MULTIPLE MOLECULAR FORMS OF ATP:HEXOSE 6-PHOSPHOTRANSFERASE FROM RAT LIVER*

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Several groups of investigators, using different approaches, have been able to distinguish and partially characterize two ATP:hexose 6-phosphotransferases from rat liver (Viñuela et al., 1963; Walker, 1963). Using ammonium sulfate fractionation, Viñuela et al., (1963) described one of these enzymes, with a low K_m for glucose (0.01 mM), as a typical animal hexokinase (EC 2.7.1.1), and the other, with a high K_m for glucose (10 mM), as glucokinase (EC 2.7.1.2). The distinction between both enzymes is of special interest, since it appears that the activity of the high-K_m enzyme changes markedly with dietary and hormonal alterations (Viñuela et al., 1963; Sharma et al., 1963; Salas et al., 1963) and also during development (Walker, 1963; Oliver and Cooke, 1964).

Since we have found that total glucose phosphorylating activity from rat liver decreased with fasting or with a high fat, carbohydrate free diet (Niemeyer et al.,1962b,1963; Pérez et al.,1964) it seemed of interest to investigate whether the two phosphotransferases would vary under the different dietary conditions previously

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studied. Preliminary experiments showed that the simultaneous assay of both activities was not entirely reliable, thus a more direct approach was undertaken, i.e., isolation of the enzymes in order to perform separate assays on each enzyme. During these studies, four fractions with glucose phosphorylating activity were separated from rat liver by means of ion exchange chromatography. These results are reported here.

Male albino rats weighing approximately 200 g were used. The animals were fed on different diets or were deprived of food for several days before death. The composition of the diets has been already reported (Niemeyer et al., 1962a).

The results of typical chromatographic separations performed with liver extracts from rats deprived of food or fed different diets are shown in Fig.1. Three peaks of activity (A, B, D) were detected when 100 mM glucose was used as a substrate. When the assay was performed with 0.5 mM glucose, however, a fourth peak (C) could be unmasked. This last fraction was inhibited by excess of substrate. Peak D, which represents 88.2 ± 2.4 % of total activity in rats fed a balanced diet, showed the most striking changes after dietary alterations. In fact, it almost disappeared in animals receiving the high fat, carbohydrate free diet (Fig. 1, IV). If the areas under the peaks were integrated and the results referred to 100 g of body weight, semiquantitative information could be obtained. The variations observed in fractions A, B, and C from rats fed different diets were of little significance compared to the changes undergone by fraction D (unpublished results).

The four fractions have been repeatedly observed, even when the liver of one single animal was used. The percentages of recovery after chromatography on DEAE-cellulose varied widely (usually between 60 and 90 %). However, the relative activities of the fractions

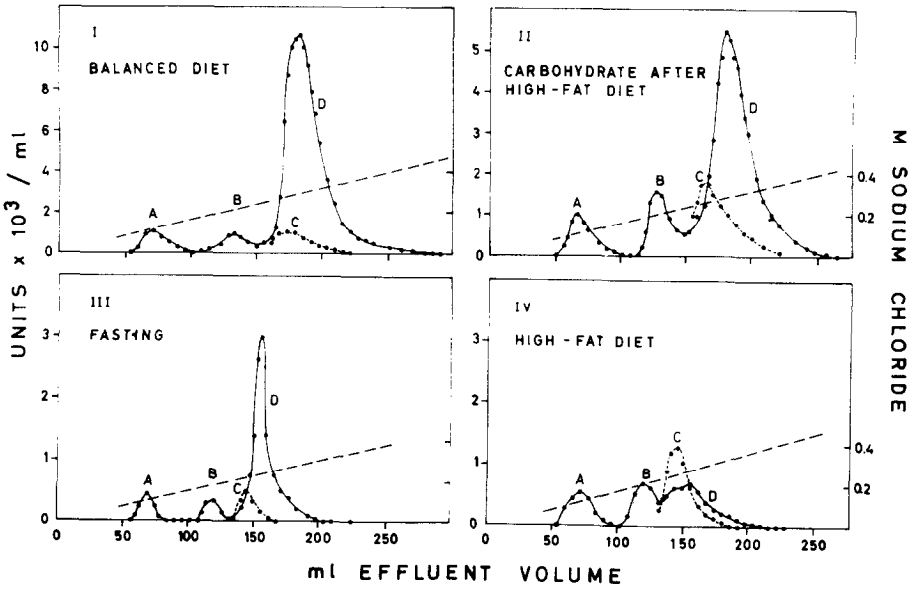

Fig. 1. Chromatographic fractionation of ATP:hexose phosphotransferase isoenzymes from livers of rats under different dietary conditions.

I. Balanced diet. Column load: 17 ml of supernatant fluid obtained after treatment with CM-cellulose (CM_S), 200 mg of protein, 0.035 units/mg of protein. II. Carbohydrate after high fat diet. 19 ml CM_S, 228 mg of protein, 0.021 units/mg. III. Fasting. 7 ml CM_S, 90 mg of protein, 0.011 units/mg. IV. High fat diet. 12 ml CM_S, 134 mg of protein, 0.005 units/mg.

Fifty percent (w/v) homogenates were prepared from the pooled livers of two or more rats in 0.01 M Tris - 0.001 M EDTA, pH 7.0, and centrifuged at 105,000 x g in a Spinco preparative ultracentrifuge. The supernatant fluids were treated batchwise with CM-cellulose at pH 7.0 to remove basic proteins. The supernatant fluid after centrifugation (CMS) , containing all the glucose phosphorylating activity, was chromatographed on a DEAE-cellulose column (1.2 x 23 cm; 3.4 g of powder). Linear gradient elution was carried out with NaCl from 0 to 0.5 M (- - -) in 0.01 M Tris - 0.001 M EDTA, pH 7.0. Flow rate was about 60 ml/hour. Fractions of 3 ml were collected and the content of each tube was examined for protein (Lowry et al., 1951) and for glucose phosphorylating activity. ATP:hexose phosphotransferase activity was measured following the reduction of NADP in a Beckman DU spectrophotometer in the presence of ATP, Mg ions, and an excess of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Pérez et al., 1964). The enzyme activities were routinely assayed at 100 mM (●), and 0.5 mM (○) glucose concentrations. The same systems with ATP omitted acted as blanks. Similar blank values were obtained when 0.1 M N-acetyl glucosamine was added to the system, independent of the presence of ATP. One unit of ATP:hexose phosphotransferase corresponds to the amount of enzyme that phosphorylates 1 μmole of glucose in 1 minute at 30°.

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were essentially the same under each experimental condition. The same pattern was also observed when the crude supernatant fluid was chro-

matographed directly on DEAE-cellulose. Nevertheless, the CM-cellulose treatment was used as a routine procedure because it stabilized the enzyme activities. Rechromatography of the peak including C and D on DEAE-cellulose under the usual conditions did not separate both fractions, which were eluted at the expected molarity of sodium chloride; furthermore, no other peaks of activity were detected. However, both enzyme activities with the kinetic properties of fractions C and D could be separated by column chromatography on hydroxylapatite.

The four fractions may correspond to isoenzymes of ATP:hexose 6-phosphotransferase, as described for yeast hexokinase (Trayser and Colowick, 1961). The possibility that one or more of the peaks correspond to erythrocyte hexokinase could be ruled out. Contamination in well-bled animals is negligible, as can be deduced from assays of phosphotransferase in rat blood (unpublished results) and from the content of residual blood in liver (Hodgson et al., 1960).

Some kinetic properties of the four isoenzymes are shown in Table I. The K_m for glucose of fraction D, as well as the ratio V_{max} fructose : V_{max} glucose were essentially the same, whether the livers were obtained from rats fed a balanced diet, or from animals deprived of food for 48 hours. Thus, in order to obtain the figures for fraction D shown in Table I, the results obtained from animals under those two conditions were averaged.

The data reported in this paper show clearly that in rat liver several molecular forms, or isoenzymes, account for the total glucose phosphorylating activity, thus partially confirming the work of other investigators. The fact that we found three low-K_m isoenzymes instead of one (Viñuela et al., 1963; Walker, 1963) could be explained by the high resolution of cellulose chromatography, not attainable either by the ammonium sulfate fractionation as used by Viñuela et al., (1963) or by the kinetic analysis as performed by Walker (1963). Their

Table I

Kinetic Properties of ATP:Hexose 6-Phosphotransferase Isoenzymes

Isoenzyme	K_m			$\frac{V_{max}\ \text{fructose}}{V_{max}\ \text{glucose}}$
	Glucose M	Fructose M	ATP M	
A	4.4×10^{-5}	3.1×10^{-3}	4.2×10^{-4}	1.09
B	1.3×10^{-4}	3.0×10^{-3}	7.0×10^{-4}	1.21
C	6.0×10^{-6}	1.2×10^{-3}	-	1.28
D	$1.8 \pm 0.03 \times 10^{-2}$*	-	4.9×10^{-4}	0.25 ± 0.02*

Phosphorylating activities were determined as reported (Pérez et al., 1964). When fructose was used as a substrate, phosphohexose isomerase was included in the incubation mixture. The figures correspond to one determination or to the average of two assays from different preparations, with the exception of those marked (*), which are the means ± standard error of 4 determinations on different preparations. To determine the ratio between phosphorylating activities for fructose and glucose, saturating concentrations of each substrate were used.

low-K_m enzyme (hexokinase) and our fractions A, B, and C have in common a high affinity for glucose and a high phosphorylating activity for fructose. Isoenzyme D would correspond to the high-K_m enzyme (glucokinase) described independently by Viñuela et al., (1963) and by Walker (1963). Our value of apparent K_m for glucose and the value for the ratio V_{max} fructose : V_{max} glucose are slightly higher than the corresponding figures given by Sols' group. Purer enzyme preparations are highly desirable in order to obtain more reliable figures.

The results presented in this communication point out the difficulties, and the possibilities of misinterpretation, inherent to the simultaneous assay, in crude extracts, of different forms of one enzyme. Under different physiological conditions, the major changes of glucose phosphorylating activity occur in fraction D, which represents a high proportion of the total activity in well fed animals

(Fig. 1). Therefore, the determination of total enzyme activity in crude preparations, seems to be a reasonable approach to the study of the variations of liver ATP:hexose 6-phosphotransferase in rats submitted to dietary or hormonal alterations.

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