INHIBITION OF ENZYMES BY LIPOPROTEINS

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In previously reported experiments[1,2] it was shown that lipoproteins derived from diabetic plasma, liver, muscle and the pituitary inhibited glucose utilisation by the rat diaphragm and by cell free muscle extracts. The inhibition of the rat diaphragm was partly reversible by insulin, although the possibility of a normal insulin effect on a lower basic rate of glucose utilisation could not be excluded. Occasional insulin reversals of the inhibition in cell free extracts were also observed. Therefore, it appeared that these observations could have considerable bearing on the problem of control of carbohydrate metabolism. Accordingly, it appeared of importance to determine the specificity of the inhibition in regard to other enzymes and enzyme systems.

EXPERIMENTAL

Two sources of lipoprotein were used. The first was plasma of severely diabetic human subjects*, and the second the plasma of alloxanised rats in which the blood sugar was at least 600 mg/100 ml.

The plasma was fractionated by Method 10 of COHN *et al.*[3]

Fraction III_0 was then dissolved in 0.6 *M* sodium chloride and freed of zinc by passage through an I.R.C.50 column. This passage was accompanied by considerable purification of the material, with no appreciable loss in activity. There was little change in the total lipid content, but a variable loss in the total protein, resulting in a change in lipid-protein ratio from 0.6 to 1.1–1.6 after passage through the column. The material was then dialysed against 0.15 *M* sodium chloride for 2 hours.

Lipids were determined gravimetrically, after extraction with chloroform-butanol (3:1 v/v), and protein by the method of LOWRY[4]. All glucose estimations were done by the method of SOMOGYI[5]. Two standard systems were used to determine the inhibitory activity of the lipoprotein so obtained.

1. The muscle glycolytic system previously described .

2. The utilisation of glucose was determined spectrophotometrically by reduction of TPN at 340 mμ in the following system[6]:

Glucose 0.5 μ*M*, ATP 2 μ*M*, Mg 20 μ*M*, TPN 0.5 μ*M*, glycyl glycine buffer 0.04 *M* 0.25 ml, Zwischenferment, muscle extract, and water to 3 ml. Where lipoprotein was added, this displaced portion of the water.

* I should like to thank Dr. H. P. TAFT of the Royal Melbourne Hospital and Drs. EWEN DOWNIE and B. HUDSON of the Alfred Hospital for their co-operation in this investigation.

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The results of a number of experiments using lipoprotein from various sources are presented in Table I*.

TABLE I

LIPOPROTEIN INHIBITION OF GLUCOSE UTILISATION BY MUSCLE EXTRACTS

Expt.	*Source lipoprotein*	*Lipoprotein added (as ml original plasma)*	*Protein content (μg)*	*Glucose* disappearance (μM/30 min)*	
				Control	*With lipoprotein*
1	A.D.R. (LP6)	0.4	—	0.46	0.37
2	A.D.R. (LP6)	0.4	—	0.98	0.72
3	A.D.R. (LP8)	0.37	—	0.98	0.35
4	A.D.R. (LP9)	0.20	—	1.46	1.08
5	A.D.R.+ (LP10)	0.55	—	1.00	0.28
6	N.H. (LP11)	2.00	207	1.40	0.89
7	N.H. (LP11)	0.50	51	1.22	1.16
8	A.D.R. (LP14)	0.40	—	1.04	0.67
9	I.R.D.[1] (LP18)	0.08	—	1.11	1.03
		0.20	—		0.98
		0.40	—		0.83
10	I.R.D.[2] (LP19)	0.04	—	0.81	0.74
		0.10	—		0.59
		0.20	—		0.46
11	I.R.D.[2] (LP20)	0.05		1.55	1.44
		0.14			1.34
		0.28	81		1.22
12	I.R.D.[2] (LP21)	0.05		1.02	0.92
		0.125			0.88
		0.25	82		0.80
13	I.R.D.[2] (LP22)	0.03		1.55	1.36
		0.09			1.19
		0.18	176		0.89
14	D.H. (LP23)	0.10		1.55	1.33
		0.25			1.14
		0.50	186		0.83

* All glucose estimations carried out in triplicate by the technique of SOMOGYI.
A.D.R. – alloxan diabetic rat. A.D.R.+ – alloxan diabetic rabbit.
N.H. – normal human. I.R.D.[1] – insulin resistant diabetic human.
I.R.D.[2] – insulin resistant diabetic human. D.H. – diabetic human.
(LP–) indicates the preparation used.

In the investigation of other enzyme systems, the lipoprotein used was always tested on one of the above muscle systems. It was then added in a concentration sufficient to produce 50% inhibition of glucose use by a muscle extract. Wherever possible, all enzyme systems investigated conformed to those described in the literature.

The first series investigated was a group of purified and crude hexokinases derived from brain, heart, yeast and red blood cells.

Purified brain and heart hexokinase were prepared according to the technique of CRANE AND SOLS[7].

* Dr. M. E. KRAHL informs me that he has prepared lipoprotein fractions with inhibitory activity toward muscle hexokinase from plasma of alloxan-diabetic rats and from plasma of depancreatised dogs. Values for five samples of fraction III_0 from diabetic rat plasma were (as μg glucose used/hour/ml muscle extract) without and with inhibitor, respectively: 3860, 854; 2080, 1640; 1760, 316; 940, 490; 1760, 1110; the concentration of III_0 lipoprotein/ml test medium was in each instance equivalent to 0.7–0.9 ml diabetic plasma. Values for three samples of lipoprotein prepared by flotation from diabetic dog plasma were: 1650, 530; 700, 455; 715, 587; the concentration of lipoprotein/ml test medium was in each instance equivalent to 0.2 ml diabetic plasma.

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The results (Table II) show that only the solubilised heart hexokinase was inhibited. In order to determine the specificity of the inhibition the following enzymes and enzyme systems were investigated.

TABLE II

EFFECT OF LIPOPROTEIN ON HEXOKINASES AND HEXOKINASE-CONTAINING SYSTEMS

Enzyme	*No. of Expts.*	*Mean glucose utilisation (μM/30 min)*		*Percentage inhibition*
		Control	*With lipoprotein*	
Yeast hexokinase	4*	1.42	1.40	0
(purified)	2**	0.26	0.25	0
Brain hexokinase	4*	1.26	1.23	0
(purified)	2**	0.29	0.27	0
Heart hexokinase				
(purified and solubilised)	3**	0.34	0.25	27
Crude brain	3**	0.16	0.17	0
Crude red cell	3**	0.20	0.18	0

* Glucose estimated by method of SOMOGYI.
** Glucose utilisation determined spectrophotometrically.

1. Red cell cholinesterase[8*].

Red cell haemolysate 1 in 90, acetyl choline 0.006 *M*, sodium bicarbonate 0.025 *M*, lipoprotein, total volume 3 ml, were gassed with 5 % carbon dioxide in nitrogen. Evolution of carbon dioxide was measured manometrically.

2. Brain cholinesterase[8*].

Brain homogenate, acetyl choline 0.006 *M*, sodium bicarbonate 0.025 *M*, lipoprotein, total volume 3 ml, were gassed with 5 % carbon dioxide in nitrogen. Evolution of carbon dioxide was measured manometrically.

3. Plasma pseudocholinesterase[8*]

Plasma diluted 1:90, acetyl choline 0.06 *M*, sodium bicarbonate 0.025 *M*, lipoprotein, total volume 3 ml, were gassed with 5 % carbon dioxide in nitrogen. Evolution of carbon dioxide was measured manometrically.

4. Intestinal alkaline phosphatase[9].

Intestinal alkaline phosphatase**, phenyl phosphate 0.002 *M*, phosphate buffer pH 7.85 0.1 *M*, lipoprotein, water to 2 ml. Incubated at 37° C for 1 hour. Production of phenol was measured with Folin-Cocialteu reagent.

5. Transaminase[10].

1 ml mitochondrial preparation (rat liver) in 0.1 *M* phosphate buffer pH 7.4, 1 ml 0.1 *M* glutamic acid in 0.05 *M* phosphate buffer pH 7.4, 1 ml 0.05 *M* sodium pyruvate in 0.05 *M* phosphate buffer pH 7.4, lipoprotein, water to 4 ml. Incubated 1 hour at 37.8° C. Deproteinised with trichloracetic acid. Aliquots chromatographed in phenol-ammonia, glutamic acid and alanine eluted, the colour developed with ninhydrin and read at 580 mμ.

6. Adenosine triphosphatase.

0.25 ml methionine activating enzyme[12] with ATPase activity, ATP 10 μ*M*, bicarbonate buffer 0.154 *M* pH 7.4, gas phase 5 % carbon dioxide in oxygen. Incubated 1 hour at 37° C. Free phosphate was estimated by the technique of WEIL MALHERBE[11].

* I should like to thank Mr. L. AUSTIN for carrying out the cholinesterase studies.
** I should like to thank Dr. R. K. MORTON for making available partially purified intestinal phosphatase.

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7. Methionine activating enzyme[12].

The technique described by CANTONI was used, lipoprotein and water being added to a fixed volume. Inorganic buffers were used as it was found that "Tris" interfered with the inhibition (Fig. 1).

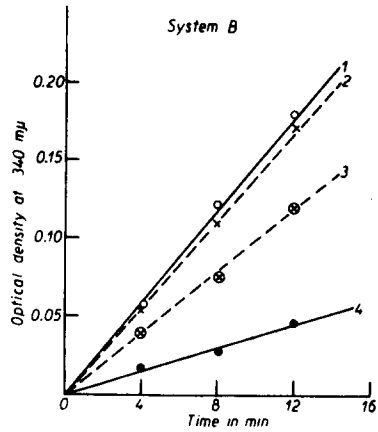

Fig. 1. Interference of Tris buffer with inhibition of methionine activating enzyme. 1. Control PO_4-buffer; 2. Control Tris buffer; 3. Lipoprotein PO_4-buffer; 4. Lipoprotein Tris buffer.

8. Succinic dehydrogenase[13]*.

Mitochondrial preparation 0.2 ml, cyanide 0.02 *M* 0.1 ml, 2:6-dichlorphenolindophenol 0.2 ml, succinate 0.6 *M* 0.3 ml, lipoprotein or water, buffer 0.2 *M* sucrose + 0.2 *M* phosphate, pH 7.4 2 ml.

9. Yeast glucose-6-phosphate dehydrogenase[14].

Glucose-6-phosphate 0.5 μM, Zwischenferment, TPN 0.5 μM, Mg^{++} 20 μM, 0.04 *M* glycyl glycine buffer pH 7.4 0.25 ml, lipoprotein, water to 3 ml. Reduction of TPN followed spectrophotometrically at 340 mμ.

10. Yeast invertase.

Sucrose 20 μM, invertase, lipoprotein, water to 1 ml. Incubated at 21° C for 20 minutes. Aliquots removed for glucose and fructose analysis at 5 minute intervals.

11. Prior to this investigation the effect of lipoprotein on muscle fructokinase was investigated by Dr. BARBARA ILLINGWORTH BROWN of the Department of Biological Chemistry, Washington University, St. Louis.

The results of these experiments are summarised in Table III.

TABLE III

EFFECT OF LIPOPROTEIN ON ENZYME SYSTEMS NOT CONTAINING HEXOKINASE

Enzyme	*No. of expts.*	*Mean percentage inhibition**
Red cell cholinesterase	3	0
Brain cholinesterase	2	0
Pseudocholinesterase	3	22
Intestinal alkaline phosphatase	4	21
Transaminase	3	54–77
Adenosine triphosphatase	4	6–33
Methionine activating enzyme	4	21–85
Succinic dehydrogenase	2	0
Glucose-6-phosphate dehydrogenase	8	0
Invertase	2	0
Fructokinase—some degree of inhibition was observed in three out of ten experiments. The source of the lipoprotein was pituitary.		

* Where the variation in inhibition was greater than can be accounted for by variations in analytical procedures, the range of inhibition observed is given.

DISCUSSION

In experiments involving the use of intestinal phosphatase, it was found that a very high blank was obtained from lipoprotein incubated with the enzyme preparation

* I should like to thank Mr. E. MARTIN for carrying out these experiments.

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used. This was found to be due to the presence of tyrosine in the preparation. Similarly, tyrosine was demonstrated chromatographically in experiments on transamination of pyruvate to alanine, thus emphasizing the need for adequate controls in experiments using this substance.

Consideration of the results in Table I shows that lipoprotein derived from normal serum will inhibit glucose utilisation by muscle extracts but that the equivalent of a much larger amount of serum is required to obtain a similar inhibition. It is also seen that the quantity of lipoprotein expressed as ml original plasma required to produce a given inhibition varies considerably, although the variation is not as marked in terms of μg of protein added.

In experiments 10, 11 and 12 Table I, the blood was taken when the patient was receiving 4800 units of insulin daily, whereas in experiment 13 the patient was temporarily deprived of insulin therapy. From these results it appears that the administration of insulin diminished the quantity of inhibitory material per unit plasma, although the addition of insulin *in vitro* had no effect on the inhibitions observed in any of the four experiments. This would suggest that the inhibitory material accumulates in the circulation in the absence of insulin, although its effects are not necessarily reversed by that hormone. Over seventy experiments were carried out to test the effect of insulin *in vitro* in such inhibited systems; although some reversals were observed, these were so irregular that at the present time they cannot be regarded as being of any significance.

Table II shows that not all systems utilising glucose are inhibited by lipoprotein in the concentrations used, the most notable exceptions being hexokinases derived from yeast, brain and red cells, whereas those derived from muscle were inhibited.

It is also observed that all systems in which inhibition has been observed, with the single exception of pseudocholinesterase, are either dependent on an organic phosphate coenzyme or hydrolyse organic phosphate esters. Two possible conclusions can be reached from this finding. The first is that the added lipoprotein binds organic phosphates, thus rendering them inaccessible to the enzyme. This is unlikely because it has, in the course of these and previous experiments, been repeatedly shown* that in muscle extracts the inhibition of glucose utilisation is independent of ATP concentration. A more satisfactory explanation is that the phosphatide containing lipoprotein competes with organic phosphates for an active site on the enzyme molecule. However, not all enzymes utilising ATP were inhibited. This and the inhibition of pseudocholinesterase make it necessary to regard such a hypothesis with caution as it is possible that further investigation may disclose other enzymes of different types to be inhibited by lipoproteins.

In conclusion it can be stated that the inhibition observed is not specific to muscle hexokinase, and hence the relationship of these phenomena to the problem of control of carbohydrate metabolism remains obscure.

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* Unpublished data.

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SUMMARY

1. Experiments designed to test the specificity of lipoproteins as inhibitors of glucose utilisation are presented.

2. It has been shown that the difference between normal and diabetic plasma in regard to content of this material is largely quantitative.

3. Lack of specificity is demonstrated as several enzymes other than those utilising glucose are inhibited.

4. The possibility that the inhibitor acts by competing with organic phosphates for an active site on the enzyme molecule is discussed.

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