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Research Article

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Studies of the Insulin-inhibitory Effect of Human Albumin Fractions *

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Vallance-Owen and his colleagues described an insulin inhibitor associated with human plasma albumin fractions (1–3). Inhibitory fractions were prepared by the Debro procedure (4) using plasma obtained from both normal and diabetic subjects. The existence of this inhibitor and its quantitative increase in diabetics have been confirmed by Lowy, Blanshard, and Phear (5) and Alp and Recant (6). Other investigators, notably Keen, Cameron, and Menzinger (7, 8), have failed in their efforts to demonstrate this material.

In view of the difficulties encountered by the latter group of investigators and since our laboratory previously described observations on a relatively small number of subjects, it seemed important to report on our more extensive experience with this inhibitor.

Human albumin fractions prepared by the Cohn fractionation procedure (9) as well as the Debro procedure were tested for insulin-inhibitory activity. We attempted to define more quantitatively the distribution of the inhibitor in normal subjects, in a variety of diabetics, and in a group of pregnant, nondiabetic women. Additional experiments were performed to clarify certain aspects of the mechanism of insulin inhibition produced by these albumin fractions.

Methods

Debro albumin fractions. Albumin fractions were prepared from the serum of 17 normal subjects, 21 diabetics, and 9 pregnant, nondiabetic women by the Debro procedure using trichloroacetic acid and obtaining albumin in the acid ethanol soluble supernatant. Debro preparations were also made from the urine protein of three nondiabetic nephrotic subjects and two diabetic nephrotics.

All subjects were fasted overnight unless otherwise noted. None of the patients studied were greater than 15% over ideal body weight. Four of the normal subjects and two of the pregnant subjects tested had positive family histories of diabetes. Among the normal patients, D.H., M.L., G.L., and B.S., the diabetic relatives were in the immediate family (father, mother, or siblings). In the other two instances only a distant family member was diabetic. The pregnant women were in their third trimester of pregnancy. Their glucose tolerance tests were normal, and no complications of pregnancy were noted. Follow-up studies at term revealed that all babies were of normal size, and no major complications were encountered. Diabetic subjects were not homogeneous. Clinical data are provided in Table I. Those diabetics on specific therapy were studied 24 hours after the last insulin injection or Orinase tablet unless otherwise specified.

Cohn albumin fractions (normal albumin). Eight albumin fractions prepared by method 6 of Cohn [1] represented pools of both fasted and nonfasted plasma prepared in large batches. In the text these fractions are referred to by their respective lot numbers. Three preparations of pure Fraction V were tested.[2] Five preparations of crude or unreworked Fraction V were also tested: lot no. 327 (unreworked),[2] 18866, 457, 520, and 3505.[3]

Methods. Albumin fractions were tested at concentrations varying from 1.25% to 5.0%. Insulin-inhibitory activity of these preparations was tested *in vitro* with a cut rat hemidiaphragm in a paired technique only partially detailed in a previous communication (6).

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[1] Obtained through the kindness of Dr. Karl Schmid, Professor of Biochemistry, Boston University Medical School.

[2] Lot no. 145 and 327 (reworked) obtained from the Massachusetts State Laboratories, thanks to Dr. Lewis H. Larsen, Massachusetts Department of Public Health, Jamaica Plain, Mass. The third pure Fraction V was a Swiss Red Cross albumin.

[3] The last four fractions were provided by Dr. R. B. Pennell of the Protein Foundation, Jamaica Plain, Mass.

TABLE I

*Clinical data concerning diabetic and prediabetic subjects tested**

Subject	Age	Sex	Known duration diabetes	Obesity		Family history diabetes	Abnormal G-T-T†	Symptomatic glycosuria	Prescription
				Past	Present				
A.D.	29	M	1 month	0	0	0	+	+	Diet
M.K.	50	F	9 years	0	0	1 sibling, mother	+	0	0
H.F.	64	M	5 years	++	+	Identical twin	+	0	Diet
J.F.	64	M	2 years; myocardial infarction	++	+	Identical twin	+	0	Diet
C.P.	58	M	>10 years; acromegaly	0	0	Unknown	+	+	Insulin
L.H.‡	25	F	>10 years; nephrosis	0	0	Unknown	+	+	Insulin
J.R.	80	M	1 month	0	0	0	+	+	Tolbutamide
L.R.	50	F	Discovered at time of test	0	0	Mother, 2 of sister's children	+	0	0
T.L.	49	M	6 months	0	0	0	+	+	Insulin
B.S.	58	F	9 years	0	0	2 siblings, parents	+	+	Tolbutamide
A.S.	60	M	5 years; lymphoma 3 years	0	0	0	+	0	0
D.W.	23	M	6 years	0	0	0	+	+	Insulin
W.R.	23	M	3 years	0	0	Son of L.R. above	+	+	Insulin
A.B.‡	38	F	12 years; nephrosis	0	0	Unknown	+	+	Insulin
S.S.	60	M	9 years	+++	0	Father	+	+	Diet
M.S.	39	M		0	0	Father, mother	↑1 hour	0	0
Ra.S.	37	M		0	0	Father, mother	↑1 hour	0	0
R.S.	35	F		0	0	Father, mother	↑2 hour	0	0
G.B.	35	F		0	0	Mother, 2 paternal aunts, 1 paternal uncle	?	0	0

* Five other diabetic samples were tested. These represented pooled plasma collected from groups of diabetics. Pools 1, 2, 3, and 4 were collected from insulin-treated patients. Pool 5 was collected from untreated diabetics.
† G-T-T = glucose tolerance test. At least two abnormal sugars (fasting, 1 hour, 2 hour) after 100 g glucose *per os*.
‡ Urine albumin tested; plasma albumin of L.H. also tested.

The albumin preparation to be tested was weighed out carefully on an analytical balance and diluted in *1)* Krebs-Henseleit buffer containing 250 mg per 100 ml of glucose and 200 mg per 100 ml of gelatin and *2)* buffer containing glucose and gelatin plus 1,000 μU per ml of crystalline insulin.[4] Two-ml volumes of buffer, buffer plus insulin, albumin, and albumin plus insulin were added, respectively, to the incubation vessels, which were then stoppered and chilled until the tissues were ready to be added. Contact of albumin with insulin was present from the time of the original dilution until the end of the experiment. Preparation of the tissues required about 20 minutes.

Male Sprague-Dawley rats weighing approximately 160 g were fasted overnight. The animals were guillotined without anesthesia and the hemidiaphragms removed and placed immediately in chilled buffer without glucose. Six rats were used in one experiment, and the procedure was divided into two parts so that tissues could be handled rapidly, i.e., three rats were sacrificed, tissues dissected, weighed on a torsion balance after gentle blotting, and then placed in the respective incubation vessels. After this the second group of rats was treated similarly.

The tissues were gassed gently in the closed vessels with 95% O_2–5% CO_2 for 10 minutes and then incubated an additional 70 minutes at 37° C in a Dubnoff shaker.

[4] Kindly supplied by Dr. W. R. Kirtley of Eli Lilly, Indianapolis, Ind. (5 × recrystallized beef insulin, 25 U per mg).

At the end of incubation (a total of 80 minutes), the medium was poured into test tubes and stoppered. Glucose analyses were performed immediately, in duplicate, on samples of medium diluted 1:40 in $Ba(OH)_2$–$ZnSO_4$ and water. One-ml samples of the supernatants were then analyzed in duplicate for glucose by the Nelson procedure using a Klett colorimeter (10). Duplicates of the initial or unincubated samples were simultaneously precipitated and analyzed for glucose. In some experiments glycogen analyses were made on the tissues after the incubation by the method of Walaas and Walaas (11). Free fatty acid content of various albumin fractions was measured by the method of Mendelsohn (12).

The experimental protocol was as follows:

Vessel no.		Hemidiaphragms from
1	Albumin in buffer (A)	Rat 1
2	Buffer (B)	
3	A	Rat 2
4	B	
5	A	Rat 3
6	B	
7	Albumin + insulin (A + I)	Rat 4
8	B + I	
9	A + I	Rat 5
10	B + I	
11	A + I	Rat 6
12	B + I	

TABLE II

*Detailed observations in the assay for inhibitory activity of albumin**

Vessel no.	Initial glucose	Final glucose	Δ Glucose	Glucose uptake
	μg/2 ml	*μg/2 ml*	*μg*	*mg/g tissue*
1 A	5,140	4,360	780	6.60
2 B		4,470	670	6.10
3 A		4,480	660	6.60
4 B		4,440	700	6.25
5 A		4,540	600	6.25
6 B		4,530	610	5.81
7 A + I	5,140	4,510	630	5.72
8 B + I		4,030	1,110	9.65
9 A + I		4,180	960	7.44
10 B + I		4,000	1,140	8.26
11 A + I		4,390	750	6.41
12 B + I		4,210	930	7.95

Mean	A	B	A + I	B + I	Insulin effect in B	Insulin effect in A	% inhibition
mg/g tissue	6.48	6.05	6.52	8.62	+2.57	+0.04	98.5%

* 2% albumin from diabetic patient W.R., male, age 23.
A = albumin; B = buffer; I = insulin. Statistical analysis: the standard error of the mean ($S_{\bar{x}}$) is 0.372 mg per g tissue. B + I is significantly different from A, B, and A + I, $p < 0.1$. There are no significant differences between A, B, or A + I.

Mean values were calculated for the glucose disappearance (milligrams glucose uptake/gram tissue/80 minutes) in the triplicate samples of buffer, albumin, buffer + insulin, and albumin + insulin. The insulin effect was described as the mean increment in glucose uptake over the mean of the respective base-line values. Inhibition of this insulin-induced increment was produced by certain albumin fractions and expressed as percentage of inhibition, considering the insulin effect in the buffer system without albumin to be 100%. The use of "per cent inhibition" as a quantitative expression of the inhibitory activity of albumin was adopted to permit gross comparisons between individuals, since absolute insulin effects varied from experiment to experiment. Confidence limits of the assay procedure were determined on the basis of five consecutive assays in which triplicate buffer pairs and triplicate buffer pairs + 1,000 μU per ml of insulin were compared. The mean difference between the insulin-treated pairs was 0.238 mg per g tissue with a standard error of 0.203 showing no significant difference betwen pairs. The 95% confidence limits for the difference are −0.326 to +0.802. No significant differences were found in any of the series of pairs of buffer alone. A highly significant insulin effect was observed in all assays ($p < 0.01$ to $p < 0.001$).

To indicate the type of variation encountered in the assay for inhibitory activity of various albumin preparations, Table II shows the raw data in an experiment with a highly inhibitory albumin preparation. The standard error of the mean ($S_{\bar{x}}$) is 0.372 mg per g tissue, calculated from the residual mean square in the analysis of variance. The determination of significance of the differences in insulin response was carried out by Duncan's multiple range test. In this experiment, a highly significant difference in response to insulin in the albumin sample vs. buffer is noted ($p < 0.01$).

Table III shows similar statistical data obtained in a series of individual assays and shows the rough correlation of the per cent inhibition with the individually determined p value for the glucose uptake in the insulin pairs (buffer + insulin vs. albumin + insulin). As a result of these observations, it would seem reasonable to consider a > 40% inhibition significant.

Results

I. *Insulin-inhibitory activity of Debro fractions*

Normal subjects. Table IV records data obtained with preparations of albumin from normal subjects. Section A of Table IV shows that with albumin concentrations of 3 to 3.5%, a significant inhibition of insulin action was observed in the over-all group ($p = 0.05$). Lower concentrations of albumin (section B of Table IV), 2.5% or less, produced, on the average, no significant inhibition of 1,000 μU of insulin ($p = NS$). Individual subjects, however, showed considerable variation in inhibitory activity. At 2.5% albumin, two normal subjects (one with a nega-

TABLE III

Statistical data from individual assays

Samples tested	% albumin tested	$S_{\bar{x}}$*	p for insulin-treated pairs†	% inhibition of insulin effect by albumin
	%	*mg/g*		%
Buffer 1		0.501	NS	
Buffer 2		0.453	NS	
Buffer 3		0.771	NS	
Buffer 4		0.462	NS	
Buffer 5		0.406	NS	
Albumin				
G.L., normal	2.5	0.788	NS	12
M.S., prediabetic‡	2.0	0.467	NS	29
G.B., prediabetic‡	2.0	0.554	NS	29
S.S., diabetic	2.0	0.473	NS	36
Ra.S., prediabetic‡	2.0	0.491	<0.05	46
B.S., diabetic	2.0	0.370	<0.01	53
R.S., prediabetic‡	2.0	0.484	<0.05	54
Fr.V., no. 457	3.0	0.352	<0.05	65
W.R., diabetic	2.0	0.372	<0.01	98

* Standard error of the mean calculated from the error row of the analysis of variance.
† Level of probability for which significant differences were found.
‡ Glucose uptake (mg per g) in A + I vs. B + I: M.S., 4.58 vs. 5.49; G.B., 7.8 vs. 9.09; Ra.S., 5.99 vs. 7.80; and R.S., 4.22 vs. 6.27. Data for other samples noted are in succeeding tables.

tive family history of diabetes) showed 48 and 40% inhibition, whereas another normal albumin obtained from nephrotic urine showed only 29% inhibition at 3.5% concentration of albumin. No significant degree of insulin inhibition was observed with albumin concentrations of 2% or less in the seven normal subjects tested at this concentration. Testing of 2% albumin obtained from four subjects with bilateral positive family histories of diabetes revealed significant inhibition in two (Table III). Analysis of the prediabetics as a group revealed a $p < 0.01$ for the difference between A + I vs. B + I.

Diabetic subjects. The activities of 22 albumin preparations obtained from diabetic subjects are shown in Table V. The concentrations of albumin tested ranged from 1.25% to 2.5%, at which level a highly significant inhibition of insulin action was found for the over-all group ($p < .001$). It is of interest that urine albumin obtained from two diabetic nephrotics showed insulin inhibitory action at 1.25%. Serum albumin from nephrotic patient L.H. showed 56% inhibition; her urine albumin showed a 45% inhibition when tested at the same concentration. Albumin fractions from diabetics showed quantitatively more inhibitory activity than normal albumin, activity being demonstrable at less than 2.5% concentrations. However, 6 diabetic samples of the 22 tested showed less than 36% inhibition. In addition a wide range of inhibitory activity was observed in all samples. In an effort to understand the failure of certain diabetic subjects to show more inhibitory activity at low concentrations of albumin, new albumin preparations were made from two of these patients (H.F. and A.S.). In both cases, less than 25% inhibition of 1,000 μU per ml of insulin was observed with repeat preparations. Other possible factors were examined. In the case of J. R., elevation of the base-line glucose uptake in buffer may have represented a technical error. In the cases of H.F., A.S., M.K., and the pooled samples, the experiments were technically satisfactory. It seemed possible that the severity of the diabetic state might correlate to some degree with the inhibitory activity. For this reason, inhibitory activity of albumin preparations from patients who did not require insulin was compared with inhibitory activity of those requiring treatment. The mean inhibitory activity observed was 35% in the noninsulin group vs. 66% in the insulin group. The difference between these means is statistically significant at the 0.01 probability level.

That insulin treatment per se was not necessarily the factor involved in intensifying the in-

hibitory activity may be seen in that patient T.L., who was not receiving insulin at the time of the test, as well as certain individuals in the group not requiring insulin, showed highly significant inhibitory activity.

Of special interest is the fact that H.F., who failed to show inhibition, is the identical twin of J.F., who showed significant inhibitory activity.

Pregnant women. Nondiabetic women in the third trimester of normal pregnancy were studied. Table VI shows the distribution of insulin-inhibitory activity associated with Debro albumin preparations in this group. The mean test concentration of albumin was 2.18%. In all cases the concentration was 2.5% or less. The mean inhibition observed was 40.8% ($p < 0.01$ for the difference between the insulin effect in buffer vs. albumin). This degree of inhibition at relatively low concentrations of albumin suggests that pregnancy may play a role in the development of increased insulin-inhibitory activity. All patients examined have since delivered babies. No infant was found to weigh more than 9 pounds.

When the over-all means of the normal, diabetic, and pregnant groups tested at albumin concentrations of 2.5% or less were compared by the analysis of variance and the method of least significant difference, it was found that the normals and diabetics differed with a $p < 0.01$, and the

TABLE IV

Insulin-inhibitory effect of albumin prepared by Debro method from normal subjects

Albumin sample	Concentration	Glucose uptake*				Insulin effect in		% inhibition of insulin effect
		B	A	B + I	A + I	B	A	
	%	*mg/g muscle/80 min*						%
A.†								
Serum								
X.Y.	3.5	4.44	4.92	8.37	5.87	3.93	0.95	77
H.M.	3.0	7.18	7.69	10.80	8.25	3.62	0.56	85
C.M.	3.0	6.90	6.81	8.51	7.85	1.61	1.04	35
Urine								
A.G.	3.5	4.29	4.05	8.59	7.14	4.30	3.09	29
G.H.	3.5	4.14	4.10	8.21	5.61	4.07	1.55	62
Mean ± SEM§	3.30	5.39 ±0.68	5.51 0.74	8.90‡ 0.48	6.94‡ 0.52	3.51‡ 0.49	1.44‡ 0.44	
B.†								
Serum								
G.L.	2.5	5.10	5.87	8.84	9.16	3.74	3.29	12
H.M.	2.5	6.00	5.60	7.55	6.85	1.55	1.25	19
D.H.	2.5	2.90	4.31	6.06	5.94	3.16	1.63	48
L.R.	2.5	3.22	3.51	6.68	5.59	3.46	2.08	40
C.H.	2.0	4.26	4.32	6.88	6.39	2.62	2.07	21
B.L.	2.0	4.34	4.49	8.30	7.84	3.96	3.35	15
M.L.	2.0	4.57	4.22	5.87	6.65	1.30	2.43	0
B.S.	2.0	4.51	4.38	8.52	7.53	4.01	3.15	21
J.P.	1.25	2.32	2.72	4.17	4.11	1.85	1.39	26
O.K.	1.25	5.10	5.29	9.56	9.87	4.46	4.58	0
C.M.	1.25	7.07	7.12	9.27	8.93	2.15	1.86	13
Mean ± SEM§	1.97	4.49 ±0.41	4.71 0.36	7.43‖ 0.50	7.17‖ 0.52	2.93‖ 0.33	2.46‖ 0.31	

* Each value represents the mean of three observations. B = glucose buffer; A = albumin in glucose buffer; B + I, A + I = respective addition of 1,000 μU per ml insulin.

† A refers to normal albumin preparations tested at concentrations above 3.0%. B refers to normal preparations tested below 2.5% concentrations.

‡ Significance determined by paired *t* test, $p < 0.05$ for difference between insulin effect in buffer vs. albumin at albumin concentrations of 3 to 3.5%.

§ SEM = standard error of the mean.

‖ p not significant at albumin concentrations below 2.5%.

TABLE V

Insulin-inhibitory effect of albumin prepared by Debro method from diabetic subjects

Albumin sample	Concentration	Glucose uptake*				Insulin effect in		% inhibition of insulin effect
		B	A	B + I	A + I	B	A	
	%	*mg/g muscle/80 min*						%
Serum								
A.D.	2.5	3.21	2.68	6.33	4.59	3.12	1.91	40
H.F.	2.0	3.26	3.93	7.01	6.75	3.75	2.82	24
J.F.	2.0	3.52	3.84	6.86	5.00	3.34	1.16	66
Pool 3, nonfasted	2.0	6.66	5.90	8.23	6.48	1.57	0.58	63
Pool 4, nonfasted	2.0	6.80	6.10	8.34	7.17	1.57	1.07	32
B.S.	2.0	5.26	5.99	8.02	7.27	2.76	1.28	53
A.S.	2.0	2.20	2.88	5.84	5.70	3.64	2.82	22
D.W.	2.0	4.62	4.67	5.73	4.36	1.11	−0.31	>100
W.R.	2.0	6.05	6.48	8.62	6.52	2.57	0.04	98
S.S.	2.0	1.88	2.65	6.85	5.81	4.97	3.16	36
M.K.	1.25	2.15	1.88	5.87	4.88	3.72	3.00	19
Pool 1	1.25	2.35	2.37	4.58	3.36	2.23	0.99	56
Pool 2	1.25	2.87	2.63	4.42	3.72	1.55	1.09	30
C.P.	1.25	4.39	3.90	5.26	3.50	0.87	−0.40	>100
L.H.	1.25	6.52	6.47	10.24	8.08	3.71	1.61	56
J.R.	1.25	6.10	4.75	7.34	6.15	1.24	1.40	0
L.R.	1.25	3.30	3.25	6.66	4.61	3.36	1.36	59
T.L.	1.25	3.17	4.04	7.51	4.56	4.34	0.52	87
Pool 3	1.25	6.93	6.60	9.11	6.59	2.18	−0.01	>100
Pool 5, nonfasted	1.25	4.33	4.76	6.67	6.26	2.34	1.50	36
Urine								
A.B.	1.25	5.66	5.74	8.80	6.43	3.22	0.69	78
L.H.	1.25	6.47	6.22	9.65	7.95	3.18	1.73	45
Mean ± SEM	1.78	4.44 ±0.37	4.44 0.33	7.18† 0.34	5.72† 0.30	2.74† 0.24	1.27† 0.22	

* See Table IV for explanation of symbols.
† $p < 0.001$ for the difference between insulin effect in buffer vs. albumin.

TABLE VI

Insulin-inhibitory effect of albumin prepared by Debro method from the sera of pregnant nondiabetic women at third trimester

Albumin sample	Concentration	Glucose uptake*				Insulin effect in		% inhibition of insulin effect
		B	A	B + I	A + I	B	A	
	%	*mg/g muscle/80 min*						%
R.K.	2.5	4.07	3.30	7.63	5.67	3.56	2.37	33
L.R.	2.5	2.42	2.98	6.69	5.35	4.27	2.37	44
V.H.	2.5†	3.83	4.84	6.80	6.63	2.97	1.79	73
B.H.	2.5†	5.56	6.99	7.65	8.06	2.09	1.07	48
G.C.	2.5	5.09	4.64	5.91	4.69	0.83	0.05	94
G.C.	2.0	4.76	5.78	7.61	8.51	2.85	2.73	0
I.D.	2.0	3.79	3.61	7.61	5.98	3.82	2.37	38
A.M.	2.0	3.32	5.30	6.71	5.04	3.39	−0.26	>100
M.T.	2.0	3.46	3.33	7.68	6.69	4.32	3.36	22
J.S.	1.25	5.06	4.39	8.71	7.28	3.65	2.89	21
Mean ± SEM	2.18	4.13 ±0.21	4.52 ±0.40	7.30 ±0.25	6.39 ±0.40	3.18‡ ±0.23	1.87‡ ±0.38	

* See Table IV for explanation of symbols.
† Positive family history of diabetes.
‡ $p < 0.01$ for the difference between insulin effect in buffer vs. albumin.

TABLE VII

Insulin-inhibitory effect of various albumin preparations prepared from normal plasma by method 6 of Cohn

Albumin sample	Concentration	Glucose uptake*				Insulin effect in		% inhibition of insulin effect
		B	A	B + I	A + I	B	A	
	%	*mg/g muscle/80 min*						%
No. 457	5.0	6.48	5.79	7.69	5.05	1.21	−0.74	>100
No. 457	5.0	4.86	5.29	7.24	5.26	2.38	−0.03	>100
No. 145	5.0	4.87	3.66	7.03	5.03	2.16	1.37	36
No. 145	4.0	5.99	4.60	10.70	7.95	2.87	2.11	26
Swiss Red Cross	4.0	5.47	6.05	6.75	5.77	1.28	−0.28	>100
No. 18866 D†	4.0	1.30	1.80	6.16	5.80	4.86	4.00	17.6
No. 520 D	3.5	5.42	5.76	8.28	7.09	2.86	1.33	53.5
No. 3505 D	3.5	3.34	2.29	6.91	4.46	3.57	2.17	39.0
No. 327 D (unreworked)	3.5	5.55	5.51	8.82	7.74	3.27	2.23	31.0
No. 327 D (reworked)	3.5	5.27	5.29	10.25	8.15	4.98	2.86	42.5
No. 457 D	3.0	4.85	4.63	7.24	5.09	2.39	0.46	80
No. 457 D	3.0	5.10	5.34	7.63	6.00	2.53	0.66	74
Mean ± SEM	3.9	4.87 ±0.29	4.67 0.40	7.89‡ 0.40	6.12‡ 0.37	2.86‡ 0.34	1.35‡ 0.40	

* See Table IV for explanation of symbols.
† D = extensive dialysis against distilled water.
‡ $p < 0.001$ for the difference between insulin effect in buffer vs. albumin.

normals and pregnant group differed with a $p = 0.05$.

II. *Insulin-inhibitory activity of normal Cohn albumin fractions*

Since Vallance-Owen and his associates' original report (1), studies of the albumin insulin-inhibitor have been carried out almost entirely with Debro albumin fractions except for the experiments of Jervell (13), who reported on two normal Cohn fractions, only one of which was inhibitory at 5%. Four other types of albumin preparations were actively inhibitory at 5% concentrations.

It seemed important to determine whether albumin fractions prepared by other than the Debro procedure would show inhibitory activity in our hands. For this reason a variety of Cohn albumin preparations were tested. These materials were prepared by method 6 and represented either pure or unreworked [5] Fraction V obtained from pooled normal plasma or blood. In some cases outdated plasma was the starting material. Table VII shows the results obtained with these fractions. In all cases some inhibitory activity was observed, although this varied among the fractions. Fraction 457, an unreworked preparation, appeared to be the most active. Unreworked lot 327, however, was no more active than its reworked or purer fraction.

[5] In the Cohn preparation of Fraction V, unreworked fractions have not been subjected to the final ethanol step and hence may contain α-globulins.

Starch gel electrophoresis at pH 8.6 shows the protein patterns obtained with Cohn fractions no. 457, no. 145, diabetic Debro albumin, and normal serum (Figure 1). All albumin fractions tested including the Red Cross preparations showed two to three bands but only one ultracentrifugal peak. Immunoelectrophoresis of these fractions revealed no β- or γ-globulins.

III. *Mechanism of action of insulin-inhibitory albumin preparations*

Effect on glycogen synthesis. Comparisons of the effects of the inhibitor on insulin stimulation of glucose uptake and glycogen synthesis in muscle were made. Table VIII shows eight albumin preparations (Debro and Cohn), with varying degrees of inhibitory activity on glucose uptake, which were examined with regard to these two parameters. A high degree of statistical correlation between inhibition of insulin effects on glucose uptake and glycogen was found. The r was 0.970 with $p < 0.001$. The fact that inhibition of glycogen synthesis was not greater than inhibition of glucose uptake suggests that there

was no obvious impairment of phosphorylation of the glucose that was taken up by the tissue.

Free fatty acid content of albumin. One major question that has been repeatedly raised with regard to insulin inhibition by albumin preparations

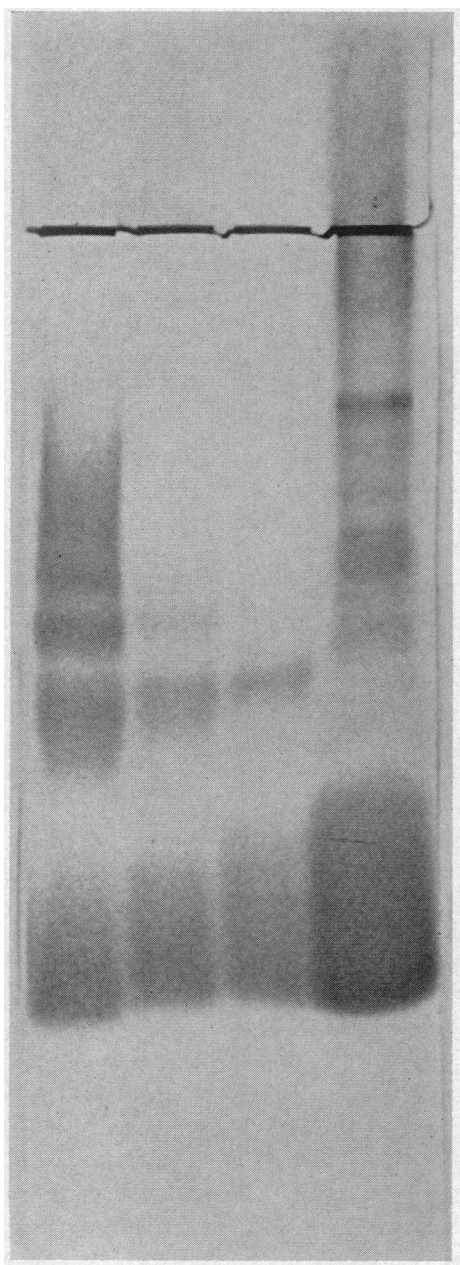

FIG. 1. STARCH GEL ELECTROPHORESIS, pH 8.6, SHOWING FROM LEFT TO RIGHT, COHN FRACTIONS V, NO. 457 AND NO. 145, DIABETIC DEBRO ALBUMIN, AND WHOLE NORMAL SERUM. The multiple bands probably represent polymers of albumin. (Courtesy of Dr. Hugh Chaplin.)

TABLE VIII

*Correlation between insulin-inhibitory effect of albumin on glucose uptake and glycogen synthesis**

Albumin preparation	Concentration	Glucose uptake†						% inhibition of insulin effect	Glycogen levels						% inhibition of insulin effect
						Insulin effect in							Insulin effect in		
		B	A	B + I	A + I	B	A		B	A	B + I	A + I	B	A	
	%	*mg/g muscle/80 min*						%	*mg/g muscle*						%
Debro															
A.S.	2.0	5.26	5.99	8.02	7.27	2.76	1.28	53	2.67	3.15	5.01	4.57	2.34	1.42	39
B.L.	2.0	4.34	4.49	8.30	7.84	3.96	3.35	15	3.23	3.09	5.72	5.24	2.49	2.15	13
D.H.	2.5	2.75	2.93	5.21	3.59	2.46	0.66	73	2.33	3.04	4.91	4.53	2.58	1.49	42
L.R.	2.5	3.22	3.51	6.68	5.59	3.46	2.08	39	2.34	2.81	4.37	4.19	2.03	1.38	32
Cohn Fraction V															
No. 457	3.0	3.91	4.56	6.25	4.49	2.34−0.07		> 100	2.40	2.56	4.48	3.49	2.08	0.93	55
No. 457	3.0	6.31	6.35	8.43	6.30	2.24−0.05		> 100	3.39	3.25	5.63	4.03	2.24	0.78	65
No. 457 D‡	3.0	5.64	5.67	7.42	6.07	1.78	0.40	78	2.78	2.72	4.35	3.56	1.57	0.84	47
No. 520 D	3.5	4.52	5.76	8.28	7.09	2.86	1.33	54	3.14	2.96	4.93	4.12	1.79	1.16	35

* The $r = 0.9698$, $p < 0.001$, indicating highly significant correlation.
† See Table IV for explanation of symbols.
‡ D = extensive dialysis against distilled water.

TABLE IX

*Relation between FFA concentration and insulin-inhibitory activity of albumin**

Albumin preparation	Test concentration of albumin	% inhibition	Test concentration of FFA
	%	*1,000 μU/ml insulin*	*μEq/L*
Vallance-Owen Fraction			
Normal (D.H.)	2.50	48	563
Normal (G.L.)	2.50	12	431
Diabetic (D.W.)	1.25	100	244
Pooled diabetic	1.25	100	216
Cohn Fraction V			
Red Cross	4.00	100	1,650
No. 145	4.00	26	385
No. 18866	4.00	17	822
No. 520 (u)†	3.50	54	1,100
No. 3505 (u)	3.50	39	980
No. 327 (R)‡	3.50	31	674
No. 325 (u)	3.50	42	650
No. 457 (u)	3.00	80	934

* The r = 0.013; p, NS.
† u = unreworked Fraction V.
‡ R = reworked Fraction V.

relates to the free fatty acid (FFA) content of these preparations. In Table IX the FFA content of various preparations is compared with insulin inhibitory activity of the albumin. The r is 0.013, which indicates that there is no correlation between FFA content and inhibition. One would further expect that if FFA were involved in the inhibition of insulin action, they might also inhibit the base-line uptake of glucose (14). This certainly did not appear to be the case in that Red Cross albumin and Fraction 457, which had very high FFA contents, did not impair glucose uptake (Table VII).

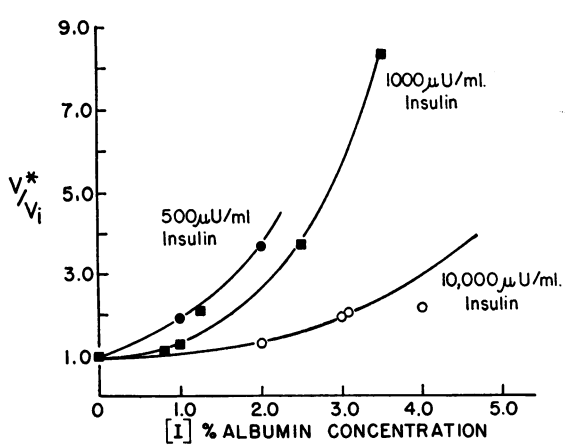

FIG. 2. THE EFFECT OF INCREASING CONCENTRATIONS OF INSULIN ON THE INHIBITORY ACTIVITY OF AN ALBUMIN PREPARATION. V = velocity of the uninhibited reaction, or the increment in glucose uptake per gram diaphragm per 80 minutes in the presence of varying concentrations of insulin (500, 1,000, 10,000 μU per ml). V_i = velocity of the reaction in the presence of albumin and insulin. $V/V_i = 1.0$ if no inhibition is present. The ratio is increased with inhibition of insulin action. Note that inhibitory activity at any one concentration of albumin is least at the highest concentration of insulin.

Relationship of inhibitory activity to concentration of albumin. Five albumin preparations were tested at more than one concentration.

In Table VII the inhibitory activity of preparation 145 at 4% and 5% is shown. Four per cent concentration of 145 showed 26% inhibition, whereas 5% showed 36% inhibition. Fraction 457 was tested at 1.25%, 3%, and 5%. Duplicate assays performed at 3% and 5% are also shown in Table VII; inhibitory activity was greater at 5%. At 1.25% concentration no significant inhibition was found: the insulin effect was + 2.77 in albumin, whereas it was + 2.98 mg glucose per g tissue in buffer. Debro albumin prepared from patient H.M. (Table IV) was tested at 2.5 and 3.0% concentrations with 85% inhibition in the latter and only 19% in the former. C.M., also shown in Table IV, showed 13% inhibition at 1.25% and 35% at 3.0%. These observations suggest that with any one albumin preparation, there is a relationship between increasing albumin concentration and increased inhibitory activity. The data are too few to permit mathematical description of this relationship.

Effect of inhibitory albumin at different concentrations of insulin. Figure 2 shows the results of studies of various concentrations of an inhibitory albumin fraction tested in the presence of 500, 1,000, and 10,000 μU of insulin per ml. V is the maximal increment in glucose uptake stimulated by insulin in buffer, and V_i is the increment in glucose uptake stimulated by insulin in albumin. If no inhibition is obtained, the ratio is $V/V_i = 1.0$. If inhibition occurs, the ratio is greater than 1.0. At higher concentrations of insulin, the inhibitory effect of the same concentration of albumin is decreased. There are too few points to provide a significant kinetic analysis of what appears to be some type of competitive inhibition.

TABLE X

Binding and degradation of insulin by albumin fractions with differing inhibitory activities

Cohn Fraction V* samples	Insulin-I^{131} in medium†			% degradation of insulin-I^{131}
	Total	TCA precipitate‡	Supernatant§	
	cpm	*cpm*	*cpm*	%
145	12,222	11,788	434	3.59
3505	12,313	11,883	430	3.49
520	12,338	11,908	430	3.48
457	12,315	11,875	440	3.57
No albumin	12,510	12,125	385	3.07

* The insulin inhibitory activities of these fractions as derived from Table VII are as follows: no. 145, 26%; no. 3505, 39%; no. 520, 53.5%; no. 457, 74 to 80%.

† Two ml of 3% albumin in glucose buffer containing 1,000 μU per ml cold insulin and insulin-I^{131} as a marker were incubated at 37° C for 80 minutes. After incubation the per cent degradation of insulin was determined.

‡ Trichloroacetic acid (TCA) precipitate (10%) or undenatured insulin.

§ Degraded insulin.

This competition of inhibitor with insulin does not appear to be manifested by an impairment of gross binding of insulin to the hemidiaphragm. When I^{131}-insulin incubated with tissue for 2 hours in insulin-glucose buffer alone was compared with tissue in 3% active albumin and in 3% inactive albumin, the cpm I^{131}-insulin per 100 mg diaphragm did not differ significantly. In addition, there is no evidence that inhibitory preparations contain insulin-destroying activity. This is indicated in Table X by the preservation of trichloroacetic acid precipitable insulin after incubation at 37° C with buffer and variously active albumin preparations.

Discussion

The results reported confirm the basic observation of Vallance-Owen that there is a potent inhibitor of insulin associated with the albumin fraction of plasma. This inhibitor can be obtained from albumin fractions prepared by different methods. The Debro procedure according to Schwert (15) is a highly satisfactory method for preparation of ultracentrifugally pure albumin utilizing an acid alcohol extraction at room temperature. The sedimentation constant, electrophoretic mobility, and patterns suggest considerable purity. Cohn's method 6 differs in that lower temperatures, varying alcohol concentrations, and an increasingly acid pH are combined in preparation of Fraction V. Clearly, both procedures yield albumin that has insulin-inhibitory properties. From the data presented here it is not possible to say whether albumin itself or a molecule associated with albumin is the inhibitor. Vallance-Owen and associates have found that the inhibitor can be separated from albumin and is a dialyzable substance (1). However, there are no data to directly support their suggestion that the inhibitory material is the B chain of insulin, since this substance has not yet been isolated from inhibitory albumin preparations (16).

Whatever the nature of the inhibitory activity, comparisons of albumin from groups of normal individuals with groups of diabetic subjects reveal a significantly greater degree of inhibitory activity in the diabetics. Since the biological assay for the demonstration of the insulin inhibitor is subject to the statistical variations noted in this report, it seems wise at this time to consider the data as semiquantitative. There appears to be a considerable spread of activity through the normal and the diabetic groups, although a separation can thus far be made between the two, in that albumin from normal subjects has not, in our laboratory, been inhibitory at concentrations of 2% or less. (It must of course be noted that the number of observations is small.) On the other hand, most but not all diabetic albumin preparations have been inhibitory at concentrations of 2% or less. Our observations suggest that there may be a relationship between increased inhibitory activity and the type of diabetes. In general, diabetics requiring insulin have shown higher levels of inhibitor. This effect cannot be ascribed to the presence of insulin-binding antibodies in the Debro albumin preparations. Ob-

viously much work remains to be done to clarify this problem. Perhaps the most important is the development of a more precise method of quantifying inhibitory activity. The effects of a variety of factors (insulin, diet, starvation, steroids, growth hormone, body weight changes) upon this inhibitory activity must also be studied systematically. Although Vallance-Owen and coworkers have described a pituitary-adrenal dependence of this inhibitor (1), Lowy and coworkers could not confirm their observations (5). Our finding that the third trimester of pregnancy appears to be associated with increased levels of inhibitor and that albumin obtained from C.P., an acromegalic diabetic, was one of the most inhibitory preparations studied suggests, indeed, that this factor may be influenced by known diabetogenic hormonal substances. The elevated levels of steroids, possibly growth hormone, placental lactogen (17), and insulin (18) in pregnancy support the existence of a "diabetogenic state" in pregnancy (19).

Perhaps the most perplexing and important question relates to the basic role of this inhibitor. Is it primary or secondary to the development of the diabetic state? What correlations, if any, exist between the inhibitory activity, endogenous plasma insulin levels, and the progression from prediabetes to overt diabetes? Vallance-Owen's description of this factor as a genetic dominant (3) seems difficult to accept. In our experience two diabetics, who showed little insulin inhibitory activity, had diabetic siblings, one of whom was an identical twin who showed considerable inhibitory activity. It should be noted that the latter twin had suffered myocardial infarction 8 months before our test. Vallance-Owen has related insulin-inhibitory albumin in such individuals to a genetic diabetic state. Two of four prediabetics noted in Table III were not significantly inhibitory at 2%. In addition, family history of diabetes did not seem to play a role in those pregnant women who showed greater than normal inhibitory activity. Because of such discrepancies in terms of this genetic hypothesis, and because of the deficiencies in the assay system, it would seem wise to clarify first the variety of nongenetic factors that may influence this inhibitory activity. Further, it is difficult on a theoretical basis to assign an all or none characteristic to a material that is present to varying degrees in most subjects. Thus one must conclude that at this time it is premature to characterize this factor as a genetic marker.

Perhaps one of our clinical observations deserves particular mention. The finding of highly significant levels of inhibitory activity in nephrotic urine albumin may be important in the understanding of the alterations in carbohydrate metabolism in nephrosis. In patient L.H. (Table V), serum and urine albumin preparations showed similar inhibitory activity. Certainly the loss of a potent insulin inhibitor in the urine of diabetic nephrotics might well play a role in the decreased insulin requirements of these patients. Other possible explanations for improved carbohydrate tolerance in diabetics with nephrosis have been reported (20, 21).

With regard to the mechanism of action of this inhibitor, certain points can be made. Although insulin-inhibitory activity is demonstrable *in vitro* with separated albumin fractions from normal subjects, whole normal serum containing equivalent amounts of albumin does not show *in vitro* insulin-inhibitory activity (3). Since some normal albumin preparations at *in vitro* concentrations of 5% can totally impair the activity of 1,000 μU per ml insulin (a concentration of insulin at least 10- to 100-fold that found in normal plasma by immunoassay), it is difficult to understand how insulin can act *in vivo* in the normal individual if this inhibitor indeed has a physiologic action. It may well be that this insulin inhibitor is specifically concentrated by the fractionation procedures used in preparing albumin, that it may be coprecipitated, and that we are in no position to quantify the actual concentration of insulin inhibitor in the circulation or available to the tissues.

In an earlier paper Alp and Recant (6) demonstrated that these albumin preparations had no insulin-inhibitory effects upon adipose tissue. In fact these preparations contained measurable amounts of endogenous insulin plus the capacity to enhance the action of added insulin on the glucose metabolism of fat. Keen and associates also stated that Debro preparations of albumin contained insulin (7, 8). Despite the presence of insulin, these preparations in our hands may still

be actively inhibitory of insulin action on muscle. In fact preparations of diabetic albumin contained as much or more insulin than normal preparations (6). However, the insulin content of these preparations tested at 2% concentrations is insignificant (0 to 30 μU per ml by immunoassay) relative to the 1,000 μU per ml of insulin that is added. It would seem likely that the inhibitor competes with insulin. This competition does not appear to be for gross binding of insulin to the tissue. Why adipose tissue and muscle behave so differently with regard to this inhibitor cannot be definitely answered. In view of the observations of Froesch and Ginsburg (22) and Frerichs and Ball (23) with regard to effects of insulin on the metabolism of fructose and glucose by adipose tissue and the inhibitory effects of phlorizin on insulin action, it is difficult to accept a basic difference in membrane response to insulin in muscle and fat cells. On the other hand, totally different responses of adipose tissue to substances known to inhibit insulin action in muscle have been observed. FFA clearly inhibit muscle glucose metabolism, and they may stimulate glucose responses to insulin in adipose tissue (24, 25). It may be that adipose tissue possesses enzymes capable of altering inhibitors. There is precedence in the reports of Antoniades and Gundersen (26) and Shaw and Shuey (27) for such a hypothesis. These investigators have shown that adipose tissue and adipose tissue extracts release insulin from a complexed form to a free form, but no such activity can be attributed to muscle. It might be postulated that some destruction of the albumin inhibitor is produced by adipose tissue. In fact, Ensinck and Vallance-Owen reported findings suggestive of this (28). In our preliminary experiments, partial loss of inhibitory activity occurs after incubation of albumin preparations with adipose tissue. However, a similar loss of activity occurs after incubation with muscle.

Although there are a multitude of unanswered questions concerning this inhibitor associated with plasma albumin, it is likely that further studies, particularly in the direction of separation and identification of the active substance, may provide more precise knowledge of the origin and role of this material in the diabetic state.

Summary

Human plasma albumin fractions prepared either by the Debro procedure or Cohn method 6 were associated with significant insulin-inhibitory activity when tested *in vitro* with rat diaphragm muscle. Diabetic subjects showed quantitatively more inhibitory activity than normals. Pregnant women in the third trimester appeared to have more inhibitory activity than normal but probably less than diabetic. The mechanism of insulin inhibition appears to be competitive and is not quantitatively related to the free fatty acid content of albumin preparations.

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