Sulphate content and activities of the different LDH-isoenzymes in various fractions of mouse liver

Fraction	Sulphate % of dry weight	LDH-isoenzyme number as % of total LDH-activity 1	2	3	4	5	Cathodally migrating band (see [10])
Whole liver	0.12 ± 0.005	0.36 ± 0.1	1.4 ± 0.3	3.0 ± 0.5	4.4 ± 0.5	90.7 ± 7.0	0.12 ± 0.06
Liver with connective tissues removed	0.096 ± 0.002	0.2 ± 0.08	0.31 ± 0.1	0.34 ± 0.1	1.8 ± 0.5	95.6 ± 8.0	1.7 ± 0.05
'Connective tissue'	0.83 ± 0.01	7.2 ± 1.0	7.5 ± 1.0	9.0 ± 4.0	20.0 ± 4.0	58.0 ± 5.0	

pieces. The connective tissues were removed by pressing the whole livers through a nylon gauze. The fractions were then separately freeze-dried for 60 h under a pressure of approximately 10 mm Hg, and were then analysed for their sulphate content according to the method of WAINER and KOCH[8] after hydrolysis with formic acid. The amount of sulphate in each tissue was taken as an approximate measure of the content of connective tissue. The LDH-isoenzymes of the different fractions were separated by starch electrophoresis and were rendered visible and evaluated as described by KARLSSON and KJELLBERG[9].

The sulphate content (the content of connective tissues) and the relative activities of the various liver fractions are shown in the Table. A marked diminishing in the activity of LDH-5 was noted, accompanied by an increase in LDH1, 2, 3 and 4 as the fraction becomes more dominated by the connective tissues. The sixth, cathodally migrating band, as described by AGRELL and KJELLBERG[10] shows no correlation with the sulphate content of the material and will not be considered further here. In an attempt to evaluate the total activity of LDH in the various fractions, no significant difference was found.

From the results related it seems likely that the liver cells show only LDH-5 activity while the other tissues contribute to all 5 LDH-isoenzymes. The material also suggests that the 'connective tissues' in this report is not a separate entity but contains material of several cell types, as the values do not approximate to a binomial distribution. This heterogenity is, however, to be expected as the fraction contains not only the connective tissue cells but also other cells e.g. cells from blood vessels.

Zusammenfassung. Mit einer einfachen Methode wird demonstriert, dass Lebergewebe von Mäusen in 2 Fraktionen verschiedener Menge an Bindegewebe zu trennen ist. Die an Bindegewebe reiche Fraktion zeigt Enzymaktivität in LDH 1, 2, 3, 4 und 5. Nach Entfernung des Bindegewebes wird die Aktivität der LDH 1, 2, 3 und 4 Isoenzyme des restierenden Lebergewebes reduziert.

L. PALMER and B. KJELLBERG

Zoophysiological Institute, University of Lund (Sweden), 25th April 1967.

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Some Aspects of the Hydrolysis of Phosphomonoesters by Homogenates of Growing and Regressing Mesonephroi in Chick Embryos[1]

Several investigators have obtained results which indicate that acid phenylphosphatase and acid β-glycerophosphatase activities are associated with different enzyme molecules. In a study of the hydrolysis of different phosphomonoesters by the microsomal fraction of rat liver preincubated at 37 °C at pH 5, BEAUFAY and DE DUVE[2] concluded that the enzyme glucose-6-phosphatase acts upon phenylphosphate, but is unreactive towards β-glycerophosphate. A study with homogenates of rat liver and spleen by MACDONALD[3] led that author to believe that an enzyme other than glucose-6-phosphatase is responsible for the hydrolysis of phenylphosphate. He compared the activities of glucose-6-phosphatase, acid phenylphosphatase, and acid β-glycerophosphatase, and examined their stability after preincubation at 37 °C at pH 5 for 1 h. As a result of his investigation, he suggested that each of the above enzymic activities is attributed to a different enzyme molecule. The study reported here on homogenates of growing and regressing chick mesonephroi indicates that acid phenylphosphatase and acid β-glycerophosphatase activities are associated with the same enzyme molecule.

The embryonic kidneys were homogenized in ice-cold distilled water, and then diluted with sodium acetate buffer (pH 5) to give 0.4% homogenates. The enzymatic activities of the untreated homogenates and homogenates preincubated for 1 h at 37 °C were then determined. The assay employed for β-glycerophosphatase activity was based on that described by FISKE and SUBBAROW[4]; that

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employed for phenylphosphatase activity was a modification of the KING-ARMSTRONG method (MOOG[5]). β-glycerophosphatase activities were determined at pH 4.5–4.6 and phenylphosphatase activities, at pH 5.2 since these were the optimal pH ranges for these enzymatic activities in growing and regressing chick mesonephroi.

As shown in the Table, acid β-glycerophosphatase activity appears to be an unstable enzyme because in mesonephroi from embryos at 13 days of incubation, there is a 42% inactivation of the enzyme after preincubation, and in mesonephroi from embryos at 17 days of incubation, 31% inactivation. With respect to acid phenylphosphatase activity, there is a 43% decrease after preincubation in mesonephroi from embryos at 13 days of incubation., and a 26% decrease in mesonephroi from embryos at 17 days of incubation. The results of this study on the effect of preincubation at 37 °C at pH 5 on the activities of acid β-glycerophostase and acid phenylphosphatase thus indicate that the same enzyme may be responsible for the hydrolysis of phenylphosphate and β-glycerophosphate, the proportion of the 2 enzymatic activities lost after heating being about the same at 13 days of incubation (growth period of mesonephros) and at 17 days of incubation (regressive period of mesonephros). Since this is not sufficient evidence to warrant the conclusion that the same enzyme is hydrolyzing phenylphosphate and β-glycerophosphate, further studies were performed that substantiate this view. The above results may also be due to the activities of 2 different enzymes which are both inactivated by heating at 37 °C for 1 h at pH 5. Since the activities were measured on crude homogenates, other factors may also have been involved.

The effect of the disodium salt of ethylene diaminetetraacetic acid (EDTA) on the hydrolysis of β-glycerophosphate and phenylphosphate was examined. WEBER and NIEHUS[6] had observed that EDTA lowers the K_m of acid β-glycerophosphatase in tail homogenates from growing and metamorphosing *Xenopus* larvae. WEBER, WEBER and NIEHUS[7] also observed lower Michaelis-Menten constants of acid β-glycerophosphatase activities in *Xenopus* liver extract and wheat germ in the presence of EDTA. These authors believe that the activation by EDTA might be a general characteristic of acid phosphatase. In chick mesonephroi, results were obtained that are not in agreement with this view. Figure 1 indicates that EDTA (10 or 1 m*M*) does not have any effect on the activity of this enzymatic activity during periods of mesonephric growth (Fig. 1a) and mesonephric regression (Fig. 1b). The K_m is the same (about 0.5 m*M* Na-β-glycerophosphate) with or without the addition of EDTA to the 0.4% (4 mg wet tissue/ml) homogenates in 0.25 *M* sucrose. Other studies indicate that the K_m of acid phenylphosphatase also is not affected by the addition of EDTA (10 or 1 m*M*) to the 0.4% homogenates in 0.25 *M* sucrose at

The effect of preincubation at pH 5 on the hydrolysis of phenylphosphate and β-glycerophosphate

	β-glycerophosphatase activity (μg phosphorus released/ml)		Phenylphosphatase activity (μg phenol released/ml)	
	13 days	17 days	13 days	17 days
Control	6.0	10.2	38.5	35.6
Preincubated at 37 °C for 1 h at pH 5	3.5	7.0	22.0	26.3
% loss due to heating	42	31	43	26

β-glycerophosphate activity determination: 0.25 ml of enzyme (4 mg wet tissue/ml) and 1.25 ml of substrate-buffer in final reaction mixture; substrate concentration, 20 m*M* Na-β-glycerophosphate; sodium acetate buffer, pH 4.5; 10 min reaction period, 37.5 °C. Phenylphosphate activity determination: 0.3 ml of enzyme (4 mg wet tissue/ml) and 1.5 ml of substrate-buffer in final reaction mixture; substrate concentration, 20 m*M* Na_2-phenylphosphate; sodium acetate buffer, pH 5.2; 10 min reaction period; temperature, 37.5 °C.

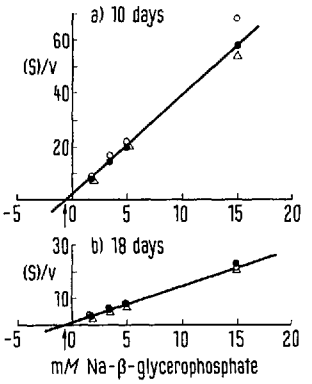

Fig. 1a and b. The effect of EDTA on acid β-glycerophosphatase activity in sucrose homogenates of chick mesonephroi at 10 and 18 days of incubation respectively. β-glycerophosphatase activity is expressed in μg phosphorus liberated. 0.25 ml of enzyme (4 mg wet tissue/ml) and 1.25 ml of substrate-buffer in the final reaction mixture; substrate, Na-β-glycerophosphate; sodium acetate buffer, pH 4.5; 10 min reaction period; temperature, 37.5 °C. The varying concentrations of Na-β-glycerophosphate are expressed on the abscissa, and (S)/v, where (S) is equal to the substrate concentration and v is equal to the velocity of reaction, is expressed on the ordinate. The following conditions were employed: o, 0.25 *M* sucrose; •, 0.25 *M* sucrose plus 1 m*M* EDTA; and △, 0.25 *M* sucrose plus 10 m*M* EDTA. Arrow indicates the $-(K_m)$ value. For description of graph, see DIXON and WEBB[8].

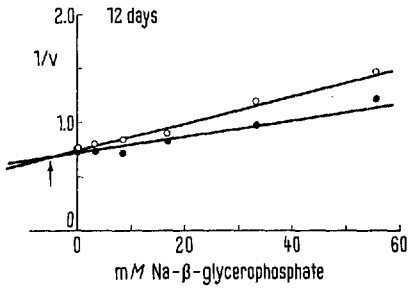

Fig. 2. The effect of β-glycerophosphate on the hydrolysis of phenylphosphate in aqueous homogenates of chick mesonephroi at 12 days of incubation. Arrow indicates point of intersection for the 2 lines determined at different substrate concentration: o, 20 m*M* phenylphosphate and •, 40 m*M* phenylphosphate. Abscissa: series of different inhibitor concentrations (Na-β-glycerophosphate). Ordinate: 1/velocity of reaction (v) which is expressed in μg phenol liberated/ml/min. Enzyme determination: 0.3 ml of enzyme (2 mg wet tissue/ml) and 1.5 ml of substrate-buffer in final reaction mixture; substrate, Na_2-phenylphosphate; sodium acetate buffer, pH 5.2; 10 min reaction period; temperature, 37.5 °C. For description of type of graph, see DIXON and WEBB[8].

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10 days and 18 days of incubation. The values for the K_m are about 1.0 mM phenylphosphate in the absence and presence of EDTA. The constancy of the K_m values for activity with phenylphosphate and β-glycerophosphate during growth and regression in the presence or absence of EDTA lends no support to the idea that these 2 substrates are hydrolyzed by 2 different enzymes.

The effect of β-glycerophosphate on the hydrolysis of phenylphosphate was also studied in order to determine if the hydrolyses of these 2 substrates are independent reactions or dependent reactions in some way. If the same enzyme molecule hydrolyzes 2 different substrates, the activity towards one substrate may be inhibited by the presence of the other. It is possible, then, to determine this inhibition by application of the Michaelis-Menten equation and the Lineweaver-Burk plotting method (DIXON and WEBB[8]). Fig. 2 indicates that the enzymatic liberation of phenol from phenylphosphate is competitively inhibited by β-glycerophosphate during growth (12 days of incubation). This was also observed at 17 days of incubation which is during mesonephric regression. The interpretation of these data is that the inhibitor (β-glycerophosphate) and the substrate (phenylphosphate) are competing for the same site on the same enzyme molecule.

Thus, from the above studies, it seems reasonable to assume that acid phenylphosphatase and acid β-glycerophosphatase activities are associated with the same enzyme molecule in homogenates of growing and regressing mesonephroi in chick embryos[9].

Résumé. L'activité de la phosphatase acide envers le β-glycérophosphate et le phénylphosphate a été comparée sous des conditions variées afin de constater si ces deux actions reflètent celle de molécules enzymatiques pareilles ou différentes pendant la croissance et la régression du mésonéphros chez l'embryon de poulet. Pour élucider cette question, on a étudié les effets de la température au pH 5, et du versène, et l'influence d'un substratum sur l'hydrolyse. Les résultats de ces recherches indiquent que la même enzyme pourrait être responsable de l'hydrolyse de ces 2 substrats.

VIRGINIA BROADDUS PATEL[10]

Biology Department, Washington University, St. Louis (Missouri USA), 13th April 1967.

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[10] Present address: Department of Biology, State University of New York at Buffalo, Buffalo (New York, USA).

Der Einfluss von 2-Deoxy-D-glucose auf die Substratoxydation in Ascites-Tumorzellen

Deoxyglucose hemmt das Wachstum und die Glykolyse[1] von Tumorzellen. Versuche mit Ascites-Carcinomzellen ergaben, dass Deoxyglucose in Anwesenheit hoher Glucosekonzentrationen die Milchsäurebildung partiell hemmt, die der Glykolyse folgende, über den Zitronensäure-Zyklus verlaufende Oxydation des Glucose-Kohlenstoffes hingegen stimuliert[2,3]. Die folgenden Experimente wurden mit der Absicht durchgeführt, einen Einblick in den Mechanismus zu erhalten, der bedingt, dass Glucose als oxydatives Substrat bevorzugt wird. Es wurde untersucht, in welcher Weise Deoxyglucose in Anwesenheit hoher Glucosekonzentrationen die Oxydation ^{14}C-markierter Substrate des Zitronensäure-Zyklus beeinflusst.

Experimentelles. Die Versuche wurden in der gleichen Weise durchgeführt, wie sie an anderer Stelle beschrieben wurde[3].

Resultate und Diskussion. Zunächst wurde untersucht, welchen Einfluss Deoxyglucose in Anwesenheit von Glucose auf die Oxydation von (2-^{14}C)- und (3-^{14}C)-Pyruvat hat. Glucose setzt, wie bekannt und wie auch aus Tabelle I zu ersehen ist, als kompetitives Substrat die Oxydation von exogenem Pyruvat herab. Sie hemmt auch die O_2-Aufnahme der Tumorzellen[4,5]. Wenn von jenen Werten ausgegangen wird, die bei Anwesenheit von Glucose und Pyruvat vorliegen, so zeigt sich, dass Deoxyglucose die Oxydation von Pyruvat C-2 und C-3 verhältnismässig mehr als die O_2-Aufnahme steigert. Deoxyglucose beeinflusst also die Oxydation von Pyruvat in gleichem Sinne wie die Oxydation von Glucose C-6 (ref. [3]) (Tabelle IV).

Es wurde in Betracht gezogen, dass Deoxyglucose durch Einflussnahme auf das NAD-abhängige Redoxsystem auf die Regulation der Substratoxydation einwirkt. Daher wurde untersucht, in welcher Weise Deoxyglucose die Oxydation des Succinats beeinflusst, eines Substrates, dessen Redoxreaktion im Zitronensäure-Zyklus an das Flavoprotein gekoppelt ist. Die Ergebnisse der Tabelle II zeigen, dass die Oxydation des Succinats C-1,4 zu CO_2 durch Deoxyglucose nicht stimuliert wird.

Tabelle I. Der Einfluss von Deoxyglucose auf die Pyruvatoxydation in Anwesenheit von Glucose

Zusätze	O_2-Aufnahme (μMole)[a]	μ Atome Pyruvat zu CO_2[a] C-2	C-3
Keine	15.6	2.63	1.96
Glucose	11.0	0.81	0.62
Glucose + Deoxyglucose	11.7 (+ 6%)	1.17 (+ 45%)	0.96 (+ 55%)

Pro Ansatz wurden 10^8 Ehrlich-Lettré-Ascites-Tumorzellen der Maus in Ca^{2+}-freiem Krebs-Ringer-Puffer (30 mM Phosphat, pH 7.4) im Warburg-Apparat inkubiert. Gasphase, Luft. Temperatur-37 °C. Endvolumen, 3 ml. Die Reaktion wurde durch Zukippen einer Lösung, welche die Substrate und die Deoxyglucose enthielt, gestartet und durch Zugabe von Säure beendet. Alle Ansätze enthielten 10 mM Pyruvat. Endkonzentration der übrigen Zusätze: 10 mM D-Glucose, 15 mM 2-Deoxy-D-glucose. [a] In Klammer: Änderung gegen die glucosehaltigen Ansätze.

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