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Comparative Studies on Glucose Phosphorylating Isoenzymes of Vertebrates

Identification and Characterization of Amphibian Liver Hexokinases[1]

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Glucose phosphorylating activities were measured in liver extracts from two urodeles and twenty-six anurans. Fractionation on diethylaminoethyl-cellulose columns of liver extracts from these amphibians permitted the recognition of four hexokinases which are called A, B, C, and D. However, any given amphibian displays only three liver hexokinases and the profiles so far observed are either of the type A-B-D or C-B-D. The distribution of the amphibians in either type of pattern does not show any simple taxonomic relationship. A wide generic and specific, but not individual, variation of the relative proportion of each isoenzyme was observed. Hexokinases A and B were shown to be low $K_{m\ glucose}$ isoenzymes (0.06 and 0.15 mM glucose, respectively) with normal hyperbolic kinetics. Hexokinase C, also a low K_m isoenzyme (0.05 mM) was found to be inhibited by excess substrate at physiological levels of glucose. Hexokinases A, B, and C were able to phosphorylate fructose, mannose, and 2-deoxyglucose at equal or higher rates than glucose when assayed at saturating sugar levels. Hexokinase D was found to be a high K_m isoenzyme ($K_{0.5} \simeq 2$ mM) with sigmoidal saturation curves for glucose (Hill coefficient $\simeq 1.6$). Fructose and mannose were also phosphorylated by this isoenzyme at about 70% of the glucose rate when studied at saturating sugar concentrations. The properties of the amphibian hexokinases are thus similar, although not identical, to those of mammalian hexokinases.

Previous reports from this laboratory have presented evidence for the presence of multiple glucose phosphorylating enzymes (ATP:D-hexose 6-phosphotransferases, EC 2.7.1.1; trivial name, hexokinases) in the liver of mammals (2-5), birds (6, 7), and reptiles (1). A minimum of two and a maximum of four such phosphotransferases (hexokinases A, B, C, and D)[4] have been found in those vertebrates. The relative proportions, adaptive features, and chromatographic and kinetic properties of these isoenzymes show striking differences in some taxa (for reviews see Ureta (8) and Niemeyer *et al.* (10)).

A few reports have dealt with this isoenzymic system in amphibians. Sharma *et al.* (11) and Lauris and Cahill (12) were able to detect a high $K_{m\ glucose}$ phosphotransferase (isoenzyme D) in crude extracts from frog liver. The presence of this so-called glucokinase[4] in the liver of *Rana catesbeiana* was documented by starch gel electrophoresis by Pilkis *et al.* (13). Also, an additional glucose phosphotransferase

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[4] Nomenclatural aspects have been discussed elsewhere (8, 9).

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of the low $K_{m\ glucose}$ type was observed by the same procedure (13). The kinetics and allosteric regulation of a low K_m isoenzyme from the liver of the leptodactylid frog *Calyptocephalella caudiverbera* has been recently reported (9).

We have now expanded the knowledge of the amphibian glucose phosphotransferases through the study of their levels, number, and relative proportions in the liver of 26 anuran and two urodelan species. In some of these animals the chromatographic procedure used for the assessment of isoenzymic profiles yielded enzyme preparations with high enough activity to study several parameters which allow a fair comparison with previously reported data on vertebrate hexokinases (for reviews see 8, 10, 14–19). Preliminary reports have been previously published in abstract form (20–22).

MATERIALS AND METHODS

Materials. ATP, ADP, NADP, NADH, pyruvate kinase (containing lactate dehydrogenase), phospho*enol*pyruvate, glucose 6-P dehydrogenase, and Sephadex G-100 and G-200 were obtained from Sigma Chemical Co. DEAE-cellulose[5] (DE-52) was a Whatman product. Uniformly labeled [^{14}C]- and [^{3}H]glucose were purchased from New England Nuclear. Phosphogluconate dehydrogenase was prepared from rat liver as previously described (4). Other reagents were of the highest purity commercially available.

Animals. The species used and their classifications and sources are listed in Table I. Whenever possible the animals were sacrificed shortly after capture. In a few cases, however, animals were kept in a terrarium or aquarium and forcibly fed small pieces of rat liver.

Enzyme assay. Hexokinase activity was measured by one of the following procedures:

(a) Measurements of glucose 6-P formation were made by coupling the hexokinase reaction with $NADP^+$ and an excess of glucose 6-P dehydrogenase and 6-phosphogluconate dehydrogenase. NADPH formation was followed at 340 nm in a Gilford spectrophotometer thermostatted at 30°C in a medium containing (final concentrations): 10 mM KCl, 100 mM Tris–HCl buffer (pH 7.5), 12.6 mM $MgCl_2$, 5 mM ATP, 1.6 mM EDTA, 0.5 mM $NADP^+$, an excess of both dehydrogenases, and 50 to 100 μl of suitably diluted enzyme preparation. Usually, two glucose concentrations (100 and 0.5 mM) were used. Assay systems with ATP omitted were used as blanks. When analyzing column effluents 6-phosphogluconate dehydrogenase was omitted from the assay mixtures.

(b) Small amounts of enzymes, such as occur during chromatography of liver extracts from very small animals, were measured by the radioassay of Radojković *et al.* (23) using labeled glucose as substrate. The reaction mixtures were as described in method a except for the omission of the dehydrogenases and $NADP^+$ and the inclusion of 0.5 mM glucose containing about 500,000 cpm of labeled sugar and enzyme in a final volume of 0.5 ml. After incubation, the labeled product was separated from unreacted glucose by filtration on small Dowex-1 columns.

(c) Measurement of ADP formation was made by coupling the phosphotransferase reaction to the pyruvate kinase and lactate dehydrogenase system. NADH oxidation was followed at 340 nm in a Gilford spectrophotometer. A system without substrate was used as a blank. One unit of ATP:hexose phosphotransferase activity is defined as the amount of enzyme catalyzing the phosphorylation of 1 μmol of glucose in 1 min at 30°C.

Preparation of liver extracts. The animals were decapitated without anesthesia, thoroughly bled, demedullated, and the liver excised, weighed, and placed in cracked ice. Ten percent homogenates were prepared with a small piece of liver in a Potter-Elvehjem apparatus in a medium containing 100 mM KCl, 50 mM Tris–HCl buffer (pH 7.5), 6 mM EDTA, and 6 mM $MgCl_2$ (buffer A). High-speed supernatant fluids were obtained by centrifugation at 105,000*g* in a Spinco ultracentrifuge for 60 min. Aliquots of the homogenates and of the supernatant liquids were assayed for glucose phosphorylating activity as described above. The total homogenate was treated with Triton X-100 (final concentration, 0.05%) before being assayed.

Separation of isoenzymes. The liver was homogenized in 1 vol of 10 mM Tris–HCl (pH 7), 1 mM EDTA, 1 mM dithiothreitol, and 100 mM glucose (buffer B). The homogenates were centrifuged at 105,000*g* for 60 min. The isoenzymes in the supernatant liquid were resolved by chromatography on DEAE-cellulose columns using a linear gradient of KCl from 0 to 0.5 M in a solution similar to buffer B except for the omission of glucose. Fractions were collected in a Gilson microfractionator and assayed for enzyme activity. The linearity of the gradients was checked by conductimetry. Two different columns were used throughout this study: (a) A column made from a Becton-Dickinson 2-ml syringe for up to 1 g of liver. This column will be referred to as the 2-ml column. Fractions of 300 μl were collected. The gradient volume was 34 ml. (b) A column (0.63 × 19 cm) hereafter referred to as the 14-ml column. Gradient volume was 240 ml and fractions of 2 ml

[5] Abbreviations used: DEAE-, diethylaminoethyl-; EDTA, ethylenediaminetetraacetate.

TABLE I

GENERAL CHARACTERISTICS OF THE AMPHIBIANS STUDIED[a]

Species	Body weight (g)	Liver weight (g)	Liver protein (mg/g)	Liver glycogen (g/100 g)	Source[b]
Caudata					
Taricha torosa (3)	8.6 ± 0.7	0.42 ± 0.06	159 ± 18	8.3 ± 0.6	California[1]
Ambystoma mexicanum (4)	94.0 ± 14	2.64 ± 0.19	183 ± 22	4.6 ± 0.8	Mexico[2]
Anura					
Pipidae					
Xenopus laevis (3)	77.0 ± 20	2.62 ± 0.85	234 ± 16	8.6 ± 0.8	California[3]
Leptodactylidae					
Ceratophrys ornata (1)	176.0	3.28	143	2.1	Tucumán[4]
Calyptocephalella caudiverbera (14)	141.3 ± 16	3.42 ± 0.73	345 ± 16	5.1 ± 1.7	Santiago[5, 6]
Telmatobius halli (5)	7.5 ± 0.6	0.11 ± 0.01	223 ± 9	1.9 ± 0.3	Bolivia[7]
Telmatobius peruvianus (2)	9.6; 13.1	0.25; 0.43	–	–	Bolivia[7]
Batrachyla taeniata (4)	2.3 ± 0.6	0.27 ± 0.08	–	–	Isla Mocha, Chile[8]
Batrachyla leptopus (6)	2.9 ± 0.4	0.05 ± 0.007	–	–	Llanquihue, Chile[6, 7]
Eupsophus roseus (4)	7.1 ± 1.0	0.15 ± 0.01	328 ± 24	1.0 ± 0.2	Santiago[5, 6]
Eupsophus vertebralis (1)	3.9	0.06	–	–	Llanquihue, Chile[6]
Alsodes nodosus (4)	14.4 ± 2.0	0.29 ± 0.05	178 ± 21	4.3 ± 0.6	Santiago[5, 6]
Alsodes monticola (3)	16.2 ± 3.2	0.32 ± 0.09	–	–	Llanquihue, Chile[6]
Odontophrynus americanus (4)	11.2 ± 0.5	0.19 ± 0.02	246 ± 18	3.9 ± 0.5	Sao Paulo[10]
Pleurodema thaul (10)	4.7 ± 0.7	0.14 ± 0.02	345 ± 49	0.1 ± 0.03	Santiago[5, 6]
Pleurodema bufonina (3)	9.6 ± 1.1	0.24 ± 0.01	–	–	Maule, Chile[6]
Leptodactylus chaquensis (3)	33.3 ± 7.3	0.76 ± 0.34	263 ± 37	3.4 ± 0.9	Tucumán[4]
Leptodactylus bolivianus (1)	43.1	0.97	334	1.5	Caracas[11]
Bufonidae					
Bufo spinulosus (15)	48.3 ± 4.2	1.70 ± 0.20	361 ± 23	5.6 ± 1.1	Santiago[5]
Bufo arenarum (3)	117.0 ± 6.0	5.05 ± 1.04	113 ± 10	4.6 ± 0.6	Tucumán[4]
Bufo marinus (2)	96; 99	1.77; 1.99	280; 206	9.7; 9.9	Sao Paulo[10]
Bufo paracnemis (1)	117	2.10	288	7.3	Tucumán[4]
Bufo rubropunctatus (2)	15.1; 13.6	0.34; 0.27	181; 187	6.3; 4.3	Concepción, Chile[6, 9]
Bufo variegatus (2)	4.2; 4.1	0.13; 0.10	–	–	Llanquihue, Chile[6]
Bufo atacamensis (2)	33.6; 36.5	0.70; 0.92	–	–	La Serena, Chile[6]
Rhinodermatidae					
Rhinoderma darwinii (2)	2.0; 1.4	0.05; 0.02	–	–	Valdivia, Chile[6, 12]
Ranidae					
Rana pipiens (4)	31.2 ± 1.0	0.71 ± 0.06	124 ± 10	4.7 ± 1.0	Caracas[13]
Hylidae					
Hyla punctata (2)	6.9; 6.8	0.15; 0.15	–	–	Bogotá[14]
Hyla pulchella (2)	1.1; 1.2	–	–	–	Montevideo[7]

[a] The results are given as the means ± SE. Figures in parentheses indicate the number of animals in each group. A dash indicates measurement not performed.

[b] Notes: 1, A gift from Dr G. Sato (La Jolla, California); 2, Courtesy of Dr. C. Mohar (Universidad Nacional de Méjico); 3, purchased from the Hermosa Reptile Farm, California; 4, donated by Dr. F. D. Barbieri (Universidad Nacional de Tucumán, Argentina); 5, purchased from professional collectors; 6, captured by one of us (N.D.); 7, contributed by Dr. A. Veloso (Universidad de Chile); 8, a gift from Dr. J. Pefaur (Universidad de Chile); 9, courtesy of Dr. R. DonosoBarros (Universidad de Concepción, Chile); 10, donated by Dr. W. Becak (Istituto Butantán, Sao Paulo); 11, courtesy of Dr. G. Scorza (Universidad Central de Carcas); 12, contribution of Dr. R. Formas (Universidad Austral de Chile); 13, from a colony kept at IVIC, Caracas; 14, courtesy of Dr. J. Peñaranda (Universidad de Bogotá).

were collected. Extremely good reproducibility of the elution patterns was always observed when the same extract was chromatographed separately in the two columns.

Characterization of isolated isoenzymes. Hexokinase preparations used were enzyme fractions from the DEAE-cellulose columns employed for the assessment of isoenzyme patterns. In some cases, active fractions were pooled and used without further purification for the measurement of kinetic parameters. Dilute preparations were concentrated with an Amicon ultrafilter apparatus. Since the toad *B. spinulosus* and the frog *C. caudiverbera* are easily available Chilean species, enzyme fractions from those animals were isolated in preparative DEAE-cellulose columns and further purified by gel filtration on Sephadex G-100 or G-200 columns. Due to the peculiar kinetic features of the hexokinase isoenzymes its individual levels in crude supernatant liquids cannot be measured even approximately. Therefore, purification factors or yields cannot be ascertained. Specific activities of the different isoenzymes varied widely, the highest being those of hexokinase D and the lowest those of hexokinase A. The absence of enzymes which could interfere in the enzyme assays was checked in all preparations by the use of proper blanks.

Sugar substrate specificity studies of the different hexokinases were performed at 100 mM sugar concentrations (except in the case of hexokinase C) by estimation of the rate of ADP formation (assay procedure c).

Molecular weight measurements of hexokinase D were performed by gel filtration through calibrated Sephadex G-100 columns. In a few cases, sucrose gradient centrifugation according to Martin and Ames (24) was employed using yeast alcohol dehydrogenase as standard.

Other assays. Protein was measured by the method of Miller (25) and glycogen by the procedure of Montgomery (26).

RESULTS AND DISCUSSION

General Characteristics of the Amphibians Studied

Body and liver weight and protein and glycogen content of the liver homogenates from the animals studied are listed in Table I.

Enzyme Levels in Crude Extracts

As happens in other vertebrates (1, 4, 7, 13), glucose phosphotransferase levels varied widely in the livers of amphibians (Table II). The highest activity levels in the supernatant liquids were observed in *P. thaul* males (5.5 units/g of liver), a value which is higher than those found in fed rats (about 2.5 units/g). The lowest activities were recorded in *C. ornata* (0.27 units/g). Values of 1.1 units/g have been recently reported by Cristea (27) for *Rana esculenta*. No relationship was observed between glucose phosphotransferase levels and the body or liver size and glycogen content of the liver or the taxonomic classification of the animals.

The glucose phosphorylating activity from amphibian liver seems to be exclusively located in the soluble cytoplasm (Table II). In fact, the activity levels measured in high-speed supernatant liquids were usually higher than those found in the corresponding homogenates, which suggests the presence of inhibitors that are removed by centrifugation. The only exception was found in the individual *T. peruvianus* studied in which only 50% of the activity was recovered in the high-speed supernatant fluid. The problem was not further explored. It is interesting to recall that the glucose phosphotransferases from mammalian liver are usually recovered in the soluble fraction. Contrariwise, in most avian and reptilian species a sizeable fraction of glucose phosphorylating activity remains in the centrifuged pellet (1, 7).

Parallel assays of the enzyme activity of crude extracts at a high (H = 100 mM) and low (L = 0.5 mM) glucose concentrations, a procedure widely used to detect high K_m glucose phosphotransferase activity (28), revealed that the majority of amphibians do have such a high K_m enzyme since H/L ratios well above unity are seen (Table II). In general, the H/L ratios observed are not as high as those observed in most mammals (about 10), which suggests a relatively lower proportion of a high K_m isoenzyme in the amphibian liver.

Enzyme activity levels were about the same in animals of different sex. Nevertheless, *P. thaul* males displayed significantly ($P < 0.0005$) higher enzyme levels than females (Table II). A similar difference was observed by Ureta *et al.* (4) in the case of hamsters (*Mesocricetus auratus*).

TABLE II

GLUCOSE PHOSPHORYLATING ACTIVITY IN LIVER HOMOGENATES AND SUPERNATANT FLUIDS OF SEVERAL AMPHIBIAN SPECIES[a]

Species	Homogenates		Supernatant fluid		Percentage in supernatant fluid
	units/g of liver	H/L	units/g of liver	H/L	
T. torosa (3)	0.96 ± 0.12	1.99 ± 0.09	0.87 ± 0.08	2.48 ± 0.22	93 ± 7
A. mexicanum (4)	2.03 ± 0.41	1.53 ± 0.21	3.00 ± 0.06	1.52 ± 0.16	161 ± 33
X. laevis (3)	0.85 ± 0.17	2.68 ± 0.50	1.21 ± 0.44	3.58 ± 0.77	136 ± 30
C. ornata (1)	–	–	0.27	1.40	–
C. caudiverbera (14)	0.60 ± 0.09	1.12 ± 0.10	0.79 ± 0.09	1.41 ± 0.08	132 ± 6
T. peruvianus (1)	1.31	1.58	0.66	1.43	50
B. taeniata (1)	0.59	2.62	0.59	2.73	100
B. leptopus (2)	1.37; 0.24	2.34; 1.21	1.37; 0.48	2.31; 2.60	100; 200
E. roseus (1)	–	–	2.18	–	–
E. vertebralis (1)	2.05	1.83	3.04	2.81	148
A. nodosus (4)	1.46 ± 0.15	4.56 ± 0.32	1.71 ± 0.10	4.67 ± 0.25	119 ± 5
A. monticola (1)	–	–	1.89	1.48	–
O. americanus (2)	1.23; 1.17	1.35; 1.78	1.25; 1.08	1.39; 1.71	102; 92
P. thaul (5) male	2.89 ± 0.19	7.02 ± 0.56	5.48 ± 0.27	7.60 ± 0.68	190 ± 18
(5) female	1.53 ± 0.14	7.06 ± 0.48	2.91 ± 0.08	7.00 ± 0.62	190 ± 13
P. bufonina (1)	0.27	1.52	1.01	1.65	374
L. chaquensis (3)	–	–	3.06 ± 0.47	3.15 ± 0.13	–
L. bolivianus (1)	0.99	1.72	1.61	1.75	95
B. spinulosus (7)	1.47 ± 0.09	2.73 ± 0.14	1.55 ± 0.09	3.30 ± 0.22	106 ± 3
B. arenarum (3)	0.43 ± 0.13	1.27 ± 0.19	0.59 ± 0.14	1.32 ± 0.24	140 ± 9
B. marinus (2)	1.05; 1.14	2.28; 1.65	1.29; 1.01	3.00; 2.02	123; 89
B. paracnemis (1)	0.81	2.03	1.06	2.26	131
B. rubropunctatus (1)	1.73	2.03	1.93	2.06	112
B. variegatus (1)	0.29	1.00	0.70	1.11	241
B. atacamensis (1)	0.55	1.29	1.86	3.92	338
R. pipiens (4)	1.31 ± 0.06	4.08 ± 0.97	1.29 ± 0.09	3.38 ± 0.32	98 ± 6
H. punctata (1)	–	–	2.19	4.35	–

[a] Suitably diluted aliquots from homogenates and supernatant liquids were assayed for enzyme activity at 100 and 0.5 mM glucose using method (a) described under Materials and Methods. The results under "units/g of liver" are the values obtained at 100 mM glucose. The ratio of the activities measured at both concentrations of substrate is expressed as "H/L". The results are given as means ± SE. Figures in parentheses indicate the number of animals studied. Dashes indicate measurements not performed.

Since the levels of glucose phosphotransferases in several, but not all, rodents diminish markedly upon fasting (4) the possible effect of short-term food deprivation on the liver glucose phosphorylating activity was studied in a group of toads (*B. spinulosus*) captured at the same time and place (Table III). Enzyme activity and liver glycogen levels were not affected after 96 h of fasting.

Chromatographic Studies

The isoenzymic patterns presented in Appendix Figs. 1 to 9 are typical results obtained by DEAE-cellulose chromatography of crude supernatant liquids from 28 amphibian species. The profiles have been grouped together according to the taxonomic classification listed in Table I. Whenever replicate chromatographic runs were possible, the same patterns were always observed except for minor quantitative variations.

A cursory glance at Appendix Figs. 1 to 9 reveals the presence of three glucose phosphorylating isoenzymes in all the amphibian species studied. The major qualitative difference within the chromatographic patterns resides in the chromatographic mobility and kinetic properties of the first-eluting glucose phosphotransferase. Thus, in the species listed in Fig. 1, bottom (see also Appendix Figs. 4 to 7) this isoenzyme was found to elute at about

TABLE III

THE EFFECT OF FASTING ON THE LIVER GLUCOSE PHOSPHOTRANSFERASES FROM *Bufo spinulosus*[a]

Condition	Body weight (g)	Liver weight (g)	Phosphotransferase activity		Glycogen (%)	Protein (mg/100 g body wt)
			100 mM Glucose (units/100 g body wt)	0.5 mM Glucose (units/100 g body wt)		
Fed controls (12)	75 ± 5	2.3 ± 0.2	3.09 ± 0.13	1.62 ± 0.10	6.3 ± 1.1	399 ± 28
Fasted 48 h (3)	59 ± 2	1.9 ± 0.1	3.37 ± 0.29	1.28 ± 0.18	9.3 ± 1.3	476 ± 45
Fasted 72 h (4)	73 ± 5	2.0 ± 0.1	3.65 ± 0.29	1.68 ± 0.14	9.9 ± 0.5	507 ± 46
Fasted 96 h (4)	72 ± 6	2.0 ± 0.2	3.20 ± 0.15	1.75 ± 0.22	4.2 ± 0.7	486 ± 94

[a] All animals were forcibly fed about 500 mg of rat liver daily to make their nutritional status uniform. After 6 days some toads were fasted (water *ad libitum*) and groups of three to four animals were sacrificed after 48, 72, or 96 h. Fed, control animals were sacrificed after the same intervals. Very similar enzyme levels were found in these control toads; therefore, the results were pooled. Glucose phosphotransferase activities in high-speed supernatant fluids were measured using method (a) at two glucose concentrations (100 and 0.5 mM) and are expressed as the total activity of the liver per 100 g body wt. Protein levels were measured in whole homogenates and expressed as milligrams in the whole liver per 100 g body wt. Glycogen was measured in homogenates. The results are given as the means ± SE. Figures in parentheses indicate the number of animals in each group.

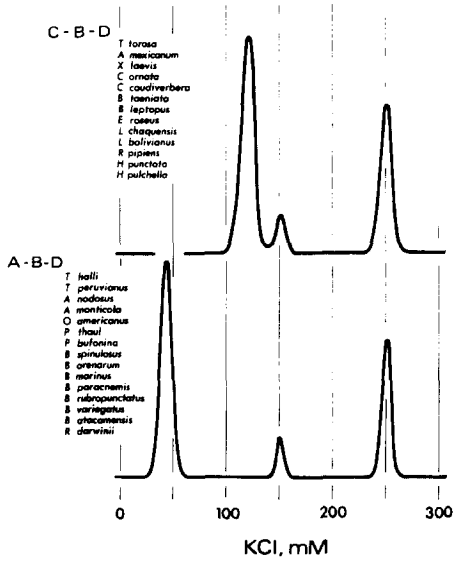


FIG. 1. A summary of the glucose phosphotransferases chromatographic patterns of the class Amphibia. Mean chromatographic mobilities (KCl concentration at the tube with maximal activity of each isoenzyme, cf. Table IV) of the amphibian hexokinases are depicted. The idealized activity profiles shown do not represent any particular species but a rather approximate average of the relative proportion of the isoenzymes (see Appendix Figs. 1 to 9). *Top*: The C-B-D pattern. *Bottom*: The A-B-D pattern.

40 mM KCl, whereas in the species listed in Fig. 1, top (see also Appendix Figs. 1 to 5, 8 and 9) the first isoenzyme came off the column at about 114 mM KCl. In addition to the different chromatographic mobilities, these first-eluting enzymes displayed quite distinct kinetic properties: The isoenzyme which elutes at 40 mM KCl (isoenzyme A) presented normal hyperbolic saturation curves for glucose while the isoenzyme eluting at 114 mM KCl was usually[6] strongly inhibited by glucose concentrations within physiological levels (isoenzyme C). None of the animals (75 chromatographic runs) showed both the enzyme eluting at about 40 mM KCl and the substrate-inhibited enzyme eluting at about 114 mM KCl.

The second-eluting isoenzyme in all the animals studied was present in very small amounts and no characterization was possible except to ascertain that it is a low $K_{m\ \text{glucose}}$ enzyme with straight hyperbolic kinetics (see below). Its chromatographic mobility was about 150 mM KCl. This phosphotransferase will be referred to as hexokinase B inasmuch as its properties are very similar to those of mammalian hexokinase B.

[6] In the case of a few small animals (*T. torosa*, *B. taeniata*, *E. roseus*, *L. bolivianus*, *H. punctata*, *H. pulchella*) the enzyme activity eluting at 120 mM KCl was insufficient to ascertain the presence and extent of inhibition by excess substrate. Also, it must be borne in mind that the inhibition by glucose, at least in the case of the isoenzyme from *C. caudiverbera*, cannot be observed when assaying diluted enzyme preparations (9).

The last fraction usually came off the columns at about 260 mM KCl and it is always a high K_m isoenzyme (about 2 mM glucose). Other properties are very similar to those of isoenzyme D from mammals although a few differences are apparent (see below). Because of the resemblances to the mammalian "glucokinase" this isoenzyme will be referred to as isoenzyme D.

A summary of the chromatographic mobilities of the isoenzymes in the several amphibians studied is presented in Table IV.

The relative proportion of each isoenzyme in the different species studied varied widely (Appendix Figs. 1 to 9). Animals in which isoenzyme A is present showed that isoenzyme is the by far predominant form, with the sole exception of *R. darwinii*. In the case of animals in which hexokinase C is present, the relative proportions of isoenzymes C and D were very variable. In all cases, isoenzyme B was present in trace amounts.

TABLE IV

CHROMATOGRAPHIC MOBILITIES OF THE GLUCOSE PHOSPHOTRANSFERASES FROM AMPHIBIAN LIVER[a]

Isoenzyme	KCl (mM)
A (39)	42 ± 7
C (36)	114 ± 32
B (75)	147 ± 11
D (75)	258 ± 20

[a] Chromatographic mobility is defined as the concentration (mM) of KCl at the tube of DEAE-cellulose columns with maximal activity of the isoenzyme. The results of observations performed on 28 species of amphibians were pooled. Figures in parentheses indicate the number of chromatographic runs. The results are given as the means ± SE.

Characterization of Amphibian Hexokinases

Hexokinase A. Substrate specificity: Hexokinase A preparations (i.e., the isoenzyme eluting at about 40 mM KCl) for *B. spinulosus* were able to phosphorylate several sugars (Table V). The highest maximal velocities were obtained with fructose and 2-deoxyglucose. Hyperbolic saturation curves were observed with all sugars tested (not shown). The lowest apparent K_m value was found with glucose (0.063 mM). Michaelis constant values for glucose were also measured with hexokinase A preparations from *O. americanus* (0.065 mM) and from *T. halli* (0.054 mM). In these cases, the enzyme preparations were the peak tubes of hexokinase A activity from the DEAE-cellulose columns.

Hexokinase B. Substrate specificity: Relative maximal velocities of the *B. spinulosus* isoenzyme B (i.e., the second-eluting isoenzyme) against several sugars are shown in Table V. The rate of fructose phosphorylation was about the same as that of glucose but the $K_{m\ \text{fructose}}$ value was 100-fold higher. The velocity of phosphorylation of 2-deoxyglucose and of mannose was much higher than that of fructose or glucose. Due to the insufficient amount of

TABLE V

SUBSTRATE SPECIFICITY OF HEXOKINASE A AND B FROM *Bufo spinulosus*

Sugar	Hexokinase A		Hexokinase B	
	V_{rel}[a]	K_m (mM)[b]	V_{rel}[a]	K_m (mM)[b]
D-Glucose	1.00	0.063	1.00	0.150
D-Fructose	1.62	9.8	1.14	15.2
2-Deoxy-D-glucose	1.44	0.28	1.84	–[c]
D-Mannose	1.04	0.54	1.74	–[c]
D-Glucosamine	0.08	–[c]	0.10	–[c]
D-Galactose	<0.05	–[c]	0.14	–[c]
N-Acetyl-D-glucosamine	<0.05	–[c]	<0.05	–[c]

[a] $V_{hexose}/V_{glucose}$. All substrates at 100 mM concentration. ATP level was 5 mM. Assay procedure was method c described under Materials and Methods. Fructose phosphorylation was also tested with method a with the addition of phosphoglucose isomerase.

[b] Calculated from double-reciprocal plots.

[c] Measurements not performed.

available material no K_m values could be measured for 2-deoxyglucose and mannose.

Saturation curves for glucose of hexokinase B from the liver of several amphibians were always hyperbolic. Apparent K_m values were 0.090 mM (*R. pipiens*), 0.093 mM (*L. chaquensis*), 0.068 mM (*L. bolivianus*), and 0.069 mM (*X. laevis*). No other characterization was possible.

Hexokinase C. Most of the measurements on hexokinase C (i.e., the isoenzyme eluting at about 114 mM KCl) to be reported were obtained with enzyme preparations from the liver of *C. caudiverbera*. A detailed account of studies on the substrate inhibition of hexokinase C has been recently reported (9).

Substrate specificity: The most outstanding feature on hexokinase C is the inhibition of the enzyme activity by relatively high concentrations (above 0.2 mM) of sugar substrates (Fig. 2). Velocities at 0.2 and 100 mM sugar concentrations showed inhibition of about the same extent by the higher hexose level with glucose, mannose, and 2-deoxyglucose (not shown). Fructose displayed a quite different behavior since inhibition of its own phosphorylation did not occur at any concentration up to 100 mM, the highest level studied.

Saturation curves for glucose at three fixed levels of ATP are shown in Fig. 2, top. The shaded area in the figure corresponds to an estimation of amphibian blood glucose levels (29). From the data of Fig. 2, a K_m value of 0.034 mM glucose can be estimated using the data points obtained at presumably noninhibitory sugar concentrations (Fig. 2, bottom). The Michaelis constant for fructose was 1.25 mM. No data are available for mannose and 2-deoxyglucose.

ATP saturation curves of hexokinase C from *C. caudiverbera* are nonlinear at any glucose concentration (9). The $K_{0.5}$ value (ATP concentration at observed half-maximal velocity) at 0.2 mM glucose is 1 mM (9). Other nucleoside triphosphates (GTP, CTP, ITP, TTP) were not phosphoryl donors (not shown).

Michaelis constant values for glucose

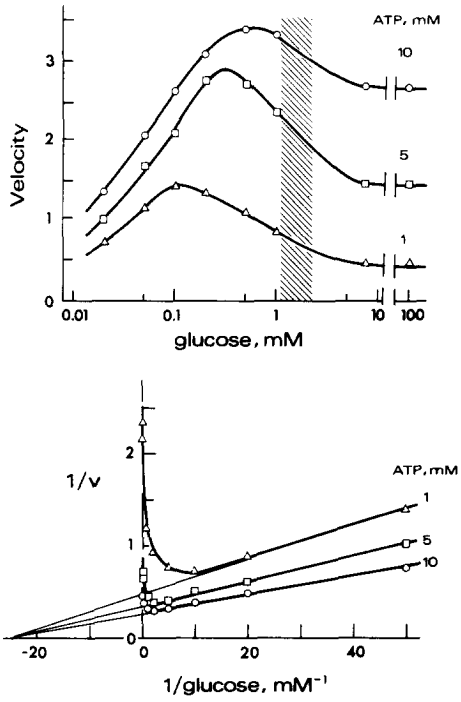


FIG. 2. The effect of glucose concentration on the velocity of hexokinase C from the liver of *C. caudiverbera*. The enzyme preparation was obtained from a DEAE-cellulose column and further purified by Sephadex G-200 gel filtration. Enzyme assay was the spectrophotometric procedure a as described under Materials and Methods. Three fixed ATP concentrations were used as indicated. $MgCl_2$ levels were 1 mM in excess over the nucleoside triphosphate. Velocity is expressed as milliunits per milliliter of enzyme preparation. *Top*: Direct representation. The shaded area indicates the range of normal blood glucose concentrations in amphibians (see text). *Bottom*: Double-reciprocal plot of the same data.

were measured using hexokinase C preparations from *A. mexicanum, X. laevis, L. chaquensis, L. bolivianus,* and *R. pipiens*. The values obtained were similar (0.044 ± 0.005 mM) to those of the enzyme from *C. caudiverbera*.

Hexokinase D. As already stated in the introduction, the presence of hexokinase D in amphibian liver has long been known but, to the best of our knowledge, no description of its properties has been published. The most extensive studies have been performed with the enzyme from *C. caudiverbera* and from *B. spinulosus* but some analyses have been also possible

with enzyme fractions from *A. mexicanum, X. laevis, R. pipiens, C. ornata,* and *L. bolivianus.*

Substrate specificity: Glucose was the best phosphoryl acceptor although fructose and mannose were also phosphorylated at a velocity of about 70% that of glucose (Table VI). 2-Deoxyglucose was about 40% as effective as glucose. This substrate specificity pattern of amphibian hexokinase D would be very similar to that of mammalian hexokinase D were it not for the fact that fructose phosphorylation with the amphibian enzyme is about 70% that of glucose. In the case of the rat enzyme fructose phosphorylation is not higher than 20% of glucose (Table VI) (2, 3, 30, 31).

TABLE VI

SUBSTRATE SPECIFICITY OF AMPHIBIAN AND RAT LIVER HEXOKINASES D[a]

Sugar	$V_{hexose}/V_{glucose}$	
	X. laevis[b]	Rat[c]
D-Glucose	1.00	1.00
D-Fructose	0.70	0.20
D-Mannose	0.64	0.82
2-Deoxy-D-glucose	0.31	0.39
D-Galactose	<0.05	<0.06

[a] All substrates at 100 mM concentration. ATP level was 5 mM. Assay was method c. Fructose phosphorylation was tested also with method a with the addition of phosphoglucose isomerase.

[b] Measurements with hexokinase D from *C. caudiverbera, B. spinulosus,* and *R. pipiens* gave identical results.

[c] Data from González *et al.* (3).

Figure 3, left, shows saturation curves for glucose of hexokinase D from *C. caudiverbera* at two fixed ATP concentrations. Sigmoidal curves were obtained as also seen in Fig. 3, right, in which the data are shown as Hill plots. Hill slopes (n_H) of 2.32 and 1.77 were obtained at 10 and 1 mM ATP, respectively. The $K_{0.5}$ values of about 1.4 mM glucose were calculated in both cases. Sigmoidal saturation curves for glucose at 5 mM ATP were also obtained with hexokinase D preparations from *A. mexicanum, X. laevis,* and *C. ornata* (Table VII). In these cases, n_H values were lower than those obtained in the experiment illustrated in Fig. 3. $K_{0.5}$ values of about 1.5 mM were obtained in all cases. The observation of the sigmoidicity of the saturation curve for glucose displayed by hexokinase D from the several amphibians confirms and expands a previous report by Niemeyer *et al.* (32). At least in the case of *C. caudiverbera* hexokinase D, the dependency of the sigmoidicity on the ATP concentration seems to be more marked than in the rat enzyme (33, 34) which may point to frogs as the best enzyme source for the study of the mechanism of the sigmoidicity. Besides its

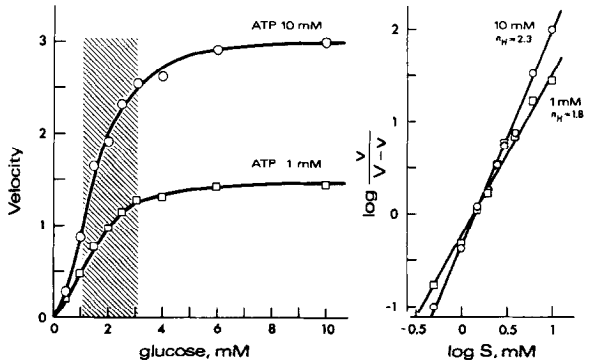


FIG. 3. Saturation functions for glucose at two fixed ATP concentrations of hexokinase D from *C. caudiverbera*. The enzyme preparation was obtained by DEAE-cellulose chromatography and further purified by Sephadex G-100 gel filtration. Enzyme assay was procedure a described under Materials and Methods. $MgCl_2$ was present in 1 mM excess over the indicated ATP concentrations. Velocity is expressed as milliunits per milliliter of enzyme preparation. *Left*: Direct representation. The shaded area represents an estimation of the normal blood sugar concentrations in amphibians (see text). *Right*: Hill plot of the same data.

TABLE VII

HILL COEFFICIENTS (n_H) AND $K_{0.5}$ VALUES FOR GLUCOSE OF HEXOKINASE D FROM AMPHIBIAN LIVER[a]

Species	n_H	$K_{0.5}$ (mM)
A. mexicanum	1.62	3.4
X. laevis	1.76	2.6
C. ornata	1.70	2.6
C. caudiverbera	1.77	1.4

[a] Velocities were measured with procedure a as described under Materials and Methods. ATP and $MgCl_2$ concentrations were 5 and 6 mM, respectively. Enzyme preparations were active fractions from DEAE-cellulose chromatograms. Nine glucose concentrations ranging from 0.5 to 10 mM were used.

relevance for the regulation of glucose utilization, the sigmoidal behavior of hexokinase D has the additional interest of being one of a very few cases of sigmoidicity in an enzyme which is monomeric and remains so in the assay conditions (34).

On the other hand, the variation of fructose concentration on the velocity of hexokinase D resulted in hyperbolic plots at three different fixed levels of ATP (data not shown). Replots of slopes and intercepts gave a $K_{m\ \text{fructose}}$ value of 0.43 M.

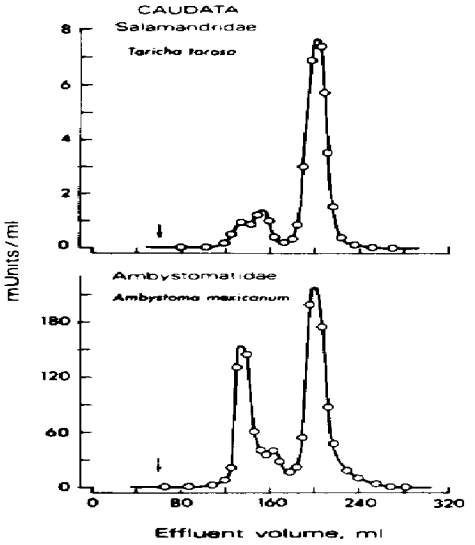
Michaelis constant values for ATP in the presence of 100 mM glucose were also measured (data not shown). Hyperbolic curves were obtained with an apparent $K_{m\ \text{ATP}}$ value of 2 mM, a value which is four times higher than the one measured with the rat enzyme (30, 31, 33). A parallel experiment using 100 mM fructose as the fixed level phosphate acceptor resulted also in a $K_{m\ \text{ATP}}$ value of 2 mM (not shown).

Apparent molecular weight: Sephadex G-100 filtration through a calibrated column (2 × 50 cm) was performed with enzyme preparations from *C. caudiverbera* and *B. spinulosus*. Values of 52,500 and 57,000 daltons, respectively, were obtained (data not shown). Sucrose gradient centrifugation at 36,000 rpm during 15 h resulted in values of 55,000 daltons for the *C. caudiverbera* and the *A. mexicanum* enzymes (data not shown). These values are very similar to those reported for rat hexokinase D (8, 34–38).

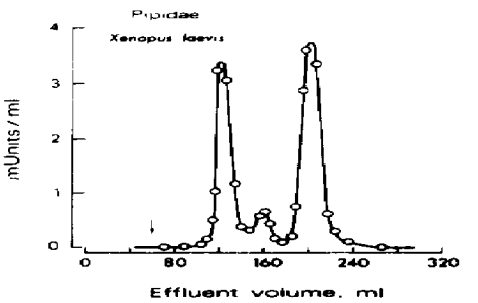
Although the properties of amphibian liver hexokinase D are very similar to those of the mammalian enzyme (3, 30–32, 35–38) two differences merit special mention. First, the amphibian enzyme can utilize fructose as substrate at 70% of the rate of glucose phosphorylation (Table VI). The poor metabolic significance of this observation notwithstanding, the fact may point to differences at the active site of the enzymes and also clearly places this so-called "glucokinase" as a multisubstrate hexokinase (see also Ref. 17). Second, the amphibian hexokinase D has a slightly but significatively lower $K_{0.5}$ value for glucose (1.5 mM) than the enzyme from mammals (about 6 mM). The observation correlates well with the fact that blood glucose levels in mammals lie between 5 and 15 mM whereas the corresponding values in amphibians are somewhat lower (1 to 5 mM (29)).

To summarize: Four glucose phosphotransferases (A, B, C, and D) very similar to those mammals can be found in the livers of amphibians. However, any given amphibian possess only three isoenzymes and the combinations found so far are of two types: C–B–D and A–B–D (Fig. 1). In the present study in which 75 animals were analyzed, none was ever found to present the pattern A–C–B–D. In other words, hexokinases A and C are never found to coexist in the same amphibian. The marked kinetic differences observed between the two isoenzymes do not support the simple notion that both are functionally interchangeable and that their presence or absence in a given species is irrelevant to the utilization of glucose by that species. Pending further work on the regulation of carbohydrate metabolism of amphibians we are at a loss in furnishing correlations or explanations with respect to the possible functional significance of the difference in the hexokinase isoenzymic pattern (see, however, Ref. 39). It seems interesting that the C–B–D type of pattern (Fig. 1, top) is shared by amphibians, close relatives of which have a well-documented fossil registry. This correlation, assuredly tenuous, may suggest that the C–B–D type of pattern should be considered as the "ancestral" glucose phosphotransferase pattern of the Amphibia.

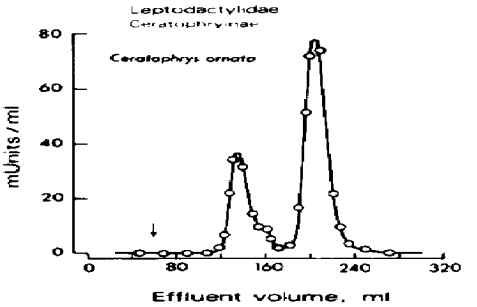
APPENDIX



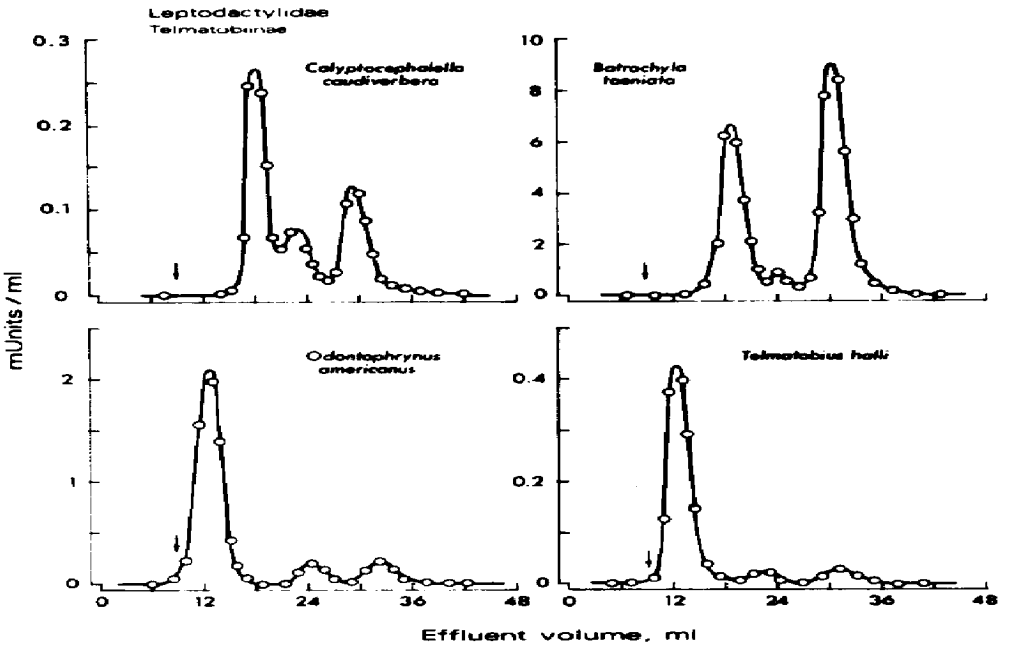
Appendix Fig. 1. DEAE-cellulose chromatography of the glucose phosphotransferases from the liver of two species of Urodeles. Top, T. torosa. The livers of three adult males were pooled (1.4 g) and processed as described under Materials and Methods. *Bottom, A. mexicanum.* A piece of 1.8 g from the liver of one adult male was processed. In both cases the 14-ml column was used. Enzyme activity was measured in the two experiments with assay (a) at 100 and 0.5 mM glucose. Only the activities measured at the high concentration are plotted for simplicity. The arrow (↓) indicates the beginning of gradient elution.



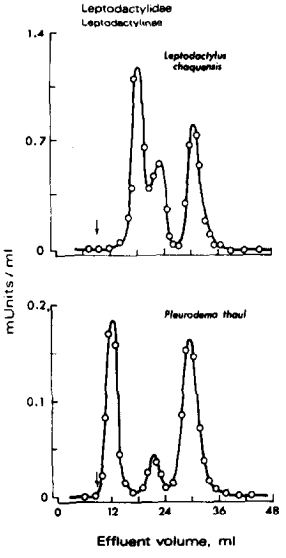
Appendix Fig. 2. DEAE-cellulose chromatography of the glucose phosphotransferases from the liver of a male adult clawed frog, X. laevis. A piece of 3.2 g of liver was used. The 14-ml column was employed. Enzyme activity was measured with procedure (a). Other details as in the legend to Appendix Fig. 1.



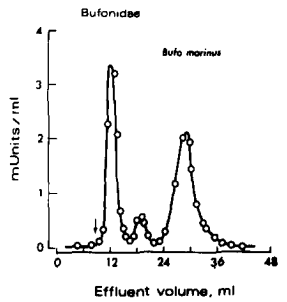
Appendix Fig. 3. DEAE-cellulose chromatography of the glucose phosphotransferases from the liver of a female adult Ceratophrys ornata. A portion of 3.3 g of liver was processed. Other details as in the legend to Appendix Fig. 1.



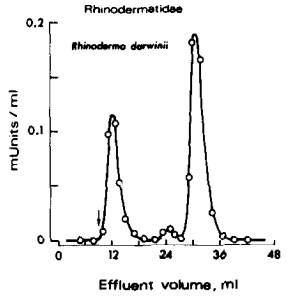
Appendix Fig. 4. DEAE-cellulose chromatography of the glucose phosphotransferases from leptodactylid frogs of the sub-family Telmatobiinae. Top left. Male adult "chilean frog" *C. caudiverbera.* Two g of liver were processed. *Top right. B. taeniata.* The livers of two adult males and two adult females were pooled (269 mg). *Bottom left. O. americanus.* The livers of one adult male and one adult female, both tetraploids, were pooled (379 mg). *Bottom right. T. halli.* The livers of three adult males were pooled (487 mg). The 2-ml column was used in the four experiments. In all cases the enzyme activity was measured with procedure (b) using [^{14}C]glucose as substrate. Selected tubes were assayed with procedure (a) at 100 and 0.5 mM glucose. Other details as in the legend to Appendix Fig.1.



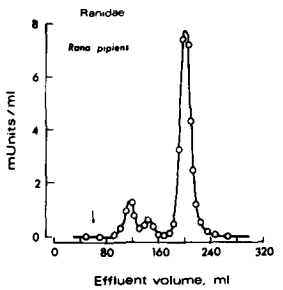
Appendix Fig. 5. DEAE-cellulose chromatography of the glucose phosphotransferases from leptodactylid frogs of the sub-family Leptodactylinae. Top, L. chaquensis. A 400 mg piece of liver from a male adult was used. *Bottom, P. thaul.* The livers of eight male adults (0.8 g) were pooled and processed. In both experiments the 2-ml column was used. Enzyme activity was measured with procedure (b) using [^{14}C]glucose as substrate. Selected tubes were also assayed with procedure (a) at 100 and 0.5 mM glucose. Other details as in the legend to Appendix Fig. 1.



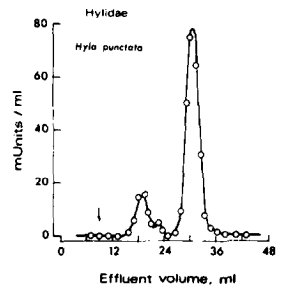
Appendix Fig. 6. DEAE-cellulose chromatography of the glucose phosphotransferases of a male adult bufonid toad, B. marinus. A piece of 1 g of liver was used. The 2-ml column was employed. Enzyme activity was measured with procedure (b) using [^{3}H]glucose as substrate. Selected tubes were also assayed with method (a) at 100 and 0.5 mM glucose. Other details as in the legend to Appendix Fig. 1.



Appendix Fig. 7. DEAE-cellulose chromatography of the glucose phosphotransferases from R. darwinii. The livers of three adult males were pooled (240 mg) and processed. Other details as in the legend to Appendix Fig. 6.



Appendix Fig. 8. DEAE-cellulose chromatography of the glucose phosphotransferases of the leopard frog R. pipiens. From the pooled livers of three adult males and one adult female a portion of 1 g was processed. The 14-ml column was employed. Other details as in the legend to Appendix Fig.6.



Appendix Fig. 9. DEAE-cellulose chromatography of the glucose phosphotransferases of H. punctata. The livers of two adult females were pooled (295 mg) and processed. Other details as in the legend to Appendix Fig. 6.

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