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An optimized assay of phosphorylase kinase in crude liver preparations

Phosphorylase kinase converts inactive phosphorylase *b* into the active *a*-form. Being activated in response to cyclic AMP as well as Ca^{2+}, it plays a central role in the regulation of glycogenolysis [1]. The activity of the purified enzyme can be determined unambiguously by the incorporation of radioactivity from [γ-^{32}P]ATP into added phosphorylase *b* from rabbit skeletal muscle, which is easily crystallized. However, the application of this assay to crude homogenates requires the separation of labelled phosphorylase *a* from other labelled tissue proteins. In the latter conditions, the activity is therefore routinely derived from the rate of activation of added phosphorylase *b*. The *gsd/gsd* rat [2] is an animal model of the human hepatic phosphorylase kinase deficiency with autosomal recessive inheritance [3]. Our local *gsd/gsd* colony presents the typical inabilities to activate phosphorylase and to degrade glycogen in the liver in response to a variety of glycogenolytic stimuli [4,5]. However, their hepatic phosphorylase kinase activity seemed unrealistically high, and prompted investigation.

Livers of fed Wistar or *gsd/gsd* rats, aged 3–4 months, were homogenized in a Potter–Elvehjem tube with 9 vol. of 50 mM-imidazole (pH 7.4), 0.5 mM-dithiothreitol, 0.3 mM-phenylmethanesulphonyl fluoride, 0.5 mM-benzamidine and 0.25 M-sucrose. The homogenates were centrifuged for 10 min at 10000 *g*. For the assay of phosphorylase kinase, 50 μl of the resulting extract was incubated at 25 °C with 50 μl of a solution containing 1 mg (20 units) of 10-times recrystallized phosphorylase *b* from rabbit muscle [6], 6 mM-ATP plus 10 mM-magnesium acetate, and 0.1 M-NaF.

When measured with the 'conventional' assay for phosphorylase *a* (Fig. 1*a*), the phosphorylase kinase activity measured in extracts from *gsd/gsd* livers was nearly half of that recorded in preparations from normal rats. Such an elevated activity suggested an artifact in the assay. As expected, AMP accumulated gradually in the phosphorylase kinase assay mixture (not shown). After 20 min incubation in the conditions of Fig. 1(*a*) the AMP concentrations were 0.47 mM and 0.43 mM for Wistar rats and *gsd/gsd* rats, respectively; this corresponds to a final concentration of about 20 μM in the phosphorylase assay. Perhaps the accumulating AMP could progressively stimulate phosphorylase *b*, in spite of the presence of 0.5 mM-caffeine, and this would then be erroneously interpreted as a progressive conversion of phosphorylase *b* into *a*?

To test the plausibility of this hypothesis the 'conventional' assay of phosphorylase *a* was performed after the enzymic conversion of any AMP to IMP, which stimulates phosphorylase *b* rather weakly [7]. The latter procedure decreased the apparent phosphorylase kinase activity in Wistar livers by half and in *gsd/gsd* livers by 80% (compare Figs. 1*a* and 1*b*). This indicates that the accumulation of AMP causes a general overestimation of the phosphorylase kinase activity in crude liver extracts. Fig. 2 illustrates quantitatively the interaction between AMP and caffeine on the activities of the two forms of phosphorylase from rabbit skeletal muscle. It can be seen that in the presence of 0.5 mM-caffeine plus 20 μM-AMP (which mimicks the situation at

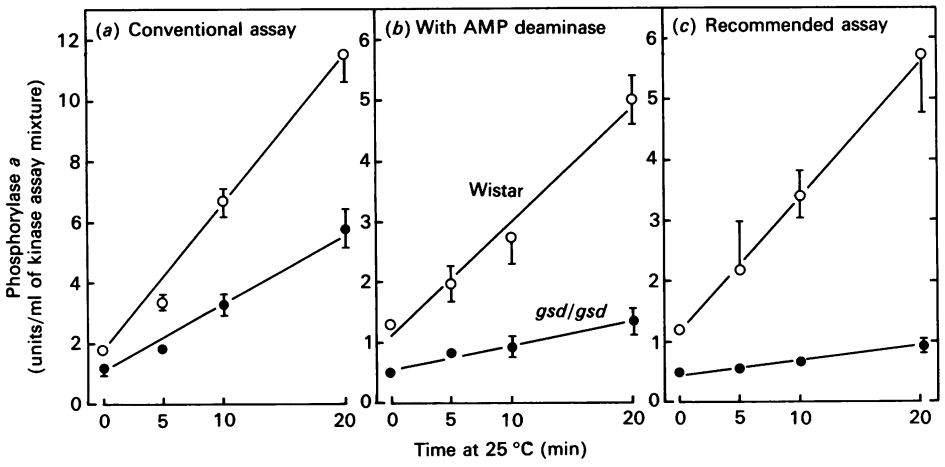

Fig. 1. Activity of phosphorylase kinase in liver extracts from Wistar rats (○) and *gsd/gsd* rats (●)

At the indicated time points samples (10 μl) were withdrawn from the kinase assay mixture and added to 190 μl of mixture for the 'conventional' assay (*a*) or the 'recommended' assay (*c*) of phosphorylase *a*. A third sample was incubated for 20 min at 25 °C with AMP deaminase, in the presence of 20 mM-EDTA and 50 mM-NaF to inhibit phosphorylation and dephosphorylation, before the 'conventional' assay (*b*). The concentrations of the 'conventional' assay components were, in the final volume of 0.2 ml: 50 mM-glucose 1-phosphate, 1% shellfish glycogen, 0.1 M-NaF, 20 mM-EDTA, 0.5 mM-caffeine, 0.1% bovine serum albumin and 20 mM-Pipes, pH 6.8 [13]. In the 'recommended' assay the concentration of caffeine was increased to 5 mM and 10 μM-AMP was also added. Data are means ± S.E.M. ($n = 4$). One unit of phosphorylase converts 1 μmol of substrate/min in the specified assay conditions. Note the distinct scale of the ordinate in (*a*) versus (*b*) and (*c*).

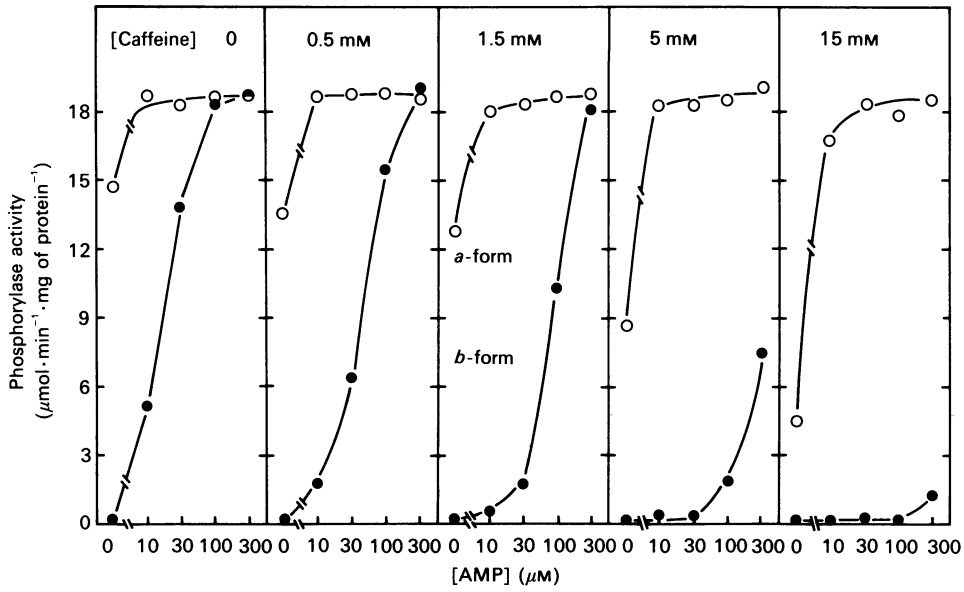

Fig. 2. Effects of caffeine and of AMP on the activities of phosphorylases *a* (○) and *b* (●) from rabbit skeletal muscle

Phosphorylase *b* was fully phosphorylated by purified phosphorylase kinase and the *a*-form was crystallized [6]. The 'conventional' assay was adapted to contain various concentrations of caffeine and AMP as indicated.

20 min in Fig. 1*a*) the *b*-form possesses about 20% of the activity of the *a*-form.

Fig. 2 shows further that the assay of muscle phosphorylase *a* can be optimized by the simultaneous inclusion of 5 mM-caffeine and 10 μM-AMP. Indeed, phosphorylase *a* is fully active in this 'recommended' assay, whereas phosphorylase *b* remains essentially inactive, even when an additional 20 μM-AMP is generated. Fig. 1(*c*) demonstrates the usefulness of the 'recommended' assay, as performed on the samples subjected also to the 'conventional' assay (Fig. 1*a*): the apparent phosphorylase kinase activity in Wistar liver extracts was 50% lower, and the activity in *gsd/gsd* livers was only 10% of that in identically assayed Wistar livers.

Two further questions deserve some attention in the context of the assay that we recommend for the determination of phosphorylase kinase activity in crude tissue extracts. The first question concerns the formation of phospho/dephospho (*ab*) hybrids during the conversion of muscle phosphorylase *b* to *a* [8]. Hybrids are fully active in the presence of 5 mM-caffeine plus 1 mM-AMP, but their activity in current assay conditions (including our 'recommended' assay) is unclear [8]. Could the deficient phosphorylase kinase in *gsd/gsd* liver extracts perhaps result in a selective accumulation of hybrids? Therefore, we have determined phosphorylase kinase in liver extracts from Wistar and *gsd/gsd* rats by monitoring simultaneously the activation of phosphorylase (measured as in Fig. 1*c*) and its actual phosphorylation (by incorporation of radioactivity from γ-^{32}P-labelled ATP into phosphorylase, subsequently re-isolated by SDS/PAGE). Either assay indicated that *gsd/gsd* livers contain about 10% of the normal phosphorylase kinase activity (not shown). This residual activity stems presumably from genuine phosphorylase kinase, since various glycogenolytic stimuli cause a small but definite increase in the concentration of phosphorylase *a* in livers and isolated hepatocytes of *gsd/gsd* rats [4,5,9,10].

Second, how does the 'recommended assay' compare with previous procedures? An alternative that has been advocated in the past is to perform 'blanks' where phosphorylase *b* is added only at the end of the phosphorylase kinase assay to correct for stimulation by AMP. This approach allowed Cohen & Cohen [11] to pinpoint a notorious overestimation of phosphorylase kinase in kinase-deficient muscle homogenates. However, as a routine matter such controls are rather cumbersome, and the precision depends on the relative importance of kinase activity versus generation of AMP. Since in the past many assays were performed without adequate controls and in the absence of any caffeine, one can safely assume that the activity of phosphorylase kinase in crude tissue extracts has often been overestimated. It may be pointed out that a gross overestimation of the activity in normal liver samples may easily go unnoticed, unless one is alarmed by the comparison with a true deficiency of phosphorylase kinase (cf. Fig. 1*a*). On the other hand, it is instructive to analyse why very low phosphorylase kinase activities have indeed been measured in liver biopsies of some patients with hepatic glycogen-storage disease [3], and in liver extracts of *gsd/gsd* rats [2]. Lederer *et al.* [3] used 0.5 mM-caffeine, but they assayed phosphorylase *a* radiochemically with a very low concentration of glucose 1-phosphate (10 mM), which disfavours the activity of phosphorylase *b* [12]. Malthus *et al.* [2], who discovered that the phosphorylase kinase activity in *gsd/gsd* livers was 8% of that in normal livers, apparently escaped the problem by inclusion of 1.7 mM-caffeine in the phosphorylase assay, plus a 2-fold higher dilution of the AMP-containing samples. Fig. 2 shows that 1.5 mM-caffeine protects indeed phosphorylase *b* against stimulation by up to 10 μM-AMP, but the assay suffers from an initial underestimation, until sufficient AMP has been produced to counteract the 30% inhibition of phosphorylase *a* by caffeine. Such problems (however small) can be avoided by the systematic inclusion of 5 mM-caffeine plus 10 μM-AMP in the assay of muscle phosphorylase *a*.

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Action of uroporphyrinogen decarboxylase on uroporphyrinogen-III: a reassessment of the clockwise decarboxylation hypothesis

In the later stages of haem biosynthesis, uroporphyrinogen-III undergoes a series of four decarboxylations to give coproporphyrinogen-III. The sequential decarboxylation of uroporphyrinogen-III by uroporphyrinogen decarboxylase could take place by 24 different pathways involving 14 possible intermediates. The intermediacy of heptacarboxylate (heptas), hexacarboxylate (hexas) and pentacarboxylate (pentas) porphyrinogens has long been recognized (Batlle & Grinstein, 1964; San Martin de Viale & Grinstein, 1968). In the mid-1970's, Jackson and coworkers undertook a detailed investigation of this process (Jackson *et al.*, 1976*a*). Four heptacarboxylate porphyrinogens are possible, depending on whether initial decarboxylation occurs at the A, B, C or D ring acetate groups (designated as heptas -a, -b, -c or -d). Six hexacarboxylate porphyrinogens (hexas) might be formed and each structure is designated by a two-letter code corresponding to the acetate units that have undergone decarboxylation. Four type III pentacarboxylate isomers (pentas) are possible and these are assigned a three-letter code which corresponds to the positions of the three methyl substituents. All fourteen of the related porphyrins were synthesized by the MacDonald condensation and the b-oxobilane approach (Jackson *et al.*, 1980*a*; see also Clezy *et al.*, 1976). Surprisingly, all 14 of the porphyrinogens were metabolized by chicken red cell haemolysates, although not all of these compounds were good substrates (Jackson *et al.*, 1976*b*; Smith *et al.*, 1976; Lash, 1979). For instance, penta-bcd was shown to be a poor substrate for uroporphyrinogen decarboxylase, while the other type III pentacarboxylate porphyrinogens were rapidly converted to protoporphyrin-IX. Hence, the nature of the decarboxylation pathway between uroporphyrinogen-III and coproporphyrinogen-III could not be deduced from the substrate specificity of uroporphyrinogen decarboxylase. Hepta, hexa and penta fractions were known to accumulate in the urine and faeces of rats poisoned with hexachlorobenzene. These components were isolated and shown, on the basis of europium shift reagent proton n.m.r. studies and comparison to synthetic samples, to primarily consist of single porphyrin isomers (Jackson *et al.*, 1976*a*). These fractions corresponded to hepta-d, hexa-da and penta-dab (Scheme 1). In addition the heptacarboxylate fraction isolated from the urine of a patient suffering from porphyria cutanea tarda (PCT) was also shown to be indistinguishable from hepta-d (Jackson *et al.*, 1976*b*; Ryder, 1977). These results suggested that uroporphyrinogen-III underwent initial decarboxylation at the D ring, followed by successive decarboxylations at the A, B and C positions. It is tempting to suggest that the porphyrinogen undergoes a series of rotations on the enzyme surface; however, there is some evidence to suggest that more than one active site is involved (cf. de Verneuil *et al.*, 1980). Given that this sequence takes place in a clockwise progression around the periphery of the conventional 'Fischer' porphyrinogen structures, this process was considered to be a clockwise decarboxylation pathway (Jackson *et al.*, 1976*a*). In an independent study, Battersby and coworkers isolated a heptacarboxylate porphyrin from incubations of porphobilinogen (PBG), the precursor of uroporphyrinogen-III, with avian red cell haemolysates at high salinity (Battersby *et al.*, 1976). The high salt content was required to induce an accumulation of the heptacarboxylate fraction. N.m.r. studies and comparisons with synthetic samples again demonstrated that a single isomer, hepta-d, had been formed. It should be noted that hepta-d, hexa-da and penta-dab were all excellent substrates for uroporphyrinogen decarboxylase and the presence of the corresponding porphyrins cannot be rationalized as being due to the preferential accumulation of these isomers. Penta-dab is also believed to be a precursor to the isocoproporphyrin series (Elder, 1972; Stoll *et al.*, 1973), a group of abnormal metabolites that have been isolated from the faeces of porphyrics and hexachlorobenzene-poisoned rats. The corresponding porphyrinogens may be intermediates in an alternate route from penta-dab to harderoporphyrinogen under abnormal conditions (Elder & Evans, 1978; Jackson *et al.*, 1980*b*).

In order to analyse trace amounts of porphyrins from natural sources, partial h.p.l.c. separations of hepta, hexa and penta isomers were developed at Cardiff in the late 1970's (Jackson *et al.*, 1980*a*). Analysis of the penta fractions from normal and porphyric urine showed that at least three type III penta isomers were present (Jackson *et al.*, 1980*c*). Subsequently, Lim and coworkers developed elegant h.p.l.c. separations of penta, hexa and hepta porphyrin fractions (Lim & Rideout, 1983; Lim *et al.*, 1983*a*,*b*; Li *et al.*, 1987; Lim *et al.*, 1987). In each case, they demonstrated that all fourteen of the possible hepta, hexa and penta isomers were present in normal urine in similar proportions. However, they were able to confirm that the major hepta in the urine of PCT patients was hepta-d (Luo & Lim, 1990). In addition, h.p.l.c. studies at Cardiff had confirmed that the hepta, hexa and penta fractions from the urine and faeces of hexachlorobenzene-poisoned rats were single isomers, in agreement with the original n.m.r. and synthetic studies (Jackson *et al.*, 1976*a*). It seems quite likely that abnormal isomers would be excreted preferentially and the presence of trace quantities of porphyrin isomers in urine samples does not preclude the validity of Jackson's clockwise decarboxylation hypothesis.

Uroporphyrinogen-I is formed in excessive quantities in certain pathological conditions, such as congenital erythropoietic porphyria (Gunther's disease), and type I porphyrins are excreted in the urine and faeces. Uroporphyrinogen-I is metabolized by uroporphyrinogen decarboxylase to give coproporphyrinogen-I. Only one hepta and one penta can be formed in the type I series, although two hexas are possible. Analysis of the type I hexa fraction from natural sources by h.p.l.c. confirmed that both isomers were present (Jackson *et al.*, 1977). In addition, when uroporphyrinogen-I was incubated with chicken red cell haemolysates, both hexas were formed as metabolic intermediates. Hence, the decarboxylation of uroporphyrinogen-I by uroporphyrinogen decarboxylase appears to be random in nature.