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The Concentration and Control of Cytoplasmic Free Inorganic Pyrophosphate in Rat Liver *in vivo*

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The concentration of cytoplasmic free pyrophosphate was calculated in freeze-clamped livers of rats from the measured concentration of reactants and $K_{eq.}$ of the UDP-glucose pyrophosphorylase reaction (UDP–α-D-glucose 1-phosphate uridylyltransferase, EC 2.7.7.9). The $K_{eq.}$ of the UDP-glucose pyrophosphorylase reaction was redetermined at 38°C, pH7.0, $I = 0.25$mol/l and free $[Mg^{2+}] = 1$mM, and was 4.55 in the direction of glucose 1-phosphate formation. The activity of UDP-glucose pyrophosphorylase in rat liver was between 46 and 58μmol of glucose 1-phosphate formed/min per g fresh wt. in the four dietary conditions studied. A fluorimetric assay with enzymic cycling was developed for the measurement of glucose 1-phosphate in $HClO_4$ extracts of rat liver. The calculated free cytoplasmic PP_i concentration in nmol/g fresh wt. of liver was 2.3±0.3 in starved, 3.8±0.4 in fed, 4.9±0.6 in meal-fed and 5.2±0.4 in sucrose-re-fed animals. These values agree well with recently determined direct measurements of total PP_i in rat liver and suggest that there is not a large amount of bound or metabolically inert PP_i in rat liver. The cytoplasmic [ATP]/[AMP][PP_i] ratio is 10^3 times the cytoplasmic [ATP]/[ADP][P_i] ratio and varies differently with dietary state. The reaction $PP_i + H_2O \rightarrow 2P_i$ catalysed by inorganic pyrophosphatase (EC 3.6.1.1) does not attain near-equilibrium *in vivo*. PP_i should be considered as one of the group of small inorganic ions which is metabolically active and capable of exerting a controlling function in a number of important metabolic reactions.

Inorganic pyrophosphate is a common reactant in nearly all of the major metabolic pathways of the mammalian liver cell: fat metabolism through the conversion of acetate into acetyl-CoA via acetyl-CoA synthetase [acetate–CoA ligase (AMP), EC 6.2.1.1] and by the enzymes which activate both medium-chain and long-chain fatty acids [acid–CoA ligase (AMP), EC 6.2.1.2 and EC 6.2.1.3]; urea synthesis through the activity of argininosuccinate synthetase [L-citrulline-L-aspartate ligase (AMP), EC 6.3.4.5]; amino acid activation through the action of a number of specific ligases forming aminoacyl-tRNA (EC 6.1.1.–); DNA synthesis through the action of DNA polymerase (EC 2.7.7.7); and glycogen synthesis through the UDP-glucose pyrophosphorylase (UDP–α-D-glucose 1-phosphate uridylyltransferase, EC 2.7.7.9) reaction. In addition to its participation in the major pathways, PP_i has been shown to be a substrate in a number of other reactions, the physiological significance of which remains obscure. Among these reactions are the pyrophosphatase and transphosphorylation reactions catalysed by glucose 6-phosphatase (EC 3.1.3.9) (Nordlie & Arion, 1965) and alkaline phosphatase (EC 3.1.3.1). PP_i has been found to be the sole phosphate donor for the phosphoenolpyruvate-forming enzyme phosphopyruvate carboxylase [pyrophosphate–oxaloacetate carboxylyase (phosphorylating), EC 4.1.1.38] in propionic acid bacteria (Siu & Wood, 1962) and is the major product of photophosphorylation in *Rhodospirillum rubrum* (Baltscheffsky *et al.*, 1966). Although a physiologically important role for PP_i in mammalian tissue has been proposed previously (Baltscheffsky, 1967), this view has not been widely accepted.

The rather large amounts of PP_i that must be produced in liver synthesis of urea, RNA, DNA, protein and glycogen, and fatty acid activation have been assumed to be hydrolysed by a pyrophosphatase. Liver microsomal fractions have been shown to contain a pyrophosphatase activity which is apparently an activity of glucose 6-phosphatase (Stetten & Taft, 1964). However, if PP_i hydrolysis in liver were very efficient and proceeded far toward equilibrium, the intracellular concentration of PP_i would be about

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1×10^{-8}M since the intracellular P_i concentration is 4mM (Veech *et al.*, 1970) and the $\Delta G'$ for PP_i hydrolysis is -19.2kJ/mol at $I = 0.25$mol/l, 38°C, $[Mg^{2+}] = 1$mM and $[K^+] = 200$mM (J. W. R. Lawson, unpublished work). Contrary to this expectation total concentrations of PP_i 2 to 3 orders of magnitude higher were found in blood (Russell *et al.*, 1971), in mouse liver (Reich *et al.*, 1968) and very recently in freeze-clamped rat liver (H. Flodgaard, personal communication).

This discrepancy with the predicted PP_i concentrations calculated on the basis of a presumed near-equilibrium in the pyrophosphatase reaction raises the possibilities that the measured total PP_i is segregated (see Reich *et al.*, 1968), that large amounts of PP_i are bound and metabolically inactive or that the pyrophosphatase reaction does not catalyse a near-equilibrium state. The present paper investigates this problem and attempts to determine the free PP_i concentration in the cytoplasmic compartment of rat liver in different dietary states. The approach has been the same as used previously to determine the ratio of free $[NAD^+]/[NADH]$ (Hohorst *et al.*, 1959) or the free $[ATP]/[ADP][HPO_4^{2-}]$ (Veech *et al.*, 1970) in rat liver cytoplasm. The cytoplasmic enzymes for which PP_i is a substrate were surveyed, their activity and equilibrium constants ascertained and the suitability of the measurement of the relevant metabolites was determined with regard to the question of metabolite binding or compartmentation. An obvious choice for an enzyme reaction that could be used to calculate the cytoplasmic free $[PP_i]$ is that of UDP-glucose pyrophosphorylase. The enzyme is strictly cytoplasmic and of high activity and easy reversibility (eqn. 1):

$$\text{Glucose 1-phosphate}^{2-} + \text{UTP}^{4-} = \text{UDP-glucose}^{2-} + PP_i^{4-} \quad (1)$$

If the reaction can be assumed to be near equilibrium *in vivo*, it would be possible to calculate the cytoplasmic free $[PP_i]$ by measuring the other components of the reaction in freeze-clamped liver (eqn. 2):

$$\text{Free } [PP_i] = \frac{[\text{glucose 1-phosphate}][\text{UTP}]}{[\text{UDP-glucose}]K_{\text{UDP-glucose pyrophosphorylase}}} \quad (2)$$

This approach to the estimation of the cytoplasmic free $[PP_i]$ has been investigated below and the results have been compared with reported measurements of the total $[PP_i]$ in rat liver.

Experimental

Animals

Male Wistar rats (Carworth, Kensington, N.J., U.S.A.) weighing 200–300g were used. The animals were divided into four groups: (1) starved for 48h; (2) meal-fed (8–11 a.m.) on the NIH stock diet (5% fat, 23.5% crude protein, 59% carbohydrate); (3) fed on the same stock diet *ad libitum*; and (4) starved for 72h and then re-fed *ad libitum* for 48h on a 58% (w/w) sucrose fat-free diet ('Fat-Free Test Diet'; Nutritional Biochemicals, Cleveland, Ohio, U.S.A.). All groups received water *ad libitum*. The meal-fed animals were killed at the end of the meal. The livers were freeze-clamped and $HClO_4$ extracts of the tissue were prepared as described by Veech *et al.* (1972).

Reagents

Standard analytical-grade laboratory reagents were obtained from Mallinckrodt Chemical Co., St. Louis, Mo., U.S.A. Florisil (100–200 mesh) was from Fisher Scientific Co., Fairlawn, N.J., U.S.A., and α-D-glucose 1-phosphate, UDP-glucose and UTP were from Sigma, St. Louis, Mo., U.S.A. All other chemicals were obtained from Boehringer (Mannheim) Corp., New York, N.Y., U.S.A.

Enzymes

UDP-glucose pyrophosphorylase was prepared from fresh calf liver and purified through the first crystallization step by the method of Albrecht *et al.* (1966). Contaminating enzymes such as hexokinase, isocitrate dehydrogenase, glutamate dehydrogenase and inorganic pyrophosphatase have been found in commercially available UDP-glucose pyrophosphorylase. UDP-glucose pyrophosphorylase was assayed in tissue and in the purified preparations by the method of Albrecht *et al.* (1966).

UDP-glucose dehydrogenase (EC 1.1.1.22) and inorganic pyrophosphatase (EC 3.6.1.1) were purchased from Sigma, St. Louis, Mo., U.S.A. Glucose 6-phosphate dehydrogenase (type I) (EC 1.1.1.49), phosphoglucomutase (EC 2.7.5.1) and all other enzymes were obtained from Boehringer (Mannheim) Corp.

Determination of metabolites

ATP, ADP, AMP and glucose 6-phosphate were measured enzymically as described by Veech *et al.* (1972). The enzymic P_i assay (method 1) described by Guynn *et al.* (1972) was modified in that the crystalline suspensions of the enzymes used in the assay were centrifuged together at 45000*g* for 15min at 0°C and the supernatant, containing $(NH_4)_2SO_4$, was discarded. The enzymes were resuspended in 0.3M-Tris–HCl, pH7.6, and used immediately in the assay. It has been found that recent commercial batches of enzymes differ in their suitability for the P_i assay. The elimination of $(NH_4)_2SO_4$ decreases the length of time for completion of the assay.

UDP-glucose was measured spectrophotometrically with UDP-glucose dehydrogenase by the method of Strominger *et al.* (1957). Identical results were

obtained when the UDP-glucose pyrophosphorylase assay system of Albrecht *et al.* (1966) was used as an assay for UDP-glucose. Recovery of standard UDP-glucose added to frozen liver powder and taken through the $HClO_4$ extraction was 99%.

UTP could theoretically be measured in the same cuvette at the completion of the UDP-glucose assay by addition of UDP-glucose pyrophosphorylase and glucose 1-phosphate. However, the preparation of UDP-glucose dehydrogenase hydrolysed UTP, and prevented a sequential assay of UDP-glucose and UTP. Therefore in a separate sample the UTP was converted into UDP-glucose and assayed with UDP-glucose dehydrogenase, the difference between this result (UTP + UDP-glucose) and the previously determined UDP-glucose being the UTP concentration. Reaction mixtures for determination of UTP + UDP-glucose contained 67mM-Tris–acetate, pH7.8, 3mM-$MgCl_2$, 1.5mM-NAD^+, and 3mM-glucose 1-phosphate. Inorganic pyrophosphorylase and UDP-glucose pyrophosphorylase were added to give activities equivalent to 0.1μmol of product formed/min in a final volume of 3ml. After incubation for 15min at room temperature (25°C) the total UDP-glucose was determined as described above. Recovery of UTP added to frozen liver powder was 103%.

Measurement of glucose 1-phosphate for the determination of $K_{eq.}$ and the preparation of standards was performed spectrophotometrically (Lowry & Passonneau, 1972). In the determination of the equilibrium constant, PP_i was measured in the same cuvette after completion of the determination of glucose 1-phosphate by addition of 0.2 unit of UDP-glucose pyrophosphorylase and 1μmol of UDP-glucose.

Glucose 1-phosphate was measured in tissue extracts by cycling the NADPH formed in the phosphoglucomutase and glucose 6-phosphate dehydrogenase reactions after elimination of glucose 6-phosphate from the tissue extracts and reagents. Volumes of 20μl of either samples or glucose 1-phosphate standards (0.2–4nM) were pipetted into 10mm×75mm disposable Corning culture tubes (Scientific Glass Co., Washington D.C., U.S.A.). The glucose 1-phosphate standards were prepared in saturated neutralized $KClO_4$. A reagent mixture (50μl) which consisted of 80mM-Tris–acetate, pH8.0, 0.2mM-$NADP^+$, 0.005mM-glucose 1,6-diphosphate and glucose 6-phosphate dehydrogenase (0.35 unit/ml of reagent mixture) was added to each tube. After 10min at room temperature the NADPH formed was destroyed by incubation with 10μl of 1M-HCl at room temperature for 3min. Each tube was neutralized with 10μl of 1M-NaOH. Another reagent mixture (100μl) which consisted of 118mM-Tris–HCl, pH8.0, 2.4mM-$MgCl_2$, 2.4mM-EDTA, 1.2mM-dithiothreitol, glucose 6-phosphate dehydrogenase (0.08 unit/ml of reagent mixture) and phosphoglucomutase (0.48 unit/ml) was then added to each tube. Duplicate samples which served as blanks were treated in the same fashion except that the phosphoglucomutase was omitted from the mixture. After 15min at room temperature the glucose 1-phosphate had reacted to give NADPH. The unchanged $NADP^+$ was then destroyed by incubation with 1M-NaOH (50μl) at 60°C for 15min (Lowry *et al.*, 1961*b*). The NADPH was measured fluorimetrically with enzymic cycling (Lowry *et al.*, 1961*a*). Where significant, the fluorescence generated in the blanks was subtracted from the fluorescence generated in the samples. Recovery of glucose 1-phosphate standard added to the frozen liver powder was 101%.

The rate of enzymic cycling was the same when glucose 1-phosphate was added to either neutralized $KClO_4$ or to $HClO_4$ extracts of liver from which glucose 6-phosphate and glucose 1-phosphate had previously been removed enzymically.

Calculations

The free cytoplasmic [ATP]/[ADP][P_i] ratio was calculated from the concentration of the individual metabolites measured in the whole liver. These measurements are reasonable estimates of the free cytoplasmic concentrations of ATP, ADP and P_i (Veech *et al.*, 1970). Although evidence has been presented that AMP may be compartmented under certain special circumstances (Veech *et al.*, 1972), the close approximation of the ratio [ATP][AMP]/[ADP]^2 measured *in vivo* to that expected at equilibrium (Veech *et al.*, 1972) indicates that the measured content of AMP is also a fair approximation of the cytoplasmic concentration. For this reason total measured AMP content has been taken to represent the free cytoplasmic concentration.

The free cytoplasmic PP_i concentration was calculated from eqn. (2) where $K_{\text{UDP-glucose pyrophosphorylase}}$ was taken to be 4.55 (see the Results section). The total measured content of UTP, UDP-glucose and glucose 1-phosphate were taken to be the cytoplasmic concentrations. No great error is likely to result from this assumption for UTP and UDP-glucose, since these compounds are similar to ATP and ADP in that they are present in high concentrations relative to the concentration of potential enzymic binding sites. The low measured content of glucose 1-phosphate in tissues makes the assumption that this value is equal to the free cytoplasmic concentration less certain (see the Results section).

The [ATP]/[AMP][PP_i] ratio is calculated from the measured content of ATP and AMP and the calculated concentration of PP_i. To achieve consistency between the phosphorylation state and the [ATP]/[AMP][PP_i] ratio it should be noted that the phosphorylation state in this paper is reported as the

[ATP]/[ADP][P_i] ratio, not the [ATP]/[ADP]-[HPO_4^{2-}] ratio as reported by Veech *et al.* (1970).

Results

Activity of cytoplasmic enzymes that have PP_i as a substrate

The cytoplasmic enzyme with the highest measured activity for which PP_i is a substrate and whose other substrates are measurable was UDP-glucose pyrophosphorylase (Table 1). The enzyme was present in rat liver in high activity with no statistically significant differences between the dietary groups studied. The activity measured in rat liver was comparable with those found in calf liver (Albrecht *et al.*, 1966). In contrast, the reported activities of argininosuccinate synthetase (Schimke, 1962) and acetyl-CoA synthetase (Söling, 1974) were much lower than those of UDP-glucose pyrophosphorylase and showed variation with dietary state. A value of 22.3 units/min per g wet wt. has been reported for inorganic pyrophosphatase in the soluble portion of fractionated rat liver (Nordlie & Lardy, 1961). However, in that work no special attempt was made to document the purity of the fractions.

Equilibrium constants of the phosphoglucomutase and UDP-glucose pyrophosphorylase reactions.

The equilibrium constant of the phosphoglucomutase reaction has previously been determined to be 17 at 25°C, pH7.0 and total magnesium = 25mM (Atkinson *et al.*, 1961). It was therefore thought to be necessary to re-determine the value of $K_{eq.}$ under more physiological conditions where $[K^+] = 125$mM, $[Na^+] = 25$mM, $[P_i] = 5$mM, $I = 0.25$mol/l, 38°C, pH7.04±0.004 and free $[Mg^{2+}] = 0.8$mM (Veloso *et al.*, 1973). Equilibrium was achieved from both directions after 2h of incubation under these conditions and the reaction was terminated with either $HClO_4$ or KOH. The nature of the agent used to terminate the reaction did not affect the measurement of either glucose 6-phosphate or glucose 1-phosphate.

The mean value of the ratio [glucose 6-phosphate]/[glucose 1-phosphate] obtained in ten experiments (five forward and five reverse) was 17.4±0.3. This value agrees well with the earlier value reported under different conditions (Atkinson *et al.*, 1961), implying that the effects of changes in free $[Mg^{2+}]$ and temperature (see Colowick & Sutherland, 1942) must approximately cancel.

For the UDP-glucose pyrophosphorylase reaction it would be expected that the $K_{eq.}$ should vary with pH and $[Mg^{2+}]$ (Turner & Turner, 1958), since the ionic species of pyrophosphate (Irani, 1961; Irani & Taulli, 1966) have binding constants for Mg^{2+} which vary over two orders of magnitude (Lambert & Matters, 1957; Wu *et al.*, 1967). Previously determined equilibrium constants of the UDP-glucose pyrophosphorylase reaction at various pH values and total magnesium concentrations varied between 2 and 8 (Albrecht *et al.*, 1966; Knop & Hansen, 1970; Gustafson & Gander, 1972), but were not determined under appropriate near-physiological conditions. Accordingly the $K_{eq.}$ was determined under the same conditions as used for the phosphoglucomutase reaction except that P_i concentration was 3mM, free $[Mg^{2+}] = 1.0$mM and pH was 7.07±0.011. Equilibrium was reached from both directions within 1h and the reactions were terminated by the addition of $HClO_4$ cooled to −25°C. After neutralization with KOH, the concentrations of all reactants were measured and the value of $K_{eq.}$ was determined. The mean value of $K_{eq.}$ in the forward direction was 4.33±0.15 (nine determinations); and in the reverse direction 4.77±0.11 (nine determinations). An average of 4.55±0.10 has been taken.

$$K_{\text{UDP-glucose pyrophosphorylase}} = \frac{[\text{UTP}][\text{glucose 1-phosphate}]}{[\text{UDP-glucose}][PP_i]} = 4.55 \pm 0.10$$

This value agrees well with the value of 3.5 (pH7.0; 30°C) of Turner & Turner (1958). No attempt was made to evaluate the constant at other pH values; however, Turner & Turner (1958) have found the constant to be relatively insensitive to $[H^+]$ between

Table 1. *Activity of UDP-glucose pyrophosphorylase in rat liver under various dietary conditions*

Activity was measured spectrophotometrically at 25°C in the direction of glucose 1-phosphate production; for details see the Experimental section. Activity is expressed as μmol of products formed/min per g fresh wt. of liver, ±S.E.M. The number of observations is given in parentheses. The activity of argininosuccinate synthetase is taken from Schimke (1962) and that of acetyl-CoA synthetase from Söling (1974). For the details of feeding each group see the Experimental section.

Group	Diet	UDP-glucose pyrophosphorylase	Argininosuccinate synthetase	Acetyl-CoA synthetase
1	Starved	54±1 (6)	4.4±0.8 (8)	0.53±0.06 (8)
2	Fed *ad libitum*	55±4 (4)	3.0±0.3 (8)	1.1±0.1 (4)
3	Meal-fed	58±4 (4)		
4	Sucrose re-fed	46±5 (5)		

pH7 and 8, reporting values of $K_{eq.}$ of 3.5 and 8.4 at pH7.0 and 7.9 respectively. Therefore a major error would not be anticipated in the use of the constant reported here at pH7.1 for the physiological range of pH values.

Metabolites in freeze-clamped liver of rats under various dietary conditions

Metabolic concentrations presented in Table 2 show that [ATP] and [P_i] were unchanged in the conditions studied, whereas the [ADP] was significantly lower in the group fed *ad libitum* and the sucrose-re-fed groups. [AMP] was significantly lower in the sucrose-re-fed group compared with the starved group. [UDP-glucose] and [UTP] in the group fed *ad libitum* and sucrose-re-fed group were increased compared with the starved animals, whereas [glucose 1-phosphate] and [glucose 6-phosphate] were increased in all fed groups compared with the starved group. The UTP and UDP-glucose concentrations found in the 48h-starved animals were approx. 50% higher than those reported in the 24h-starved animals (Burch *et al.*, 1969), although the ratio [UTP]/[UDP-glucose] was very similar. The [glucose 1-phosphate] found in the animals fed *ad libitum* was almost identical with that determined by using direct fluorimetric analysis (Henley, 1968). The ratio of [glucose 6-phosphate]/[glucose 1-phosphate] remained essentially unchanged at values between 10.6 and 12.9 even though [glucose 6-phosphate] and [glucose 1-phosphate] varied by a factor of 2.5 in the groups studied. The value of this ratio has previously been reported to be 25 in liver (Henley, 1968) and to vary between 12 and 25 in perfused rat heart (Williamson, 1965). The ratio [glucose 6-phosphate]/[glucose 1-phosphate] expected at equilibrium is 17.4. The equilibrium constant of the ratio [α-D-glucose 6-phosphate]/[α-D-glucose 1-phosphate] has been estimated to be 7 (Lowry & Passonneau, 1969). Although it is possible that the ratio found in this study reflects a disequilibrium between the α and β forms of the hexose phosphates within the liver cell, with a preponderance of the α forms accounting for the low ratio, this seems unlikely. If liver glucose phosphate isomerase (EC 5.3.1.9) possesses the same potent mutarotase activity as has been reported for the yeast (Salas *et al.*, 1965) and the rabbit muscle (Lowry & Passonneau, 1969) enzymes, a more likely possibility to account for the low [glucose 6-phosphate]/[glucose 1-phosphate] ratio found in freeze-clamped liver might be that 30–40% of the measured glucose 1-phosphate is either bound to a macromolecular cell constituent or is not free and thermodynamically active in the cytoplasmic compartment. The possibility that these deviations from equilibrium were due in all cases to flux through the phosphoglucomutase step is not likely, since that would imply that in both the fed and starved animals the flux was always in the direction of glucose 1-phosphate to glucose 6-phosphate. In any case, the deviation from equilibrium found is small and confirms that the phosphoglucomutase step does fall within the category of near-equilibrium reactions. These findings further imply that no serious error is involved in taking the total measured glucose 1-phosphate concentration to be a good estimate of the free concentration in cytoplasm of rat liver even though its concentration is relatively low.

Free cytoplasmic [PP_i] in rat liver in different dietary conditions

The free cytoplasmic [PP_i] in rat liver calculated from eqn. (2) was 1.7nmol/g wet wt. in the starved animals and increased significantly in all the fed groups (2.9–4.0nmol/g wet wt.). These values are in very good agreement with the direct measurement of total PP_i in the fed rat (6.2±0.31 nmol/g wet wt.) by

Table 2. *Concentrations of metabolites in freeze-clamped livers of rats under different dietary conditions*

Values are given in μmol/g wet wt., ±S.E.M.; six observations were made for each group. The significance of the difference between the starved and other groups as judged by Student's *t* test is indicated by $*P<0.05$ to $P<0.02$ and $**P<0.01$ to $P<0.001$. For the details of feeding each group see the Experimental section.

	Starved	Fed *ad libitum*	Meal-fed	Sucrose re-fed
ATP	2.49±0.12	2.56±0.09	2.32±0.07	2.26±0.11
ADP	1.38±0.08	1.06±0.03**	1.24±0.04	1.17±0.04*
AMP	0.343±0.024	0.313±0.022	0.384±0.028	0.239±0.016**
P_i	4.73±0.16	3.64±0.32	4.41±0.10	4.07±0.23
UDP-glucose	0.342±0.024	0.433±0.023*	0.347±0.027	0.671±0.046**
UTP	0.362±0.014	0.494±0.038**	0.443±0.039	0.648±0.074**
Glucose 6-phosphate	0.078±0.011	0.147±0.012*	0.157±0.007**	0.205±0.013**
Glucose 1-phosphate	0.0075±0.0010	0.0115±0.008**	0.0132±0.0007**	0.0190±0.001**
Glucose 6-phosphate / Glucose 1-phosphate	10.9±1.4	12.9±1.0	12.0±0.2	10.6±0.3

Table 3. *Calculated free cytoplasmic [PP_i] and the cytoplasmic pyrophosphorylation and phosphorylation state in freeze-clamped liver under different dietary conditions*

For the method of calculation, see the text. Calculated [PP_i] is given as nmol/g wet wt. ±S.E.M. The phosphorylation and pyrophosphorylation states are reported as M^{-1} ±S.E.M. assuming that 1 μmol/g is approximately equal to 1 mM. Six observations were made for each group. The significance of the difference between the starved and other groups as judged by Student's *t* test is indicated by $*P<0.05$ to $P<0.02$ and $**P<0.01$ to $P<0.001$. For the details of feeding each group see the Experimental section.

	Starved	Fed *ad libitum*	Meal-fed	Sucrose-re-fed
Free cytoplasmic [PP_i] (nmol/g wet wt.)	2.3±0.3	3.8±0.4*	4.9±0.6**	5.2±0.4**
$\frac{[ATP]}{[AMP][PP_i]}$ (M^{-1})	3.54×10^6 ±0.52×10^6	2.57×10^6 ±0.02×10^6	1.39×10^{6}** 0.19×10^6	1.95×10^{6}* ±0.30×10^6
$\frac{[ATP]}{[ADP][P_i]}$ (M^{-1})	3.66×10^2 ±0.32×10^2	7.10×10^{2}* ±0.85×10	4.2×10^2 ±0.22×10^2	4.97×10^2 ±0.65×10^2

using an isotope-dilution method (H. Flodgaard, personal communication). In the starved rat our data consistently showed a lower calculated free cytoplasmic PP_i concentration, whereas measurements of total liver PP_i (6.5±0.86nmol/g wet wt.) by the isotope-dilution method remained essentially unchanged (H. Flodgaard, personal communication). The cause of this discrepancy between the total and free cytoplasmic PP_i in the starved animals is unclear at present and cannot be explained by disequilibrium caused by a lack of sufficient UDP-glucose pyrophosphorylase activity (Table 1).

Cytoplasmic phosphorylation and [ATP]/[AMP][PP_i] ratio under different dietary conditions

The [ATP]/[AMP][PP_i] ratio calculated from the free cytoplasmic [PP_i] was 3 to 4 orders of magnitude greater than the [ATP]/[ADP][P_i] ratio (Table 3). The [ATP]/[AMP][PP_i] was a factor of 9.4×10^3 greater than the [ATP]/[ADP][P_i] in the starved group and 3.6×10^4, 3.3×10^4 and 3.9×10^4 greater in the three fed groups. The [ATP]/[AMP][PP_i] ratio reached its highest value in the starved group and decreased by a maximum factor of 2.5 in the meal-fed group. This decrease on feeding was opposite to change in the phosphorylation state, which was highest in the group fed *ad libitum* and decreased by a factor of 1.9 in the starved group.

Discussion

Our data do not agree with the widely accepted concept that *in vivo* the PP_i formed by the activation of fatty acids or amino acids or by the DNA polymerase reaction is rapidly hydrolysed by inorganic pyrophosphatase, essentially driving these PP_i-producing reactions to completion (Lehninger, 1970). The previous reports (Reich *et al.*, 1968) of substantial quantities of total PP_i in tissue did not necessarily contradict this concept, since measured total PP_i could be extensively bound or compartmentalized. However, the excellent agreement between the free cytoplasmic [PP_i] calculated from the UDP-glucose pyrophosphorylase reaction and the total measured PP_i content of rat liver (H. Flodgaard, personal communication) suggests that most of the measured total PP_i is in fact free and located in the compartment containing UDP-glucose pyrophosphorylase, namely the cytoplasm.

The ratio $[P_i]^2/[PP_i]$ in rat liver cytoplasm was 9.8, 3.2, 4.1 and 3.1 M respectively in the starved, fed *ad libitum*, meal-fed and sucrose-re-fed dietary states. The ratio $[P_i]^2/[PP_i]$ expected at equilibrium under physiological conditions of temperature, ionic strength and free [Mg^{2+}] is about 1700M (J. W. R. Lawson, unpublished work). Therefore the pyrophosphorylase reaction, like the ATPase (adenosine triphosphatase) reaction, is not near equilibrium *in vivo*. It thus seems very likely that the components of the reaction of the hydrolysis of ATP to AMP plus PP_i are near equilibrium with a number of powerful near-equilibrium systems and that the steady-state concentration of PP_i responds to changes in these linked systems rather than being primarily the resultant of the reaction of PP_i with H_2O to form $2P_i$. In turn, if it is accepted that the cytoplasmic free [PP_i] is in a state of near-equilibrium with UDP-glucose pyrophosphorylase then PP_i should be thought of as one of that small number of inorganic ions such as H^+, NH_4^+, CO_2 and P_i (see Krebs & Veech, 1969) that may act as metabolites and thus influence some of the major pathways of intermediary metabolism.

It has been customary, in attempting to determine the free concentration of a metabolite or nucleotide ratio in a particular compartment, to analyse the components of at least two enzymic reactions of high activity that are located in the same compartment (Williamson *et al.*, 1967). In attempting to determine the free cytoplasmic PP_i it was not possible to find two cytoplasmic enzymes whose reactants can be accurately measured and whose localization is known. At least two other cytoplasmic reactions with PP_i as a substrate might potentially catalyse a near-equilibrium with the cytoplasmic free PP_i, but both are unsuitable for this kind of analysis. The components

of the acetyl-CoA synthetase reaction are not suitable because of the uncertainty of the localization and the extent of the binding of acetyl-CoA and CoA. The components of the argininosuccinate synthetase reaction are not suitable because of the difficulty of measuring argininosuccinate in extracts from liver. It does appear, however, that the most active cytoplasmic enzyme with PP_i as a substrate, the UDP-glucose pyrophosphorylase reaction (Table 1), is suitable. The presumption that this reaction is near equilibrium is supported by the finding of a high activity in all dietary states (Table 1) coupled with a relatively low estimated flux of glucose 1-phosphate to UDP-glucose of only 0.3μmol/min per g wet wt. in mouse liver (Reich *et al.*, 1968).

Since the pyrophosphatase reaction does not seem to hydrolyse the cytoplasmic PP_i to the low concentrations expected at near-equilibrium, the question arises as to what consequence this might have on reactions such as amino acid and fatty acid activation. Under physiological conditions of $I = 0.25$mol/l, 38°C, free $[Mg^{2+}] = 1$mM and pH7.0, the ΔG° for the hydrolysis of the terminal pyrophosphate bond of ATP is −31.8kJ/mol (Guynn & Veech, 1973), whereas that for the hydrolysis of the inner pyrophosphate bond is −41.4kJ/mol (R. W. Guynn, L. T. Webster, Jr. & R. L. Veech, unpublished work). At the concentrations of reactants which exist in rat liver cytoplasm (Table 3), the hydrolysis of ATP to ADP and P_i would release a total of −47.3kJ/mol and the hydrolysis of ATP to AMP and PP_i would release a total of −68.2kJ/mol. Therefore even with a free $[PP_i]$ of the order calculated in the present study, reactions that are coupled to the hydrolysis of PP_i from ATP would still be driven considerably further towards completion than those coupled to the hydrolysis of ATP to P_i. The increased energy of the latter reaction derives −9.55kJ/mol more energy from the greater energy of the inner as opposed to the outer pyrophosphate bond of ATP. The additional −11.3kJ/mol comes from the difference between the cytoplasmic $[ATP]/[ADP][P_i]$ and the $[ATP]/[AMP][PP_i]$ ratios.

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