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CLEVELAND, O.	
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DISTRICT OF COLUMBIA	
George Washington University	October 2, 1958
	December 4, 1958
	February 5, 1959
ILLINOIS	
University of Illinois Med. School	March 10, 1959
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Ohio State University	October 31, 1958

Preparation and Anti-Insulin Activity of Lipoprotein Fractions from Rat Serum.* (24813)

M. E. KRAHL, M. E. TIDBALL AND E. BREGMAN
(With technical assistance of John A. Dickey)
Dept. of Physiology, University of Chicago, Ill.

Lipoprotein fractions from diabetic rat serum have been reported to inhibit glucose utilization by muscle(1) and by hexokinase in cell-free systems(2). The role of lipoproteins as regulators of enzyme activity is now under examination(3,4,5,6). These studies have been handicapped by the fact that published fractionation and test procedures require diabetic serum as starting material and are not sufficiently explicit to yield products which are always inhibitory. The present paper describes methods for preparation of uniformly inhibitory fractions; they are applicable not only to diabetic, but also to normal rat serum. Ability of inhibitory fractions to block insulin action on muscle has been estimated.

Methods and results. Measurements of glucose uptake. Fractions have been tested for ability to inhibit glucose uptake by diaphragms of normal rats, especially in presence of small concentrations of insulin. Strain, sex, weight, and feeding schedule of rats, environmental temperature, removal of diaphragms and their handling and incubation must be scrupulously standardized. Experimental de-

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tails essential for reproducibility follow. Diaphragm donors were male Sprague-Dawley rats, received as groups of 24 to 36 born on same day and kept in laboratory 7-14 days at 25°C, food available at all times. Rats used on given day were fasted 24 hours; individuals within weight range of 95-105 g were selected; animals below 90 g yielded unsatisfactory results. Weight range was constant. Insulin solutions were prepared fresh daily by dilution of Iletin (Insulin, Lilly), 80 units/ml, with Krebs-bicarbonate solution; dilutions were made to minimize loss of insulin on glass. To prevent chance insulin pollution all glass vessels were washed in chromic acid; electrodes of pH meter were washed with 1 N NaOH and then with 1 N HCl (in 50% ethanol) before each preparative run. All plastic tubes were new; all syringes and needles were soaked 24 hours or more in alkaline detergent after use. *Rat sera.* In extending original work(1) rats with severe alloxan diabetes, prepared as before (5), were used. Blood was obtained from heart, allowed to clot 5 minutes at 25°C, then centrifuged (4500 g) 15 minutes at 5°. All subsequent operations were carried out at 5° or below until incubation of diaphragms was begun. Samples representing desired degree of diabetes (as judged by serum sugar(8) level) and having desired lipide(5,9) content (see below) were pooled for fractionation. Fractions to be tested were dissolved in protein-containing medium to replace normal milieu of fraction, at first in diabetic rat serum from which inhibitory activity had been removed by repeated freezing and thawing(5, 10), then, for simplicity, in 6% solutions of Armour bovine fraction V (crude albumin) in Krebs-bicarbonate buffer. Several lots of fraction V were tried; lot No. M-12011 was satisfactory without further treatment; lot No. R-14005 was satisfactory when 7.3% solution in Krebs salt solution (without bicarbonate or CO_2) was dialysed 2 hours at 5° against the same salt solution, protein concentration being adjusted to 6% and bicarbonate concentration to that of Krebs solution before incubation; glucose concentration was 600 mg % to facilitate comparison of inhibitory effects of diabetic sera and fractions therefrom. Commercial samples of crystalline bovine albumin inhibited glucose uptake of diaphragms. Each sample of pooled serum and each precipitated fraction (in volume of Krebs salt solution without bicarbonate equal to that of parent serum) was subjected to zone electrophoresis on paper(5); results are summarized in terms of 2 fractions: albumin plus α-globulin ($A\alpha$) and β-globulin plus γ-globulin ($\beta\gamma$). Other analyses were: total protein (11), total and free cholesterol(12), phospholipide(13), total(9) and protein-bound(5) lipids. The protein-bound lipids, as here defined, averaged 83% of total lipide. *Precipitation of inhibitory fractions.* Initial reports on separation of inhibitor from diabetic rat serum stated that activity was obtained in Cohn combined fraction I, II, III(1,2). Here, the first attempts to repeat and extend these observations were unsuccessful. Diabetic rat sera were subjected to Oncley-Cohn procedure for precipitation of combined fraction I, II, III from normal human plasma(14,15). Sixteen samples of the fraction were prepared and dissolved in frozen-thawed(5) diabetic serum for test of inhibitory activity; 8 were inhibitory and 8 were inactive, average inhibition when present being 1.2 mg/g diaphragm/hour. Inhibitory activity tended to be associated with a high ratio of β- to α-lipoprotein in the fraction, and vice versa. The clue to preparation of uniformly inhibitory fractions was found in 2 modifications: preparation of fractions with all practicable speed, and use of slightly lower alcohol concentration and higher ionic strength than employed for human plasma(14,15). Electrophoretic patterns for fractions prepared from rat serum by Oncley-Cohn, and by present method are shown in Fig. 1. Procedure selected for precipitation and test of inhibitory fractions from rat sera was as follows. 1. Precipitating reagent was made up fresh daily and cooled to −5°. It contained 10 ml 95% ethanol, .15 ml .8 M sodium acetate-acetic acid buffer (pH 4), 1 ml .06 M NaCl, .08 ml 1 M acetic acid; the volume was made to 50 ml with water. 2. Samples of rat serum containing less than 700 mg % protein-bound lipide were pooled and

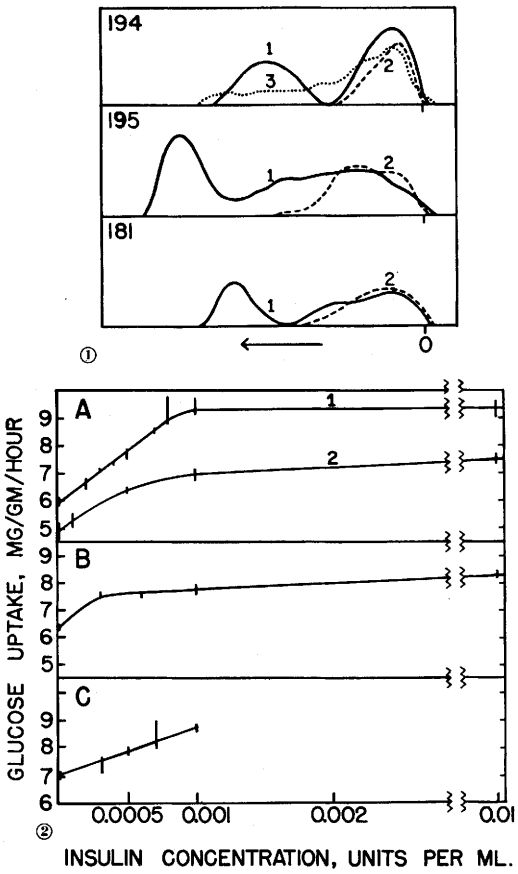

FIG. 1. Zone electrophoresis patterns for sera and inhibitory fractions after staining for lipides (5). The origin is at 0. No. 194, 195, 181 refer to same experiments as in Tables I-II. Exp. 194, fasting normal rats: 1, serum; 2, fraction prepared by present modified Oncley-Cohn method; 3, fraction from same serum by original Oncley-Cohn method; 0.02 ml serum or solution of fraction was used for each run. Exp. 195, fasting normal rats: 1, serum; 2, fraction by present method; 0.03 ml serum or fraction. Exp. 181, fasting diabetic rats: 1, serum; 2, fraction by present method; 0.013 ml serum or fraction. For other details see text.

FIG. 2. Relation of glucose uptake of diaphragms from normal fasting rats to insulin concentration in incubation medium. A, in Krebs-bicarbonate medium; B, in a solution of 6% Armour bovine fraction V in Krebs-bicarbonate; C, in diabetic rat serum which had been repeatedly frozen and thawed. Glucose concentration was 600 mg % except in A, curve 2, where it was 300 mg %. Length of each vertical line represents 2 standard errors, one on each side of mean. For other details, see text.

chilled to 0°. Samples with obvious hemolysis were excluded. The fractionation *must* be begun within less than 1 hour after blood is drawn. 3. Two-tenths ml of pooled serum was mixed with .8 ml of precipitation reagent, 4 ml of .15 M NaCl were added, pH read at 25°; if pH was not 6.2 ± 0.2, 1 M acetic acid was added to the reagent, pH test was repeated; this was continued until a reagent giving proper pH was obtained. The maximum amount of 1 M acetic acid required/50 ml was about .16 ml for diabetic serum and .23 ml for normal serum. These preliminary tests must be carried out before precipitation of main pooled serum sample is begun, and with greatest expedition, *i.e.*, in 10-15 minutes or less. 4. Four volumes of adjusted precipitation reagent were added dropwise with stirring at −5° to main pooled serum samples during 10 minutes. The mixture was stirred a further 5 minutes, then centrifuged promptly at 8000 g for 5 minutes at −5°. 5. The protein precipitate was redissolved in a solution of bovine fraction V to give a volume equivalent to that of serum from which it was prepared. By trial it was found that the precipitate contributed about 10% of final volume, and 20-25% of protein of the solution; volume and concentration of fraction V solution were adjusted accordingly to make total protein concentration 6%. 6. One ml of solution of the protein fraction to be tested was transferred to each incubation beaker at 5°. Diaphragms were then removed from donor rats, pre-soaked(7) 15 minutes at 5°, and transferred to incubation beakers containing the solution of fraction V, or fraction V plus fraction under test; each incubation beaker received 2 halves of diaphragm from one rat. All samples were then incubated with shaking at 37° for 1 hour, removed, chilled and analyzed for glucose disappearance(5). Three to 6 aliquots of medium containing the fraction were incubated in this manner concurrently with the same number of aliquots of the medium without fraction.

Inhibitory activity of fractions from diabetic rat sera (Table I) was not correlated with protein-bound lipide or glucose levels of sera. When this was settled, fractions were prepared from serum of fasting normal rats; these were just as inhibitory as fractions from diabetic rat sera. No inactive (non-inhibitory) fractions have been obtained with above

TABLE I. Inhibitory Activity of Fractions from Diabetic or Normal Rat Sera of Varying Lipide and Glucose Content. Experiments have same numbers as in Table II and Fig. 1; arranged in the order as performed. For details, see *Methods*.

Exp. No.	Glucose uptake of diaphragms, mg/g/hr ± S.E.* In albumin + 0.0003 unit insulin/ml	+ fraction	Inhibition	Protein-bound lipide of pooled serum sample, mg % Avg	Range of individual sera	Glucose of pooled serum sample, mg % Avg	Range of individual sera	pH at which fraction was made
			Fractions from diabetic rat serum					
173	7.3 ± .45	6.3 ± .17	1.0	240	230– 245	488	308– 625	5.9
176	7.9 ± .07	6.5 ± .22	1.4	728	545–1100	755	542–1122	6.0
178	8.1 ± .32	7.0 ± .12	1.1	600	275–1060	1100	885–1325	6.1
180	6.6 ± .17	5.4 ± .04	1.2	356	302– 430	1010	659–1512	6.0
181	7.9 ± .28	6.4 ± .23	1.5	285	278– 288	1015	680–1435	5.9
183	7.7 ± .07	6.8 ± .23	.9	277	255– 345	670	516– 902	6.2
184	8.0 ± .09	6.2 ± .15	1.8	344	240– 550	990	510–1545	6.0
Means	7.64 ± .13†	6.37 ± .12†	1.27					
			Fractions from normal rat serum					
190	7.5 ± .31	6.1 ± .14	1.4	260	250–266	116	114–124	6.0
191	7.6 ± .27	6.5 ± .11	1.1	252	244–261	113	97–156	6.1
193	7.3 ± .14	6.4 ± .54	.9	252	248–258	109	76–140	6.1
194	6.7 ± .12	5.8 ± .37	.9	244	237–256	104	81–140	6.2
195	7.8 ± .57	6.8 ± .27	1.0	258	222–278	99	74–124	6.2
198	7.8 ± .36	6.5 ± .36	1.3	258	244–273	115	87–130	6.1
199‡	7.8 ± .36	6.5 ± .06	1.3	255	244–278	142	115–206	6.1
Means	7.50 ± .16§	6.37 ± .12§	1.13					

* Mean ± stand. error for all control samples in albumin alone was 6.4 ± 0.04.

† P value for the difference, as estimated by Fisher procedure for small numbers, is less than 0.001.

‡ Serum donors in this experiment had food available until time of bleeding.

§ P value for difference is less than 0.001.

procedure under conditions shown. However, non-inhibitory fractions could be obtained if sera were kept at room temperature for 1 or more hours before fractionation, if the fraction was exposed to ethanol solution for 30 minutes or more, or if individual sera with protein-bound lipide values above 1100 mg % were included in serum pool. These experiments show that inhibition is not an artifact arising from the reagents. Inhibitory fractions could be stored at −5° as precipitated protein cake (before re-solution in crude albumin medium) for 4 days without loss of activity.

Inhibitory activity of β-fraction was enhanced by i.p. injection into serum donor of 2 mg growth hormone, half at 24 hours, half at 3 hours prior to sacrifice. In 2 experiments (12 diaphragms) average inhibition of glucose uptake was 3.6 mg/g/hour as compared with 1.1 for uninjected controls. Adrenocorticotrophic and thyrotrophic hormones at same dose had no effect.† It was previously shown that inhibitory activity of diabetic *serum* was related to growth hormone(6,10).

Lipides of inhibitory fractions. Inhibitory activity was clearly not dependent on presence of albumin or α-globulin electrophoretic components, as inhibitory fractions free of Aα-proteins or lipides could be prepared from either diabetic or normal rat serum (Fig. 1, Table II). In fact, inhibitory β-fractions could be obtained from normal serum which was not in itself inhibitory. The Aα-fractions thus tended to prevent inhibition by the β-fraction. Protein and lipides of inhibitory fractions of Table I were further analyzed. For solutions of βγ-fraction in a volume of solution equal to that of original serum, mean values were (g/100 ml), for fractions from diabetic serum: protein, 1.34; total lipide, .332; cholesterol, .016 free, .43 esters; phospholipides, .024. For fractions from normal

† Growth hormone (Lot no. M208), ACTH (Lot no. 212-10) and TSH (Lot no. 6) were kindly supplied by Dr. Irby Bunding, Armour Labs.

TABLE II. Distribution of Protein-Bound Lipide between $A\alpha$- and $\beta\gamma$-Electrophoretic Components of Sera and of Inhibitory Fractions. Values expressed as g of protein-bound lipide/100 ml of serum or solution of fraction in Krebs-bicarbonate medium (see *Methods*). Experiments have same numbers, arranged in the same order, as in Table I.

	Pooled serum		Inhibitory fraction	
Exp. No.	$A\alpha$	$\beta\gamma$	$A\alpha$	$\beta\gamma$
	Samples from diabetic rats			
173	.051	.193	.012	.019
176	.194	.478	.102	.438
178	.060	.486	.012	.478
180	.073	.284	.00	.185
181	.080	.205	.00	.127
183	.177	.128	.070	.066
184	.064	.325	.00	.316
Means	.100	.299	.019	.257
	Samples from normal rats			
190	.070	.195	.00	.133
191	.109	.177	.00	.063
193	.118	.174	.011	.099
194	.120	.175	.00	.100
195	.075	.202	.00	.131
198	.121	.144	.00	.163
199	.107	.195	.00	.187
Means	.103	.181	.002	.125

serum they were: protein, 1.31; total lipide, .150; cholesterol, .017 free, .034 esters; phospholipides, .030. The corresponding lipide values for normal rat serum were: total, .343; cholesterol, .032 free, .074 esters; phospholipide, .099. The fact that non-inhibitory fractions with electrophoretic patterns, not significantly different from those for inhibitory fractions, can be obtained from stored serum suggests that inhibition is associated with some component of β-lipoprotein which is quantitatively minor or with some particular molecular arrangement not revealed by methods hitherto applied.

Interaction between insulin and inhibitory fractions. Serum fractions inhibitory toward glucose uptake negated stimulatory effect of measurable amount of insulin. Dose-response curves (Fig. 2) relating concentration of insulin to glucose uptake of normal diaphragm were constructed for 3 incubation media: (a) Krebs-bicarbonate buffer, (b) 6% solution of Armour's bovine fraction V in Krebs-bicarbonate buffer, and (c) diabetic rat serum in which inhibitory activity had been destroyed by repeated freezing and thawing(5,10). Then fractions to be evaluated were dissolved in one of these media containing appropriate low concentration of insulin; glucose uptake of normal diaphragms in this solution was measured, and inhibition relative to concurrent control without the fraction was obtained. The amount of insulin corresponding to this reduction in rate of glucose uptake was estimated from dose-response curve; accuracy of the estimate is limited by flatness of curve. In the 6% fraction V solution, average inhibition induced by the fraction from 1 ml of diabetic rat serum was 1.3 mg/g muscle/hr (Table I). Fractions from normal rat serum were not significantly less inhibitory. From B (Fig. 2) this inhibition corresponded to negation 0.0003 unit of insulin/ml. In preliminary tests mentioned above, in which frozen-thawed diabetic serum was used as solvent for the inhibitory fraction the average inhibition was 1.2 mg/hr. From C (Fig. 2) this also corresponded to negation of about .0003-.0004 unit insulin/ml.

An independent estimate of amount of insulin blocked by the inhibitor of diabetic rat serum can be obtained from the difference in glucose uptake of normal diaphragms in diabetic rat serum, and in diabetic rat serum after inactivation of inhibitor by storage or freezing and thawing. In 2 series(5) values of the difference were 1.3 and 1.6, average of 1.45 mg/g/hour. From C (Fig. 2) this would correspond to freeing of .0007 unit of insulin/ml when total inhibitor in whole serum was inactivated. Since both estimates are likely to be minimal rather than maximal, it is provisionally concluded that the inhibitor in 1 ml of fasting rat serum can prevent stimulatory effect on muscle of at least .0004 to .001 unit of insulin.

The relation of these inhibitory lipoprotein fractions to the physiological action of insulin, and to other substances which interact with insulin has been discussed(6,16).

Summary. 1. Procedures for routine preparation from rat serum of fractions uniformly inhibitory to glucose uptake of rat diaphragm have been developed; it was possible to obtain such fractions not only from diabetic rat serum, as previously reported, but also from normal rat serum. Inhibitory activity of frac-

tions from normal serum was enhanced by prior treatment of serum donor with pituitary growth hormone; adrenocorticotrophic hormone or thyrotrophic hormone had no such effect. 2. Inhibitory fractions had gross properties of β-lipoproteins and could be prepared free of lipides having electrophoretic mobility of albumin or α-globulins. Average ratio of total lipide to protein was .25 for fractions from diabetic rat serum and .11 for fractions from normal rat serum; phosphatide/total lipide ratio was lower in such fractions than in parent sera. Relation of inhibitory activity to lipide content requires further clarification, as inhibitory activity may be lost by delay during preparation of fractions without significant change of lipide content or electrophoretic behavior of fractions. 3. Inhibitory fractions interfered with stimulatory action of insulin on diaphragm muscle. The fraction from 1 ml of diabetic or normal rat serum nullified the stimulatory effect of at least .0004-.001 unit of insulin.

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Net Synthesis of Heme from Protoporphyrin and Iron by Extracts of Duck Erythrocytes.* (24814)

J. RAYMOND KLEIN

Biology Dept., Brookhaven National Lab., Upton, N. Y.

Hemin obtained from incubated mixtures containing labeled ionic iron, protoporphyrin, and preparations of avian erythrocytes was found to contain appreciable labeled iron; when the porphyrin or cell preparation was omitted from mixtures, or mixtures were not incubated, little labeled iron was recovered as hemin(1-4). From these findings and others, in particular the heat-lability of the cell preparations, it was inferred that in avian erythrocytes protoporphyrin and ionic iron are the immediate precursors of protoheme and that some component(s) of the erythrocytes, participates in heme formation. The inferences are reasonable; however, the evidence, at least for formation of heme from the presumed precursors, would be supplemented by establishing net synthesis of heme from protoporphyrin and iron in the presence of red-cell preparations. In particular, net synthesis would rule out the possibility that the recovery of labeled iron as hemin indicated above arose from artifact. The present report provides evidence

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