Diabetologia 2, 86–90 (1966)

The Properties of Trichloracetic Acid-Ethanol Extracts of Human Plasma

1. Bioassay and immunoassay of plasma and extracts

J.S. CAMERON, D.R. BOYNS, R.J. JARRETT and H. KEEN
Department of Medicine, Guy's Hospital, London

Received February 28, 1966

Summary. Bioassay, using the rat hemidiaphragm and epididymal fat pad preparations, has been performed upon the plasma and upon a T.C.A.-ethanol extract of the plasma of fourteen newly diagnosed diabetics. Immunoassay of the extracts was also carried out. Both bioassay and immunoassay of three pooled T.C.A.-ethanol extracts (Dr. W.L. ASHTON) were performed. — Five of thirteen plasmas tested showed insulin-like activity (ILA) on the hemidiaphragm, while all of fourteen tested on the fat pad showed ILA. In contrast, eleven of fourteen extracts revealed considerable amounts of ILA on the hemidiaphragm. Again, all the extracts revealed ILA upon the fat pad. Immunoreactive insulin was found in all extracts, but the levels of 'insulin' measured by this method were much lower than those measured by the fat pad assay. No extract showed significant antagonism to the effects of added insulin (1 mu/ml) on the hemidiaphragm. — Two of the pooled extracts contained very large amounts of both bioassayable and immunoreactive insulin. No one of the three, however, in our experiments, showed any significant antagonism to the effects of added insulin on the hemidiaphragm.

Les propriétés d'extraits de plasma humain par l'acide trichloracétique-éthanol. 1. Dosage biologique et immunologique du plasma et des extraits.

Résumé. Le dosage biologique, utilisant les préparations d'hémidiaphragme et de tissu adipeux de l'épididyme de rat, a été réalisé sur le plasma et sur un extrait par l'acide trichloracétique (T.C.A.)-éthanol du plasma de quatorze diabétiques récemment diagnostiqués. Le dosage immunologique des extraits a été également réalisé. A la fois, le dosage biologique et le dosage immunologique de trois «pools» d'extraits par le T.C.A.-éthanol (Dr. W.L. ASHTON) ont été réalisés. — Cinq des treize plasmas testés montrèrent une activité insulinique (ILA) sur l'hémidiaphragme tandis que les quatorze plasmas testés sur le tissu adipeux montrèrent tous de l'ILA. Par contre, onze des quatorze extraits révélèrent des quantités considérables d'ILA sur l'hémidiaphragme. De plus, tous les extraits montrèrent une ILA sur le tissu adipeux. De l'insuline immunoréactive a été trouvée dans tous les extraits, mais les taux «d'insuline» mesurés par cette méthode étaient beaucoup plus bas que ceux mesurés par le dosage sur le tissu adipeux. Aucun extrait ne présentait d'antagonisme significatif pour les effets de l'insuline (1 mu/ml) ajoutée sur l'hémidiaphragme. — Deux des «pools» d'extraits contenaient de très grandes quantités à la fois d'insuline biologiquement dosable et immunoréactive. Cependant aucun des trois extraits ne montrait dans nos expériences d'antagonisme significatif sur l'hémidiaphragme pour les effets de l'insuline ajoutée.

Die Eigenschaften von Trichloressigsäure-Äthylalkohol-Extrakten des menschlichen Plasmas. 1. Biologische und immunologische Insulinbestimmung von Plasma und Extrakten.

Zusammenfassung. Plasma und Plasma-Trichloressigsäure-Äthylalkohol-Extrakte von 14 frisch entdeckten Diabetikern wurde mit biologischen Insulinbestimmungsmethoden unter Verwendung des Rattenhemidiaphragms und des epididymalen Fettgewebes untersucht. Außerdem wurde die immunologische Insulinbestimmung der Extrakte durchgeführt. Drei „gepoolte" Trichloressigsäure-Alkohol-Extrakte (Dr. W.L. ASHTON) wurden der biologischen und immunologischen Insulinbestimmung unterzogen. — Fünf der 13 getesteten Plasmen zeigten eine insulinähnliche Aktivität (ILA) am Hemidiaphragma, während alle 14 untersuchten Plasmen am Fettgewebe eine ILA aufwiesen. Im Gegensatz dazu ließen sich bei 11 von 14 Extrakten beträchtliche Mengen einer ILA am Zwerchfell nachweisen. Wieder zeigten alle Extrakte eine ILA am Fettgewebe. Immunologisch wirksames Insulin fand sich in allen Extrakten, doch lagen die mit dieser Methode gemessenen Insulinspiegel viel tiefer als die mit der Fettgewebsmethode bestimmten Werte. Kein Extrakt zeigte am Diaphragma eine signifikanten Antagonismus gegenüber der Wirkung zugefügten Insulins (1 mE/ml). — Zwei der „gepoolten" Extrakte enthielten sehr große Mengen biologisch und immunologisch bestimmten Insulins. Keiner der drei Extrakte zeigte jedoch in unseren Versuchen am Zwerchfell einen signifikanten Antagonismus gegenüber der Wirkung zugefügten Insulins.

We have previously reported our failure to detect insulin-inhibitory activity in trichloracetic acid-ethanol (TCA-ethanol) extracts of normal human plasma when tested upon the rat hemidiaphragm preparation (KEEN, 1963, CAMERON, KEEN and MENZINGER, 1964). The properties of these extracts further departed from those described by others, principally VALLANCE-OWEN and co-workers (VALLANCE-OWEN, DENNES and CAMPBELL, 1958; VALLANCE-OWEN and LILLEY 1961a and b), in that they exerted significant insulin-like activity (ILA) upon the hemidiaphragm.

We have extended our observations to a study of extracts prepared from the plasma of newly diagnosed, untreated diabetics. This paper presents the results together with those obtained using three TCA-ethanol extracts kindly supplied by Dr. W.L. ASHTON. These were pooled from three groups of prepared material characterised as 1) antagonistic 2) semi-antagonistic and 3) non-antagonistic and they were the subject of a report by ASHTON (1965), in which the sources of the extracts are described.

The accompanying paper describes our findings relating to the physical, chemical and immunological properties of some of the extracts.

Materials and Methods

Venous blood samples were collected from newly diagnosed diabetics, before treatment, in several hospitals throughout Britain, through the good offices of members of the Medical and Scientific Section of the British Diabetic Association. Blood was taken into heparin, the plasma immediately separated by centrifugation and stored frozen until used. The age, sex and clinical state of each patient are noted in Table 1.

TCA-ethanol extracts of the plasmas were prepared by the method of DEBRO et al. (1957) as described by VALLANCE-OWEN et al. (1958). Analytical grade reagents were used for the preparation. Extracts were freeze dried and later reconstituted for use in KREBS-RINGER bicarbonate buffer (UMBREIT et al., 1959).

Rat hemidiaphragm techniques have been described previously (CAMERON et al., 1964). The extracts were used at a concentration of 2 g/100 ml, except where stated.

Rat hemidiaphragm glycogen was estimated by hydrolysing the tissue in 40% KOH, precipitating the glycogen with ethanol and, after neutralisation with 10 N H_2SO_4, estimating reducing substances in an Auto-Analyser.

When experimental comparisons were made, e.g. between extract and buffer or between extract alone and extract with added insulin, they were carried out in triplicate with one hemidiaphragm in one situation and its fellow in the other.

The insulin-like activity (ILA) of the plasma and extracts were assayed on *rat epididymal fat* by a micro-incubation assay (KEEN et al., 1966) using the glucose uptake of approximately 25 mg pieces of fat in 250 μl of medium as the index of activity. The effect of biological variation between rats on the estimations was minimised by ensuring that each animal contributed a piece of fat to all of the experimental variables studied, each of which was assayed in quadruplicate.

Immunoassay of plasmas and extracts, made up in either KREBS-RINGER bicarbonate or veronal buffer, was performed using a double antibody technique similar to those described by MORGAN and LAZAROW (1963) and HALES and RANDLE (1963). Human crystalline insulin, kindly supplied by Burroughs Wellcome, was used for the standards. Insulin assayed by this method is referred to as "immuno-reactive insulin" (IRI).

Table 1. *The effect of T.C.A.-ethanol extracts from diabetic plasma upon rat hemidiaphragm*

Patient				Glucose uptake of hemidiaphragms (mg/g/90 min) incubated in:				
Initials	Sex	Age	Ketosis	a) buffer alone	b) buffer + 2 g/100 ml extract	c) buffer + insulin 1000 μU/ml	d) buffer + insulin 1000 μU/m + extract 2 g/100 ml	I.L.A. of plasma (μU/ml)
1 AF	F	15	+	8.00 ± 0.11	8.01 ± 0.49	11.86 ± 0.48	10.53 ± 1.18	0
2 RH	M	17	+	7.63 ± 0.67	11.59 ± 1.65	12.92 ± 1.79	10.11 ± 1.59	0
3 MB	M	20	++	8.35 ± 0.33	8.94 ± 0.21	10.98 ± 1.33	11.10 ± 1.40	0
4 JD	F	21	—	7.71 ± 0.34	8.91 ± 0.30	9.38 ± 1.33	10.19 ± 0.80	0
5 ABr	F	22	+++	*1.61 ± 0.31	*2.69 ± 0.40	*2.07 ± 0.26	*2.90 ± 0.24	0
6 BH	F	23	++	—	—	8.39 ± 0.46	8.38 ± 0.22	—
7 FH	M	23	++	—	—	—	—	—
8 BT	M	24	—	7.48 ± 0.10	10.99 ± 2.05	10.91 ± 2.02	8.89 ± 1.57	20
9 TB	M	25	+	*1.15 ± 0.23	*1.79 ± 0.08	*2.01 ± 0.06	*2.61 ± 0.46	0
10 JW	M	29	+	6.84 ± 0.39	6.88 ± 0.56	7.95 ± 0.44	8.93 ± 0.51	0
11 HFl	M	34	+	—	—	—	—	—
12 DC	F	35	+	4.37 ± 0.62	6.39 ± 0.40	10.86 ± 0.92	8.19 ± 0.20	20
13 ME	F	38	+	6.79 ± 0.62	7.97 ± 0.47	9.55 ± 0.82	9.56 ± 0.65	0
14 JS	F	45	++	5.67 ± 0.91	7.01 ± 0.39	9.97 ± 0.26	10.20 ± 0.36	70
15 GB	F	51	+	—	—	—	—	—
16 HF	M	54	—	—	—	—	—	—
17 DW	F	61	—	4.36 ± 0.30	6.39 ± 0.40	8.19 ± 0.36	10.86 ± 0.93	250
18 DS	F	66	—	4.37 ± 0.51	8.07 ± 0.59	9.83 ± 0.28	10.08 ± 0.38	40
19 AB	M	67	—	5.72 ± 0.27	8.83 ± 0.66	10.30 ± 0.20	9.72 ± 0.59	—

* Results in mg glycogen/g/90 minutes incubation. All results are the meacn ± S.E.M. for three hemidiaphragms. In experiments 1, 2, 3, 4, 8 and 10, the incubations were performed at glucose concentrations from 300—500 mg/100 ml. The others were at 250 mg %.

Results

(a) *Diabetics.* Rat hemidiaphragm: In the same experiments, the parent plasmas were assayed against insulin standards, while the extracts were incubated with and without added insulin. In table 1 the estimates of ILA in the parent plasma are given, together with the results obtained with the extracts. These estimates of ILA are presented for comparison only, recognising that the accuracy of the hemidiaphragm as an assay system is too low to confer much significance upon the

individual figures. In some cases (noted in the table) the blood sugars of the patients made it necessary to run the whole experiment at high glucose levels in order to avoid the possible effects of plasma dilution.

Only a few of the older patients' plasmas contained ILA detectable by the hemidiaphragm method. In contrast, eleven of the fourteen extracts tested contained considerable ILA., the glucose uptakes of the hemidiaphragms incubated with extract being significantly ($p < 0.05$) higher than those incubated in buffer alone. In three extracts, all from younger diabetics (AF, MB, JW), the effect did not differ from that of buffer alone. Three of fifteen extracts (RH, BT, DC) showed some degree of inhibition of the effect of added insulin, but this failed to achieve statistical significance. Furthermore, these extracts stimulated glucose uptake to higher levels by themselves than when insulin was added to them. No extract was prepared which both failed to stimulate glucose uptake by itself and antagonized the effect of added insulin.

Table 2. *Insulin-like activity (μU/ml) of plasmas and plasma TCA-ethanol extracts measured by rat hemidiaphragm and rat epididymal fat and by immunoassay*

Patient	Diaphragm Assay of plasma	Diaphragm assay of extract*	Fat Assay of plasma	Fat Assay of extract*	Immuno-assay of extract*
1 AF	0	0	540	520	28
2 RH	0	2000+	220	1100	36
3 MB	0	0	—	140	52
4 JD	0	800	800	1200+	12
5 ABr	0	1000	—	90	16
6 BH	—	—	—	800	16
7 FH	—	—	420	460	38
8 BT	20	60	210	—	—
9 TB	0	350	—	120	12
10 JW	0	0	1200+	1200+	—
11 HF	—	—	410	360	24
12 DC	20	40	110	160	—
13 ME	0	—	—	—	26
14 JS	70	280	40	60	28
15 GB	—	—	380	400	26
16 HF	—	—	700	700	38
17 DW	250	400	95	100	—
18 DS	40	450	650	720	18
19 AB	—	—	810	1200+	124

* The values obtained for the ILA of the extracts at 2 g/100 ml were multiplied by 2 to correct these to the normal albumin concentration in the plasma of 4 g/100 ml
\+ represents maximal stimulation of the fat pad or hemidiaphragm.

Rat epididymal fat pad: As with the hemidiaphragms, plasma and extract were assayed side by side. Of thirteen pairs of comparative observations performed (Table 2), ten showed similar values for the ILA of the parent plasma and the extract when the values for the extract were adjusted to a plasma albumin concentration of 4 g/100 ml. All seventeen extracts tested showed ILA, some maximally stimulating glucose uptake by the fat pad in 2 g/100 ml concentration.

Immunoassay: Insulin levels in the extracts, measured by immunoassay, were much lower than the levels obtained with the fat pad assay (table 2). Although IRI was found in all extracts, there was no consistent relationship between the fat — assayable and the immuno-reactive insulin in each extract.

(b) *Pooled extracts.* The three extracts (A, B and C) were investigated in ignorance of their properties in the hands of Dr. ASHTON. Each extract was tested at concentrations up to 1.25 g/100 ml on the hemidia-

Table 3. *Pooled extracts A, B and C: (I) Results of assay of I.L.A. (II) The effect of the extracts upon the glucose uptake of the rat hemidiaphragm*

		(1)	(11) *Rat Hemidiaprhagm:* glucose uptake in								
Extract conc. g/ml	Immuno-assay μU/ml	Fat Pad assay* μU/ml	Diaphragm assay** μU/ml	Buffer	Buffer + extract	t	p less than	Buffer + insulin 1000 μU/ml	Buffer + insulin 100 μU/ml + extract	t	p
Extract A.											
0.5	124	2.700	—	—	—	—	—	—	—	—	—
0.25	65	1.700	—	—	—	—	—	—	—	—	—
1.25	—	—	1000+	5.1 ± 0.91	9.59 ± 0.43	6.29	0.01	8.3 ± 0.59	8.49 ± 0.16	0.44	n.s.
1.25	—	—	1000+	***1.81 ± 0.16	3.17 ± 0.5	8.0	0.001	3.15 ± 0.12	3.09 ± 0.23	1.4	n.s.
Extract B.											
0.5	200+	10.000+	—	—	—	—	—	—	—	—	—
0.25	200+	10.000+	—	—	—	—	—	—	—	—	—
1.25	—	—	1000+	2.16 ± 0.01	3.15 ± 0.19	5.4	0.01	2.84 ± 0.27	3.17 ± 0.1	1.65	n.s.
Extract C.											
0.5	83	75	—	—	—	—	—	—	—	—	—
0.25	34	55	—	—	—	—	—	—	—	—	—
1.25	—	—	280	6.73 ± 0.35	8.43 ± 0.57	3.62	0.05	10.87 ± 0.74	9.67 ± 0.77	1.88	n.s.

* glucose uptake and ^{14}C incorporation into lipid.
** glucose uptake.
*** glycogen deposition.
Results are means ± S.E.M. of three observations. All the fat pad assays and diaphragm experiments were run in Krebs-Ringer bicarbonate buffer containing inert bovine albumin, 200 mg/100 ml, and glucose 250 mg/100 ml with the exception of the diaphragm experiments with extract B, when the glucose concentration was 60 mg/100 ml.

phragm and epididymal fat pad. The ILA of the extract was estimated and its effect on added insulin assessed. The extracts were also assayed immunologically. The results are shown in table 3.

Both extracts A and B, and particularly the latter, contained large amounts of ILA in both the diaphragm and fat pad assays. Extract B contained so much "insulin" assayable by all three methods, that, even after dilution to an albumin concentration of 0.25 g/100 ml, maximal activity in the bioassays was still obtained. Extract C contained less, but still an appreciable amount of insulin. It is interesting that, with this extract, all three methods gave similar results, whereas, with the other two extracts, the fat pad values were very much higher.

In our hands none of the extracts at a concentration of 1.25 g/100 ml inhibited the effect of added insulin (1000 μU/ml) on glucose uptake or glycogen deposition of the hemidiaphragm preparation. In Dr. ASHTON's hands, extract A at the same concentration was totally antagonistic, B was semi-antagonistic and C was non-antagonistic to the effects of added insulin.

Discussion

Using the rat hemidiaphragm preparation, we have found in most TCA-ethanol extracts from the plasma of newly diagnosed diabetics considerable ILA and no evidence of significant insulin antagonism. These results parallel our previous findings with extracts prepared from the plasma of normal subjects (CAMERON et al., 1964) and are difficult to reconcile with those of VALLANCE-OWEN and his colleagus. If the synalbumin antagonist is involved in the genesis of diabetes mellitus, it should be present at the clinical onset of the disease. Many of the patients we studied were very ill and none had received treatment. Nevertheless, from only three subjects were we able to demonstrate a doubtful diminution of the effects of 1000 μU/ml insulin on the hemidiaphragm by extracts in 2 g/100 ml concentration. In contrast, extraction of most plasmas revealed ILA active on the diaphragm, which was not evident when the plasma itself was assayed.

All of the diabetic plasmas that were without ILA when assayed upon the hemidiaphragm showed considerable activity on epididymal fat, confirming the original observations of STEINKE et al. (1961). The TCA-ethanol extracts also showed comparably high ILA when assayed on epididymal fat.

Our results with Dr. ASHTON's extracts indicate that differences in the preparation of TCA-ethanol extracts probably do not account for our failure to find insulin antagonism. The reasons for the discrepant results, using identical extracts, remain unknown.

We have suggested previously (KEEN 1963) that the process of extraction may so alter the insulin molecule that under certain conditions it acts as an insulin antagonist. We have been unable to reproduce those conditions although using extracts demonstrated, in other hands, to be antagonistic. That it is not the extraction procedure but the conditions of the hemidiaphragm incubation which may be critical, complicates appraisal of the significance of synalbumin antagonism.

If the synalbumin antagonist is insulin altered by extraction, then it would be reasonable to expect a direct relationship between the amount of antagonist in the extract and the amount of insulin in the parent plasma. In the pooled extracts, there was a relationship between the reported degree of antagonism and the amount of ILA and IRI. Presumably, the amount of insulin in the extracts was also related to the amount in the parent plasmas. Furthermore, the synalbumin antagonist has been found in greatest quantity in extracts of the plasma of diabetics, pregnant women in the third trimester of pregnancy (ALP and RECANT, 1965) and in patients following myocardial infarction (VALLANCE-OWEN and ASHTON, 1963). In all these situations, an increased circulating amount of insulin has either been described (PETERS and HALES, 1965; SPELLACY et al., 1965) or may be expected, in that the diabetics reported upon have been treated with insulin or oral hypogylcaemic agents or have been non-insulin dependent. It is of course possible that the synalbumin antagonist occurs in plasma in amounts quantitatively related to the amount of insulin present. However, as synalbumin antagonism has not been demonstrated in whole plasma or serum, it seems more likely that it arises during the extraction process. This possibility is considered further in the accompanying paper.

Acknowledgments. We are most grateful to Dr. W. ASHTON for allowing us the opportunity of examining his extracts and to the many physicians who collected plasma from diabetic patients. Burroughs Wellcome kindly supplied the purified crystalline human insulin. Technical assistance was provided by CAROL SANDERS, ANNE WIGGLESWORTH and Messrs. L. YEUNG and M. MILLER-JONES. This work was partly supported by grant No. AM 6135 MET from the National Institutes of Health, Bethesda, U.S.A. and by assistance from the British Diabetic Association.

References

ALP, H., and L. RECANT: Studies of the Insulin-inhibitory effect of human albumin fractions. J. clin. Invest. **44**, 870—882 (1965).

ASHTON, W.L.: The glucose uptake induced in rat hemidiaphragms by insulin in the presence of human albumin. J. Endocr. **33**, 103—107 (1965).

CAMERON, J.S., H. KEEN and G. MENZINGER: Insulin activity of normal plasma and plasma acid-ethanol extracts. Lancet **1964 I**, 74—76.

DEBRO, J.R., H.E. TARVER and A. KORNER: The determination of serum albumin and globulin by a new method. J. Lab. clin. Med. **50**, 728—732 (1957).

HALES, C.N., and P.J. RANDLE: Immunoassay of insulin with insulin antibody precipitate. Lancet **1963 I**, 790—791.

KEEN, H.: Properties of human albumin. A metabolic study of albumin extracts from normal and diabetic plasma. Diabetes **12**, 406—413 (1963).
— J.S. CAMERON and A.O. LURIE: A microincubation adipose tissue assay for insulin. Submitted for publication. (1966).
MORGAN, C.R., and A. LAZAROW: Immunoassay of insulin; a two antibody system. Diabetes **12**, 115—126 (1963).
PETERS, N., and C.N. HALES: Plasma insulin concentrations after myocardial infarction. Lancet **1965 I**, 1144—1145.
SPELLACY, W.N., F.C. GOETZ, B.Z. GREENBERG and J. ELLS: Plasma insulin in normal mid-pregnancy. Amer. J. Obstet. Gynec. **92**, 11—15 (1965).
STEINKE, J., K.W. TAYLOR and A.E. RENOLD: Insulin antagonism in serum from normal persons and diabetics including some observations on pig's blood. Lancet **1961 I**, 30—31.
UMBREIT, W.W., R.H. BURRIS and J.F. STAUFFER: Manometric techniques. Minneapolis. 4th Ed. 1964.
VALLANCE-OWEN, J., E. DENNES and P.N. CAMPBELL: Insulin antagonism in plasma of diabetic patients and normal subjects. Lancet **1958 II**, 336—338.
—, and W.L. ASHTON: Cardiac infarction and insulin antagonism. Lancet **1963 I**, 1226—1228.
—, and M.D. LILLEY: An insulin antagonist associated with plasma albumin. Lancet **1961a I**, 804—806.
— — Insulin antagonism in the plasma of obese diabetics and pre-diabetics. Lancet **1961b I**, 806—807.

J.S. CAMERON
Department of Medicine
Guy's Hospital
London/England