Glycogen Phosphorylase and its Converter Enzymes in Haemolysates of Normal Human Subjects and of Patients with Type VI Glycogen-Storage Disease

A STUDY OF PHOSPHORYLASE KINASE DEFICIENCY

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(Received 22 July 1974)

1. The properties of phosphorylase *a*, phosphorylase *b*, phosphorylase kinase and phosphorylase phosphatase present in a human haemolysate were investigated. The two forms of phosphorylase have the same affinity for glucose 1-phosphate but greatly differ in $V_{\max}$. Phosphorylase *b* is only partially stimulated by AMP, since, in the presence of the nucleotide, it is about tenfold less active than phosphorylase *a*. In a fresh human haemolysate phosphorylase is mostly in the *b* form; it is converted into phosphorylase *a* by incubation at 20°C, and this reaction is stimulated by glycogen and cyclic AMP. Once activated, the enzyme can be inactivated after filtration of the haemolysate on Sephadex G-25. This inactivation is stimulated by caffeine and glucose and inhibited by AMP and fluoride. The phosphorylase kinase present in the haemolysate can also be measured by the rate of activation of added muscle phosphorylase *b*, on addition of ATP and Mg^{2+} . 2. The activity of phosphorylase kinase was measured in haemolysates obtained from a series of patients who had been classified as suffering from type VI glycogenosis. In nine patients, all boys, an almost complete deficiency of phosphorylase kinase was observed in the haemolysate and, when it could be assayed, in the liver. A residual activity, about 20% of normal, was found in the leucocyte fraction, whereas the enzyme activity was normal in the muscle. These patients suffer from the sex-linked phosphorylase kinase deficiency previously described by others. Two pairs of siblings, each time brother and sister, displayed a partial deficiency of phosphorylase kinase in the haemolysate and leucocytes and an almost complete deficiency in the liver. This is considered as being the autosomal form of phosphorylase kinase deficiency. Other patients were characterized by a low activity of total (*a*+*b*) phosphorylase and a normal or high activity of phosphorylase kinase in their haemolysate.

Three patients, one boy and two girls, with glycogen-storage disease, having normal activities of glucose 6-phosphatase and amylo-1,6-glucosidase but a greatly diminished activity of phosphorylase in their livers were described by Hers (1959). This deficient phosphorylase could not be reactivated on incubation of the liver extract with ATP, Mg^{2+} and cyclic AMP. Considering the complexity of the phosphorylase system, no conclusion was drawn at that time on the precise nature of the primary defect responsible for the disease. The glycogen content was normal in the muscle of these patients. This form of glycogen-storage disease has been classified as type VI by Stetten & Stetten (1960) and often erroneously considered as a primary defect of liver phosphorylase. Subsequent examination of a larger number of patients with hepatomegalic glycogen-storage disease, in whom

types I, II and III could be excluded by the analysis of other enzymes, made it clear that all intermediary values between normal and very low activities of liver phosphorylase could be found, even among affected siblings (Hers, 1961; Illingworth, 1961). The diagnosis of type VI glycogenosis in these patients was made by exclusion of other types, and the primary defect responsible for it remained uncertain (Illingworth & Brown, 1964; Hers & Van Hoof, 1968).

Hug *et al.* (1966a) described another girl with a low activity of liver phosphorylase that could not be reactivated on incubation with ATP and Mg^{2+} , unless exogenous phosphorylase kinase was added. Although no direct measurement of phosphorylase kinase had been performed, they concluded that the cause of the disorder was the deficiency of that enzyme. Then Huijing (1967) and Huijing & Fernandes (1969)

described a series of patients with a sex-linked hepatomegalic glycogenosis in whom a deficiency of phosphorylase kinase could be demonstrated in leucocytes and erythrocytes. Although the residual activity in the leucocytes of affected boys was close to zero, further studies suggested that the $V_{\max}$ of the enzyme was normal but that its K_m for phosphorylase *b* was increased 3–30 times (Huijing, 1970). Other reports of sex-linked phosphorylase kinase deficiency have indicated an almost complete defect of that enzyme in the liver and a normal activity in the muscle (Morishita *et al.*, 1973; Schimke *et al.*, 1973). A puzzling observation was the normal hyperglycaemic response of these patients to glucagon (Fernandes *et al.*, 1973; Morishita *et al.*, 1973; Schimke *et al.*, 1973). Sex-linked phosphorylase kinase deficiency is also known to occur in I-strain mice; in these animals, the skeletal-muscle enzyme is deeply affected (Lyon & Porter, 1963) and the liver enzyme is normally active (Nesbitt, 1970).

In the present paper, some properties of phosphorylase, phosphorylase kinase and phosphorylase phosphatase present in human haemolysates are reported. Methods for recognizing the sex-linked and the autosomal forms of phosphorylase kinase deficiency are described. Patients with a normal phosphorylase kinase activity but a decreased activity of total (*a+b*) phosphorylase in their haemolysate are also reported. Part of this work has been presented in two symposia (Hers, 1974; Hers *et al.*, 1974).

Materials and Methods

Enzymes

Phosphorylase *b* was purified from rabbit muscle by the method of Fischer & Krebs (1962). Phosphorylase *b* kinase was prepared from rabbit muscle as described by Krebs (1966) and by Brostrom *et al.* (1971), and separated from traces of phosphorylase *b* by chromatography on *N*-butyl-Sepharose. Phosphorylase *b* was eluted from the column with 0.15M-NaF, and phosphorylase kinase was recovered later, on addition of a buffer containing 0.4M-imidazole and 0.05M-2-mercaptoethanol adjusted to pH 7.0 with citric acid. This behaviour of phosphorylase *b*, which differs from that described by Er-el *et al.* (1972), may be due to slight differences in the preparation of the butyl-Sepharose.

Chemicals

N-Butyl-Sepharose was prepared as recommended by Er-el *et al.* (1972) except that the CNBr-activated Sepharose was left at 20°C (room temperature) for a few minutes before being coupled with the alkylamine. [¹⁴C]Glucose 1-phosphate and [U-¹⁴C]glucose were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. 3':5'-Cyclic AMP, 5'-

AMP, glucose 1-phosphate (grade I), glucose 6-phosphate, glycogen (type II from oyster) and heparin (grade I) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. ATP was purchased from Boehringer, Mannheim, Germany, and Takamine Diazyme powder was from Miles Chemical Co., Clifton, N.J., U.S.A. The latter enzyme preparation was purified as described by Van Hoof (1967). α -Particulate glycogen was prepared from rabbit liver as described by De Wulf *et al.* (1970). 'Fiberglas' Pyrex-brand wool filtering fibre, from Corning Glass Works, Corning, N.Y., U.S.A., was used for removal of leucocytes. Before use, it had been treated for 1 h with conc. H₂SO₄ saturated with K₂Cr₂O₇ and thoroughly rinsed with distilled water. Desiccated bovine albumin was purchased from Povite, Amsterdam, The Netherlands.

Animals

Phosphorylase *b* kinase-deficient mice of the I strain were kindly provided by Dr. P. Cohen (University of Dundee).

Isolation of blood fractions

Blood (5–10 ml) was collected in a silicone-treated tube containing approx. 20 mg of heparin. Unless immediately processed this sample was kept in an ice bath, sometimes for as long as 6 h. The sample was centrifuged for 10 min at 1000g. After removal of the plasma, the tube was tilted and the leucocyte disc transferred with the aid of a spatula to another tube.

Preparation of the 'haemolysate'. The erythrocytes were washed three times with 3 vol. of 0.15M-NaCl. After the last centrifugation, the packed erythrocytes were diluted with 2.5 vol. of water. Some samples of packed erythrocytes were also kept frozen before dilution. This preparation of erythrocytes, referred to as a 'haemolysate', was found by microscopic examination to be contaminated by about 30% of the leucocytes initially present in the blood sample and about 5% of the thrombocytes.

Isolation of 'pure' erythrocytes. Packed erythrocytes (3 ml) were resuspended in an equal volume of 0.15M-NaCl at room temperature and passed down a column (diameter 0.8 mm) containing 2.5 g of glass wool, at a speed of approx. 0.5 ml/min. After filtration, the erythrocytes were washed once in 10 ml of 0.15M-NaCl. This method allows an almost complete removal of leucocytes and thrombocytes. However, it can only be applied to fresh samples that have to be handled within 1 h after collection.

Preparation of leucocytes. The leucocyte disc was washed three or four times with 5 ml of 0.15M-NaCl without centrifugation, to remove most of the adhering erythrocytes. The NaCl solution was then drained out and the leucocytes were frozen. After thawing they were homogenized in a conical

sintered-glass tissue grinder (Kontes Glass Co., Vineland, N.J., U.S.A.; Duall 23) in 1.5 ml of ice-cold 50mM-NaF. Insoluble material was removed by centrifugation at 8000g for 10 min at 0°C in a Servall centrifuge. In addition to leucocytes this fraction contains most of the thrombocytes of the blood sample.

Chemical analysis

Glycogen content of blood cells was determined as described by Van Hoof (1967). Glycogen contents in liver and muscle were measured as described by Hers & Van Hoof (1968). Haemoglobin was measured by the method of de Duve (1948) and proteins by the method of Lowry *et al.* (1951), with bovine albumin as standard.

Enzyme assays

Phosphorylase. The activity of rabbit muscle phosphorylase *b* was measured by the liberation of P_i from glucose 1-phosphate at 30°C by the method of Hers (1964). The same method was used for phosphorylase *a*, except that AMP was omitted and that 0.5mM-caffeine was added. This concentration of caffeine is known to inhibit almost completely mouse liver phosphorylase *b* (Stalmans *et al.*, 1971). One 'colorimetric' unit of phosphorylase is the amount of enzyme that liberates 1 μ mol of P_i /min under the conditions of the assay.

This method was not sensitive enough to measure the small activity of phosphorylase *a* present in the haemolysate. For this purpose, the following radiochemical procedure was used: 0.05 ml of haemolysate was mixed with 0.05 ml of a solution containing 2% glycogen, 0.2M-NaF, 1mM-caffeine and 20mM-[14 C]glucose 1-phosphate (0.1 μ Ci in each assay) adjusted to pH 6.1 with HCl and incubated at 37°C for various times, up to 60 min. At the end of the incubation, 0.5 ml of 0.6M-trichloroacetic acid, 0.2 ml of a 10% (w/v) solution of commercial glycogen, 2 ml of water and 4 ml of ethanol were added. The glycogen was redissolved in 2 ml of water and reprecipitated with ethanol. Proteins were then dissolved in 2 ml of hot 20% (w/v) KOH and glycogen was purified by two more precipitations in alcohol. Its radioactivity was determined by using the scintillation mixture proposed by Patterson & Greene (1965). One 'radiochemical' unit of phosphorylase is the amount of enzyme that catalyses the incorporation of 1 μ mol of [14 C]glucose into glycogen/min; it corresponds to 1.3 colorimetric units. The radiochemical method is about 100-fold more sensitive than the colorimetric method.

Phosphorylase *b* kinase. This enzyme was measured by the activation of either the endogenous phosphorylase present in the haemolysate or of purified muscle phosphorylase *b*. In the former assay, 0.4 ml of haemolysate was incubated at 20°C in a total

volume of 0.5 ml containing 50mM-glycylglycine, adjusted to pH 7.4 with NaOH, and, usually, 0.1% particulate glycogen and 2 μ M-cyclic AMP (see the text). At various time-intervals, samples were taken for the determination of phosphorylase *a* by the radiochemical method. One radiochemical unit of phosphorylase *b* kinase is the amount of enzyme that produces one radiochemical unit of phosphorylase *a*/min during the first 20 min of incubation. In the second method, 0.05 ml of haemolysate that had been previously frozen was incubated at 30°C in a final volume of 0.15 ml containing 6mM-ATP, 10mM-magnesium acetate, 7.5 units of purified rabbit muscle phosphorylase *b*, 50mM-Tris and 50mM- β -glycerophosphate adjusted to pH 6.8 with HCl. At various time-intervals, 0.01 ml samples of the incubation mixture were mixed with 0.2 ml of a cold solution containing 5mM-EDTA and 0.1M-NaF, and 0.05 ml of this mixture was used for the colorimetric determination of phosphorylase *a*. The reaction rate was linear with time. The same method was applied to muscle or liver, by using 0.05 ml of 1% homogenate, and to the leucocyte homogenate. One colorimetric unit of phosphorylase *b* kinase is the amount of enzyme that produces one colorimetric unit of phosphorylase *a*/min.

Other enzymes. Protein kinase was measured in haemolysates by the incorporation of 32 P from labelled ATP into histones, by the method *b* of Reimann *et al.* (1971) in the absence and in the presence of cyclic AMP at several concentrations.

Amylo-1,6-glucosidase was measured in haemolysates by the incorporation of [14 C]glucose into glycogen as described by Van Hoof (1967). One unit is the amount of enzyme that catalyses the incorporation of 0.1% of the glucose present, in 1 h. Liver glucose 6-phosphatase, liver and muscle phosphorylase, amylo-1,6-glucosidase and acid α -glucosidase were assayed in biopsies as described by Hers & Van Hoof (1966).

Description of patients

Of the 19 patients reported in this paper, 14 are boys and 5 girls. Their main clinical and biological features are summarized in Table 1, and data from the analysis of liver and muscle biopsies are shown in Table 2. Glucose 6-phosphatase, amylo-1,6-glucosidase and acid α -glucosidase were assayed in liver homogenates from most patients and found to be normal. No biochemical abnormality was ever found in the muscle.

No genetic information could be obtained from the familial history, except for cases 6 and 7, who belong to the large family described by Huijting & Fernandes (1969). Case 6 is the grandson of case V-56, and case 7 is the brother of case VII-64 in this pedigree.

Clinical expression is highly variable in this group of patients. In the less affected patient (no. 11),

Table 1. *Clinical and biological data of the patients*

The age mentioned is that of the time of the blood analysis. Abbreviations: N = normal; 0 = no response; + = present; ++ = intense; - = absent; ± = discrete; + → - = transitorily positive; ↗ = increased.

Patients	Sex	Age (years)	Mental retardation	Growth retardation	Hepatomegaly	Liver fibrosis	Hypotonia	Hypoglycaemia	Acetonuria	Anaemia	Glucagon test	Adrenaline test	Galactose test	Lipaemia	Cholesteraemia	Lacticacidaemia
1. A. P.	M	6	-	-	+ → -	-	-	-	-	-	N		N	N	N	N
2. T. W.	M	10½	-	-/±	+	-	-	-	-	-	N		±N	N	N	N
3. V. S. G.	M	3			++											
4. V. S. F.	M	4	-	-	+	-	-	-	-	-	N		N		↗	N
5. D. Z. R.	M	3	±	±	+	±	+	±	-	+	0/N		N	↗	↗	N
6. D. G. M.	M	4½	-	+	+		-	-	-		N			↗		
7. O. R.	M	4	-	+	+		-	-	-		N			↗		
8. C. C.	M	4½			++			-	-					↗	N	
9. W. P.	M	3	±	+	++	-	+	-	-	+	0			N	N	
10. C. X.	M	2	-	-	+	-	-	-	-		N	N		N		
11. C. M.	F	16	-	-	-	-	-	-	-					N	N	N
12. H. L.	M	5½	-		+	-	-	-	-		N			↗		
13. H. H.	F	3	-		+	-	-	-	-		N			↗		
14. V. B. A.	F	17½	-	-	+ → -		-	-	-		N	N	N	↗		
15. T. T.	M	4½	-	+	+ → -	-	-	-	-	-	N		N	↗	↗	±↗
16. V. P. V.	F	2	-		+	-	-	-	-		±N	±N				
17. L. G.	F	1	-	+	+	±	-	+	-	-	0				N	N
18. G. P.	M	1	-	-	+	-	-	-	-	±	±N	±N				±↗
19. B. A.	F	17½		-												

the diagnosis of type VI glycogenosis had never been suspected before the blood analysis. The only signs in that girl were failure to thrive and repeated infections during infancy. Type VI glycogenosis may also be a serious disease, as exemplified by the enormous hepatomegaly and tendency to cirrhosis in several patients, and by the fatal course of the disease in the case 9. This boy died from a uraemic nephropathy, apparently caused by deposition of crystalline material that could not be identified as urate with certainty. In agreement with previous reports (Hers & Van Hoof, 1968; Fernandes *et al.*, 1973; Morishita *et al.*, 1973; Schimke *et al.*, 1973) the hyperglycaemic response to glucagon was normal in the majority of the patients with type VI glycogenosis.

The cases listed in the tables are patients from Dr. H. Loeb, Brussels, Belgium (cases 1, 5, 14 and 19), Dr. J. C. Haworth, Winnipeg, Canada (case 2), Dr. H. Geerts, Herentals, Belgium (case 3), Dr. F. Dhondt, Duffel, Belgium (case 4), Dr. J. Fernandes, Rotterdam, The Netherlands (cases 6 and 7), Dr. H. Wolf, Kassel, West Germany (case 8), Dr. J. Mann, Birmingham, U.K. (case 9), Dr. R. De Meyer, Louvain, Belgium (cases 10, 11 and 16), Dr. O. Aagaens, Oslo, Norway (cases 12 and 13),

Dr. W. Mortier, Düsseldorf, West Germany (case 15), Dr. J. M. Van Hees, Liège, Belgium (case 17) and Dr. J. P. Farriaux, Lille, France (case 18).

Results

The two forms of phosphorylase and their inter-conversion

Activation of endogenous phosphorylase. Only a small percentage of the total phosphorylase present in a fresh haemolysate was active in our assay conditions. The enzyme became activated on incubation of the preparations at 20°C. The rate of activation was greatly increased by glycogen and somewhat less by cyclic AMP, and these two effects were additive (Fig. 1). A half-maximal stimulation by glycogen was obtained at a concentration of 0.3 mg/ml (not shown). The reaction was slightly faster at 20°C than at 37°C. It reached a plateau at a value which was usually of the order of 0.1 radiochemical unit of phosphorylase a/g of haemoglobin.

The activation of endogenous phosphorylase described in Fig. 1 occurred without addition of ATP to the system. It is likely that endogenous ATP generated by glycolysis was used for the activation of phosphorylase. The addition of ATP (1.2 mM) and

Table 2. *Biochemical analysis of tissue samples*

	Liver					Muscle			
	N = normal.								
	Glycogen (%)	Glycogen structure	Phosphorylase (units/g)		Phosphorylase kinase (units/g)	Glycogen (%)	Phosphorylase (units/g)	Phosphorylase kinase (units/g)	Amylo-1,6-glucosidase and acid α -glucosidase
			with 1mm-AMP	without AMP					
Normal values	1-6		15-30	6-15	20-70	0.5-2	30-100	30-100	
Patients									
1. A. P.	20		3.6			0.9	15		N
2. T. W.	12.5*	N*	8.1*						
3. V. S. G.	10.4		2.4			0.5	38		N
4. V. S. F.	19.7		3.0						
5. D. Z. R.	14		5.6		0	0.7	59	28	N
6. D. G. M.	6.2		0.7		30				
7. O. R.	8.2		4		30				
8. C. C.	10.5		7.7	3.8	30	0.6	64	57	N
9. W. P.	18.6†	N†	0†			1.1	53		N
10. C. X.	11.4		7.3		30				
13. H. H.	12		13.0	6.4	2.2				
14. V. B. A.‡	14		4.4	2.7		0.5	93		
15. T. T.	14.4		1.8	2.0		1.0	42		N
16. V. P. V.	10.8		2.2						
17. L. G.	11.4		11.2						
18. G. P.	7.5		6.0						
19. B. A.	16.3		8.7						

* Results from Dr. B. I. Brown (St. Louis, Mo., U.S.A.).
† Results from Dr. E. S. Shutt and Dr. A. D. Patrick (London, U.K.).
‡ Results obtained when the patient was 3½ years old (Hers, 1959).

Fig. 1. *Activation of phosphorylase*
Haemolysate (0.4ml) was incubated at 20°C in 50mm-glycylglycine buffer, pH7.4, with no additions (○), or in the presence of 0.1% particulate glycogen (●), of 2 μ M-cyclic AMP (▲), or of these two stimulators (■) in a total volume of 0.5 ml.

Fig. 2. *Inhibition of phosphorylase kinase by glucose 6-phosphate*
The haemolysate was incubated at 20°C in the presence of glycogen and cyclic AMP as in Fig. 1. Glucose 6-phosphate was added after 0, 20 and 40 min, at a final concentration of 10mm (broken lines).

Mg^{2+} (2mM) increased the rate of reaction by approx. 30%, whereas high concentrations (above 5mM) of the $ATP-Mg^{2+}$ complex were slightly inhibitory. The reaction was completely inhibited by 10mM-EDTA or 0.1M-NaF, the latter effect being presumably explained by the inhibition of glycolysis. Freezing and thawing of the haemolysate markedly decreased the amount of phosphorylase that could be activated under the conditions described above.

Fig. 2 shows that 10mM-glucose 6-phosphate completely inhibited the activation of phosphorylase. The same effect was obtained at 1mM-glucose 6-phosphate and a partial inhibition was observed at lower concentration.

Inactivation of endogenous phosphorylase. The inactivation of endogenous phosphorylase was conveniently studied after complete activation of the enzyme, performed as described in the preceding paragraph, followed by a separation of the enzymic system from small molecules by gel filtration. For doing so, 2.5ml of the incubation mixture was passed through a column (0.9cm $\times$ 25cm) of Sephadex G-25 in the presence of 0.1M-glycylglycine buffer brought to pH 7.4 with NaOH. On incubation of the filtered preparation at 20°C phosphorylase progressively lost its activity, and this inactivation was markedly faster in the presence of caffeine or glucose, but was almost completely inhibited by 1mM-AMP or 0.1M-fluoride (Fig. 3). The glucose stimulation of phosphorylase phosphatase was dose-dependent, a half-maximal stimu-

Fig. 3. Inactivation of phosphorylase

The phosphorylase present in a haemolysate was fully activated by a 2h incubation under the optimal conditions described in Fig. 1. The preparation was then filtered on Sephadex G-25 and incubated again at 20°C, either without addition (○) or in the presence of 0.5% (28mM) glucose (●), 1mM-caffeine (▲), 1mM-AMP (□) or 0.1M-NaF (■). The initial activity of phosphorylase was 72 radiochemical units/g of haemoglobin.

Fig. 4. Influence of glucose on the inactivation of phosphorylase

The same procedure was followed as in Fig. 3, with glucose present at the concentrations indicated. The inset shows the double-reciprocal plot of the glucose effect, and allows calculation of an apparent K_a for glucose of 3.3mM. The glucose effect is the difference between the amount of phosphorylase inactivated in 30min with and without glucose.

lation being obtained at 3.3mM-glucose (Fig. 4). The rate of inactivation of phosphorylase was also approximately doubled in the presence of 1mM-glucose 6-phosphate (not shown).

Properties of active and inactive phosphorylase. Fig. 5 shows some kinetic properties of the fully activated or fully inactivated phosphorylase present in a human haemolysate. The two forms of the enzyme in the presence or in the absence of 1mM-AMP displayed the same affinity for glucose 1-phosphate ($K_m = 7mM$) but greatly differed in their V_{max} . With 1mM-AMP the activity of phosphorylase *b* was approximately doubled but was still 11-fold less than that of phosphorylase *a*. The latter form was only slightly stimulated by AMP; in the presence of the nucleotide, its V_{max} was 0.26 μ mol of glucosyl units incorporated into glycogen/min per g of haemoglobin.

Activation of exogenous phosphorylase. The activity of phosphorylase kinase in a haemolysate can also be studied by using purified muscle phosphorylase *b* as a substrate. As illustrated in Fig. 6, this reaction was greatly stimulated by the presence of glycogen and, under these conditions, an optimal rate of reaction was obtained with 1mM-ATP and 1.7mM-magnesium acetate. In contrast with the observation shown in Fig. 2, the inhibition by 10mM-glucose 6-phosphate did not exceed 25% (not shown). As previously described by others for

Fig. 5. Double-reciprocal plots showing the effect of the concentration of glucose 1-phosphate and of the presence of AMP on the activity of the two forms of phosphorylase

Phosphorylase *a* was obtained by incubation of a fresh haemolysate for 4 h, under the optimal conditions described in Fig. 1. The preparation was then filtered on a Sephadex G-25 column, previously equilibrated with a solution containing 0.1 M-imidazole-HCl buffer, pH 6.6, and 0.2 M-NaF. To convert all the phosphorylase into the *b* form, the following procedure was used. Intact fresh erythrocytes were incubated for 2 h at 20°C in the presence of 0.5% glucose and 0.15 M-NaCl. They were then mixed with 2.5 vol. of water and the resulting haemolysate was filtered 10 min later on a Sephadex G-25 column, equilibrated with 0.1 M-imidazole-HCl, pH 6.6. The filtrate was further incubated for 2 h at 20°C and then mixed with 0.2 vol. of 1 M-NaF. Phosphorylase *a* (○, ●) and *b* (□, ■) were assayed by the radiochemical method except that caffeine was omitted with (●, ■) or without (○, □) 1 mM-AMP. *v* is expressed as μmol of glucosyl units incorporated into glycogen/min per g of haemoglobin.

phosphorylase kinase from muscle (Krebs *et al.*, 1964a) and from leucocyte (Huijing, 1970), the rate of reaction was greatly influenced by the concentration of phosphorylase *b* in the assay, with a K_m of the order of 50–100 colorimetric units/ml (not shown). In the conditions adopted as a routine (see the Materials and Methods section), the concentration of exogenous phosphorylase *b* was 7500-fold that of the endogenous substrate. Freezing and thawing the haemolysate did not markedly affect its ability to convert muscle phosphorylase *b* into phosphorylase *a*.

Experiments with 'pure' erythrocytes. When deprived of leucocytes by passage through glass wool, the preparation had lost more than 90% of its

glycogen and approx. 60–70% of its total phosphorylase (measured after complete activation). It still contained more than 85% of phosphorylase *b* kinase, measured on exogenous phosphorylase *b*, and of amylase-1,6-glucosidase.

Studies of patients with type VI glycogen-storage disease

The activity of phosphorylase and of phosphorylase kinase was measured in haemolysates from several patients with type VI glycogen-storage disease, defined as explained in the introduction (see Table 3). Phosphorylase kinase was measured by following the activation of either endogenous phosphorylase or added muscle phosphorylase *b*. Only the second method could be applied to haemolysates that had been frozen and thawed. This analysis has allowed us to classify patients into several subgroups, which are described in the following paragraphs. Fig. 7 illustrates the activation of endogenous phosphorylase in the haemolysate from patients belonging to each of these groups.

Profound phosphorylase kinase deficiency in boys. In the haemolysates of patients 1–9, all boys, the activity of phosphorylase kinase by either of the two

Fig. 6. Activation of muscle phosphorylase *b* by a haemolysate

The incubation mixture (0.15 ml) contained 0.05 ml of haemolysate, 3 units of muscle phosphorylase *b*, 7.5 μmol of Tris, 7.5 μmol of β -glycerophosphate adjusted to pH 6.8 with HCl, various amounts of ATP and magnesium acetate at a constant molar ratio 3:5, with (●) or without (○) 0.1% particulate glycogen. Phosphorylase *a* was measured by the colorimetric method on samples taken at 5 min intervals during the first 30 min of incubation.

methods was between 0 and 11% of the mean normal value (Fig. 7 and Table 3), whereas glycogen concentration was, as a mean, 10 times the normal value. The activity and the sensitivity to cyclic AMP of protein kinase was measured in the haemolysate of case 5 and found to be normal. In some patients of this group, the activity of phosphorylase kinase was also measured in liver or muscle biopsies (Table 2) and in leucocytes (Table 3); it was normal in the muscle, decreased to about 20% of the normal in leucocytes and close to zero in the liver. The glycogen content of the liver was elevated (mean value 13% of wet weight), whereas phosphorylase was low (mean value 3 colorimetric units/g), although partially active in most patients; other biochemical data were in the normal range. In many cases, the liver biopsy was too small to allow a complete analysis. Patients 6 and 7 had been independently studied by F. Huijting & J. Fernandes (personal communication), and reported to have a very low activity of phosphorylase kinase in leucocytes.

The analysis of haemolysates from the parents of some of these patients indicated that heterozygote

detection is difficult, mostly because of the variability of phosphorylase kinase activity in haemolysates from normal subjects, which ranges from 66 to 162% of the mean. The activity of phosphorylase kinase assayed by our two methods was as a mean 30, 59 and 97% of the mean normal value in the haemolysates of three mothers, and 60% in the haemolysate of one maternal grandmother. It was in the normal range in the haemolysates of the fathers of two patients. Glycogen concentration was elevated in the haemolysates of the mothers (83, 273, 336 $\mu\text{g/g}$ of haemoglobin).

An unexpected observation was the augmented activity of amylo-1,6-glucosidase in the haemolysate of the patients (Table 3) and of their mothers (3.8–8.9 units/g of haemoglobin, mean value 7.2), whereas the fathers had a normal activity (mean value 1.8 units/g of haemoglobin).

Partial phosphorylase kinase deficiency in boys and girls. A second group of patients included two pairs of siblings, each time brother and sister, with an activity of phosphorylase kinase in the haemolysate equal to about one-fifth of the normal value.

Table 3. Analysis of blood fractions from normal subjects and from patients with type VI glycogenosis

	Haemolysate				Leucocytes		
	Phosphorylase <i>a</i> (radiochemical munits/g of haemoglobin)			Phosphorylase kinase		Amylo-1,6- glucosidase (units/g of haemoglobin)	Phosphory- lase kinase (colorimetric units/g of protein)
	Glycogen ($\mu\text{g/g}$ of haemoglobin)	Initial activity at 0 min	Activity after 120 min at 20°C	(Activation of endogenous phosphorylase) (radiochemical munits/g of haemoglobin)	(Activation of muscle phos- phorylase <i>b</i>) (colorimetric units/g of haemoglobin)		
Normal values (mean + range)	47 (14–104) (<i>n</i> = 6)	18 (9–42) (<i>n</i> = 24)	106 (57–171) (<i>n</i> = 24)	1.5 (0.7–4) (<i>n</i> = 24)	7.4 (4.9–12) (<i>n</i> = 15)	2.3 (1.2–3.9) (<i>n</i> = 13)	527 (499–575) (<i>n</i> = 4)
Patients							
1. A. P.	350	3	9	0.09	0.36	6.4	86
2. T. W.	—	—	—	—	0.00	5.2	—
3. V. S. G.	835	8	11	0.02	0.06	7.6	107
4. V. S. F.	488	4	19	0.17	0.25	8.2	129
5. D. Z. R.	790	14	24	0.16	0.00	10.5	103
6. D. G. M.	230	6	6	0.00	0.16	3.5	—
7. O. R.	161	6	6	0.00	0.4	4.3	—
8. C. C.	—	5	7	0.10	0.5	3.7	—
9. W. P.	—	—	—	—	0.5	—	—
10. C. X.	235	4	35	0.35	2.2	3.7	177
11. C. M.	356	21	43	0.26	2.2	5.8	103
12. H. L.	123	—	—	—	1.1	3.1	—
13. H. H.	51	—	—	—	1.4	3.2	—
14. V. B. A.	23	27	41	0.47	10.6	3.3	301
15. T. T.	7	18	25	0.15	14.6	3.2	698
16. V. P. V.	86	25	53	0.96	9.1	1.9	—
17. L. G.	—	7	34	0.71	15.5	4.7	—
18. G. P.	47	11	54	0.69	12.4	2.7	—
19. B. A.	12	31	118	1.83	—	5.4	—

Fig. 7. Activation of endogenous phosphorylase in haemolysates of patients with type VI glycogenosis

The haemolysates were incubated at 20°C in the presence of glycogen and cyclic AMP as in Fig. 1. The haemolysates were from 27 normal subjects (○) and from cases 1-8 (●), cases 10 and 11 (▲), and cases 14-18 (■). Data are means $\pm$ S.E.M.

Phosphorylase could, however, be completely activated in the presence of exogenous phosphorylase kinase (not shown). Glycogen concentration was as a mean about four times the normal value and protein kinase activity, measured in the haemolysates of patients 10 and 11, was normal. In leucocytes, the activity of phosphorylase kinase was decreased to about 25% of the normal value, as in the group of patients described in the preceding paragraph. The analysis of liver biopsies from two of these patients revealed a large excess of glycogen, a subnormal activity of phosphorylase and a greatly decreased activity of phosphorylase kinase (Table 2). No muscle biopsy from these patients could be studied.

The activity of phosphorylase kinase in the haemolysate of the mother of cases 10 and 11 was about 50% of the normal, as measured by both methods, and was normal in the father of these children. Glycogen was elevated to about the same amount in the haemolysate of the mother and of the father (approximately four times the normal value).

Amylo-1,6-glucosidase activity was somewhat elevated in haemolysates from these patients (Table 3), although less than in the preceding group, and was normal in that of the parents.

Other groups. In other patients, boys and girls (cases 14-18), the activity of phosphorylase kinase

measured with purified phosphorylase *b* as substrate was either normal or elevated in the haemolysate and the leucocytes (Table 3). However, the endogenous phosphorylase of the haemolysate was only slightly activated on incubation in the presence of glycogen and cyclic AMP (Fig. 7), and was not further activated on addition of purified muscle phosphorylase kinase (not shown). The concentration in total phosphorylase of the haemolysate appears therefore to be decreased to about 30% of the normal value. In case 14, 'pure' erythrocytes were isolated and contained as much as 80% of the total phosphorylase measured in the haemolysate, suggesting that the defect concerns primarily the leucocyte enzyme. This patient is one of those initially described by Hers (1959) with a deficient phosphorylase activity in the liver. Glycogen concentration and amylo-1,6-glucosidase activity of the haemolysate were in the normal range. Liver phosphorylase kinase activity was not measured.

Finally, in the haemolysate of one patient (case 19) classified as type VI glycogenosis on the basis of the analysis of the liver biopsy, the activity of phosphorylase kinase, of phosphorylase and of amylo-1,6-glucosidase was normal.

Phosphorylase kinase-deficient mice. In these animals, the activity of phosphorylase kinase, measured with the colorimetric method, in skeletal muscle was 5% of that of control mice and in liver and haemolysate was normal.

Discussion

A haemolysate is a simple preparation, easily obtainable from human subjects and which can be kept frozen and sent in the frozen state from long distances. The use of this preparation allows a clear recognition of phosphorylase kinase deficiency. We will discuss separately the kinetic properties of the basal enzymic system and the types of glycogen-storage disease that it allowed us to investigate.

Properties of the enzymes

Phosphorylase. Contrary to what is usually observed with muscle or liver tissue, the phosphorylase present in a fresh human haemolysate is mostly in the inactive, *b*, form. This is in agreement with the general assumption that the high content of phosphorylase *a* of tissue is mostly due to adrenergic activation of the enzyme when the animals are killed. It is indeed expected that erythrocytes would be insensitive to this kind of stimulation. The conversion of phosphorylase *b* into phosphorylase *a* is accompanied by a large change in V_{max} , with no change in K_m for glucose 1-phosphate. An important property of phosphorylase *b* is that it is only

partially stimulated by AMP, being in this respect similar to the liver enzyme (see, e.g., Sutherland & Wosilait, 1956; Appleman *et al.*, 1966) and different from muscle phosphorylase (see Brown & Cori, 1961).

Separation of blood cells on glass wool indicates that less than one-half of the phosphorylase of the preparation originates from erythrocytes and the rest is probably from leucocytes or thrombocytes. According to Yunis & Arimura (1966) and Proux & Dreyfus (1973) these two types of cells contain both liver-type and brain-type phosphorylase. Yunis & Arimura (1968) found that human platelet phosphorylase resembles the muscle enzyme more than that of the liver.

Phosphorylase kinase. It is remarkable that the conversion of phosphorylase *b* into phosphorylase *a* occurred in the haemolysate without addition of ATP and Mg^{2+} . Since human erythrocytes are known to have an active glycolytic system (see Cartier, 1969), it is likely that the regeneration of endogenous ATP by glycolysis is sufficient to sustain an almost maximal rate of phosphorylase activation. Although a slightly higher rate of reaction could be obtained by increasing ATP concentration, we considered it much simpler and more reproducible to work with the endogenous system, without addition of ATP. Inhibition of muscle phosphorylase kinase by ATP concentration above 3 mM has been reported by Krebs *et al.* (1964b).

The stimulation of the conversion of endogenous phosphorylase *b* into phosphorylase *a* by cyclic AMP was somewhat unexpected, since carbohydrate metabolism is not known to be regulated by hormonal influence in erythrocytes. The stimulation of phosphorylase *b* kinase by glycogen was described for the muscle enzyme by Krebs *et al.* (1964b). These authors reported that with non-activated phosphorylase kinase, a half-maximal stimulation occurred with 0.3% glycogen, i.e. the same value that we now find with the human haemolysate. The mechanism of this stimulation was related to an increased affinity of phosphorylase kinase for its substrate phosphorylase *b*, in the presence of glycogen. This stimulation was most useful for our investigation, since it is only in the presence of glycogen that the reaction was fast enough to reach completion within a few hours.

The phosphorylase kinase present in haemolysates has also been measured with muscle phosphorylase *b* as substrate. The concentration of substrate was then about 7500-fold that used in the endogenous assay; ATP and Mg^{2+} were systematically added. Glycogen stimulated the reaction, and an excess of ATP and Mg^{2+} was inhibitory (Fig. 6). The inhibition by glucose 6-phosphate was, however, much less pronounced than in the endogenous assay, an observation which is easily explained by the fact that

inhibition of muscle phosphorylase kinase by glucose 6-phosphate is known to be of the competitive type (Tu & Graves, 1973). The interest of this assay procedure is that it is applicable to frozen erythrocytes, in which the endogenous phosphorylase *b* is difficult to activate. The disadvantage of the method is that it requires the preparation of muscle phosphorylase *b*, whose properties as substrate may be altered with aging. Further, this method does not provide information on the phosphorylase present in the haemolysate, which can be important in the classification of patients.

Since over 85% of phosphorylase kinase was still present in the preparation after passage of the blood cells through glass wool, one can conclude that the enzyme under study is really the erythrocyte phosphorylase kinase.

Phosphorylase phosphatase. The property of phosphorylase phosphatase of being stimulated by glucose, glucose 6-phosphate and caffeine, and inhibited by AMP, has been previously reported for the muscle and the liver enzyme (Sutherland, 1951; Krebs *et al.*, 1964a; Hurd *et al.*, 1966; Holmes & Mansour, 1968; Stalmans *et al.*, 1970).

Investigations of patients with type VI glycogen-storage disease

Sex-linked type of phosphorylase kinase deficiency. There is little doubt that the series of nine male patients characterized by an almost complete deficiency of phosphorylase kinase in the haemolysate were affected by the sex-linked disease initially described by Huijing (1967) and by Huijing & Fernandes (1969), and indeed patients 6 and 7 belong to the family previously studied by them. Our analysis of the haemolysates of the parents, without proving decisive, is in agreement with a sex-linked mode of inheritance.

In our hands, the phosphorylase kinase deficiency was as pronounced when exogenous phosphorylase *b* was used as substrate as with the endogenous substrate. Since there was a 7500-fold difference in substrate concentration between these two methods, this observation is not in favour of the hypothesis that the low activity of phosphorylase kinase is caused by a low affinity for its substrate, phosphorylase *b* (Huijing, 1970).

In agreement with previous observations (Morishita *et al.*, 1973; Schimke *et al.*, 1973), it appears that the muscle enzyme is normal, at least in two patients in whom we could analyse a muscle biopsy. In four patients we had the opportunity to measure the liver enzyme and found it to be nearly completely deficient. The disease clearly appears, therefore, as a liver disorder. It is thus surprising that nearly all patients did react normally to glucagon by a hyperglycaemia, despite the nearly complete

defect of phosphorylase kinase. Fernandes *et al.* (1973) have suggested that the normal response to glucagon may be due to the formation of AMP from cyclic AMP. This seems doubtful, however, since the normal concentration of AMP in the liver is higher than 0.1 mM (Start & Newsholme, 1968) and probably cannot be markedly increased by hydrolysis of the cyclic nucleotide, of which the basal concentration is close to 1 μ M.

The increased activity of amylo-1,6-glucosidase in the haemolysates of these patients and of their mothers is poorly understood; it could result from an increased stability of the enzyme in the presence of an excess of glycogen.

The autosomal type of phosphorylase kinase deficiency. Indirect evidence for phosphorylase kinase deficiency in girls was previously presented by Hug and his co-workers (Hug *et al.*, 1966a, 1969). There were also three girls in the series of 35 patients published by Huijing & Fernandes (1969), who considered them as heterozygotes for the X-linked abnormal gene.

Our series of patients includes two pairs of siblings, each time brother and sister, in whom the activity of phosphorylase kinase in the haemolysate was about 25% of the normal and similar in the girls and in the boys. Phosphorylase kinase was also measured in the liver of one boy and one girl and was found almost inactive. In one of the families, the two siblings are clinically similar to each other and they are obviously affected by the same disease. Since some of these patients are boys, they cannot be heterozygotes for an X-linked abnormal gene. We therefore conclude that these patients suffer from an autosomal recessive disorder characterized by an incomplete deficiency of phosphorylase kinase in the haemolysate, although more pronounced in the liver. These patients are similar to those studied by Hug *et al.* (1966a, 1969).

The existence of sex-linked and autosomal phosphorylase kinase deficiencies is in agreement with the previous description by several authors of the existence of three polypeptide chains in purified muscle phosphorylase kinase. One of these chains, called *b* (Hayakawa *et al.*, 1973) or β (Cohen, 1973), is phosphorylated by protein kinase. It is absent from phosphorylase kinase-deficient mice (P. Cohen, personal communication) and therefore appears to be controlled by a sex-linked gene. In mice, the deficiency concerns mostly the muscle (Lyon, 1970), whereas the liver and the erythrocytes are not affected. In man, the deficiency occurs in the liver, erythrocytes and at least in some types of leucocytes. Since different sex-linked genes control phosphorylase kinase in muscle and liver, there might exist in man another sex-linked disorder in which phosphorylase kinase is deficient in muscle and normal in liver. The autosomal phosphorylase kinase deficiency

seems to be due to an abnormality of one of the other two polypeptide chains. The precise structure of liver phosphorylase kinase is, however, still poorly known.

A major advantage of the use of the haemolysate is that it allows discrimination of the sex-linked and the autosomal forms of phosphorylase kinase deficiency, whereas the analysis of leucocytes does not. Further, the composition of leucocyte preparations may vary greatly according to the technique used for their preparation.

Other patients with type VI glycogenosis. Patient no. 14, in whom phosphorylase kinase, measured with exogenous phosphorylase *b* as substrate, was normal, is one of the first three patients initially described by Hers (1959) with a low phosphorylase activity in their liver. It appears that the phosphorylase present in the fresh haemolysate was already mostly in the active form and was only slightly further activated on incubation at 20°C. Separation of blood constituents indicated that there was a markedly decreased activity of total (*a+b*) phosphorylase in leucocytes. A similar situation was also observed in the haemolysates of four patients. This pathological condition could result from an abnormal phosphorylase. Several groups of workers (Drummond *et al.*, 1970; Hug & Schubert, 1970; Guibaud & Mathieu, 1972; Koster *et al.*, 1973) have, indeed, reported cases of glycogen-storage disease that they considered to be caused by a primary defect of liver phosphorylase.

Nomenclature. The numbering of the various forms of glycogen-storage disease types I to V was introduced by Cori (1957). It was continued by Stetten & Stetten (1960), who classified as type VI the three patients described by Hers (1959), and there is also a general agreement (Brown & Brown, 1968; Howell, 1972) to call type VII the deficiency of phosphofructokinase in muscle, described by Tarui *et al.* (1965). Other numbers, such as VIa, VIb (Huijing, 1970) and VIII to X (Hug *et al.*, 1966b), have also been used by some authors, but cannot be considered as having received general acceptance, or have been withdrawn (Huijing & Fernandes, 1970). Up to now, phosphorylase kinase deficiency has been classified as type VIa (Huijing, 1970), VIII (Howell, 1972) and IX (Hug *et al.*, 1966b).

From the historical background (see the introduction) it appears likely that at least some of the three patients initially classified as type VI were deficient in phosphorylase kinase, although not case V. B. A., included in the present study (no. 14). It seems thus reasonable to follow the proposal of Huijing (1970) to maintain in group VI the phosphorylase kinase deficiencies that affect the liver. The letter k (VIk) could indicate that the kinase is affected. A similar disorder affecting the muscle, and analogous to the disease in mice, would then be a subgroup of type V (muscle phosphorylase defi-

ency) and be type Vk. This type of disease has not yet been observed. An attractive possibility would be to designate the subgroups by the letters a, b, or c, corresponding to the affected polypeptide. In such a system, the disease in mice would be Vkb. It is clear, however, that great confusion will arise if each author uses his own numbering system. This is why we offer the advice to designate the diseases under study as sex-linked phosphorylase kinase deficiencies and autosomal phosphorylase kinase deficiency, until some greater agreement over a numerical classification is reached.

We are grateful to the physicians who have provided biopsies and blood samples and to Dr. P. Cohen for the gift of phosphorylase kinase-deficient mice. Our work was supported by the Belgian Fonds Médical Reine Elisabeth, Fonds de la Recherche Scientifique Médicale, and by the U.S. Public Health Service (research grant AM 9235). G.V.d.B. is 'Bevoegdverklaard Navorser' of the Nationaal Fonds voor Wetenschappelijk Onderzoek.

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