary to elucidate this point.

One important question within this field of research is whether diabetic and prediabetic patients have a higher insulin-antagonistic activity in their plasma albumin than normal persons. This has, as mentioned in the introduction, been reported from some laborato-

ries and VALLANCE-OWEN has from these results postulated elaborate theories concerning the importance of this insulin-antagonistic plasma factor for the etiology and pathogenesis of diabetes (e.g. VALLANCE-OWEN, 1964). In the present study albumin fractions were tested only from three diabetic patients. These patients had a mild diabetes and they had never received insulin. The TCA-ethanol prepared albumin fractions from these patients were tested in a concentration of 1.25% both in the diaphragm system and in the levator ani system. No insulin-antagonistic activity was seen in any of the test methods. With three albumin fractions, prepared by the method of MICHAEL (1962) and added to the test system in a concentration of 4%, a high ILA was found.

Since there are such varying and inexplicable results with this type of albumin fraction from normal persons, it seems to us doubtful to study this phenomenon in big groups of diabetics. A full evaluation of the eventual pathogenetic importance of the stimulating findings repeatedly published by VALLANCE-OWEN and his associates does not appear possible at the present time.

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