RELATIONSHIP OF FREE CYTOPLASMIC PYROPHOSPHATE TO LIVER GLUCOSE CONTENT AND TOTAL PYROPHOSPHATE TO CYTOPLASMIC PHOSPHORYLATION POTENTIAL

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1. Introduction

H. A. Krebs has pointed out the importance to metabolic regulation of certain small inorganic ions which are not normally thought of as metabolites. The compounds discussed were CO_2, HCO_3^-, H^+, NH_4^+ and P_i [1]. It has been appreciated subsequently that Mg^{2+}, while not subject to large intracellular variations under most physiological conditions, [2] has appreciable effects on the apparent equilibrium constants of a number of important intracellular reactions [3,4] and should be included in this list. In this paper we attempt to show that inorganic pyrophosphate (PP_i) is another small inorganic ion which plays an important role in the regulation of a number of metabolic processes in mammals as a result of hormonal or dietary changes.

Pyrophosphate has long been known to play an important role in intermediary metabolism in some microorganisms [5] and in *Entamoeba histolytica* [6], where it acts in place of ATP as an energy source in the phosphorylation of fructose 6-phosphate and in other key reactions of the glycolytic and gluconeogenic pathways. Pyrophosphate has been shown to be the product of photophosphorylation in *Rhodospirillum rubrum* [7] and may play a role in mammalian oxidative phosphorylation [8]. This view has not been generally accepted because of the widespread, but in our view erroneous, belief that pyrophosphate is rapidly hydrolyzed in cells to inorganic orthophosphate. In data not presented here, we have shown that under appropriate conditions, injection of short chain fatty acids can raise hepatic PP_i content to 2–3 μmol/g wet wt. If the bulk of the pyrophosphate were confined to mitochondrial matrix where it is generated during short chain fatty acid activation, and if PP_i were all in free solution in the matrix, the mitochondrial $[PP_i]$ would be >20 mM.

It has long puzzled pharmacologists that some of the effects of dibutyryl cyclic AMP can be mimicked by butyrate alone [9]. Certain abnormalities of transformed cells in culture can be returned toward normal by addition of butyrate to cell cultures, which raises the possibility that butyrate affects the acetylation reactions of nuclear histones. Our findings suggest that pyrophosphate, either directly administered or produced intramitochondrially by activation of short chain fatty acids, produces effects which mimic glucagon injection in that the free cytoplasmic $[NADP^+]$/[NADPH] is decreased, liver glucose is increased, ketone body concentrations are increased and there is a change in 3-phosphoglycerate/pyruvate ratios suggestive of inhibition of pyruvate kinase [11]. Since glucagon injection produces cAMP at levels of 8 nmol/g and should therefore produce PP_i in equal amounts, distinguishing cAMP effects from PP_i effects after glucagon stimulation will be difficult, particularly in view of the likely reciprocal relationship between free $[Ca^{2+}]$ and free $[PP_i]$ because of the extreme insolubility of Ca_2PP_i.

In addition to the effects of PP_i on glucose metabolism, the finding of relatively high cellular contents of PP_i raises the possibility that reactions of nucleic acid or protein synthesis might be affected. The reactions catalysed by the various DNA polymerases are of the form:

$$\text{Desoxy-XTP} + (\text{desoxy-XMP})_n \overset{Mg^{2+}}{\rightleftharpoons} (\text{Desoxy-XMP})_{n+1} + PP_i$$

Dedicated to Professor Sir Hans Krebs, FRS, on his eightieth birthday

These reactions are known to be reversible in the presence of PP_i [12]. The widely held view that PP_i is instantly hydrolyzed to ensure the inviolability of DNA needs reconsideration. Whether the concentrations of pyrophosphate achieved in this study are high enough to inhibit or reverse the reactions of DNA or RNA polymerases remains to be seen. It may, however, be significant that freshly isolated hepatocytes treated with 5–7 mM butyrate are reported to show decreased incorporation of [^{3}H]orotic acid into RNA without increasing [^{3}H]acetate incorporation into nuclear histones [13].

2. Experimental

Male Wistar rats (180–230 g body wt) were purchased from Charles River Labs. (Wilmington, MA) and received food (NIH stock diet) and water ad libitum. All animals were killed in the morning and stomach contents were checked to be certain that all animals were well fed.

Butyric acid and oleic acid were purchased from Sigma Chemical Co. (St Louis, MO). Sodium acetate was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Commercially available enzymes were obtained from Boehringer-Mannheim (Indianapolis, IN). The pyrophosphate–fructose 6-phosphate–phosphotransferase for PP_i assays was a gift of Dr W. E. O'Brien, Baylor College of Medicine (Houston, TX). All other materials were reagent grade and obtained from commercial sources.

Rats were injected intraperitoneally with glucose (20 mmol/kg), sodium phosphate (20 mmol/kg), NaCl (20 mmol/kg), sodium acetate (20 mmol/kg), sodium butyrate (20 mmol/kg), sodium oleate (3 mmol/kg) or physiological saline (2 ml) 15 min before killing. Sodium pyrophosphate (1 mmol/kg) was injected 20 min before killing. All solutions were adjusted to pH 7.0.

Rats were killed by cervical dislocation and livers were rapidly removed and frozen by clamping between two aluminium discs which had been cooled in liquid nitrogen [14]. Perchloric acid extracts of frozen liver were prepared as in [15] except that the extracts were not treated with Florisil; the extracts were adjusted to pH 5–6 rather than pH 7 in order to increase the recovery of PP_i [16].

PP_i was measured by the method of Cook et al. [16]. Inorganic orthophosphate was measured using a modification [17] of the method of Guynn et al. [18]. Lactate, malate, glucose 6-phosphate, glucose 1-phosphate, isocitrate and ATP were measured as described by Lowry and Passonneau [19]. ADP and

Table 1
Values of equilibrium constants

		Definition	Value	Ref.
K_{LDH}	=	$\frac{[\Sigma\text{pyruvate}][NADH]}{[\Sigma\text{lactate}][NAD^+]}$	= 1.11×10^{-4}	[29]
K_{ICDH}'	=	$\frac{[\Sigma\alpha\text{-ketoglutarate}][CO_2][NADPH]}{[\Sigma\text{isocitrate}][NADP^+]}$	= 1.17 M	[34]
K_{HBDH}	=	$\frac{[\Sigma\text{acetoacetate}][NADH]}{[\Sigma\beta\text{-hydroxybutyrate}][NAD^+]}$	= 4.93×10^{-2}	[29]
$K_{G+GK_{LDH}}$	=	$\frac{[\Sigma 3PG]}{[\Sigma DHAP]/22} \frac{[\Sigma ATP]}{[\Sigma ADP][\Sigma P_i]} \frac{[\Sigma\text{lactate}]}{[\Sigma\text{pyruvate}]}$	= $1.65 \times 10^7\ M^{-1}$	[4]
K_{MYK} at pH 7.2	=	$\frac{[\Sigma ATP][\Sigma AMP]}{[\Sigma ADP]^2}$	= 1.12	[4]
K_{PP_i}–Glucose phosphotransferase	=	$\frac{[\Sigma\text{glucose-6-P}][\Sigma P_i]}{[\Sigma\text{glucose}][\Sigma PP_i]}$	= 45.9	[20]
$K_{UDPG\ PP_i ase}$	=	$\frac{[\Sigma UTP][\Sigma\text{glucose-1-P}]}{[\Sigma\text{UDP-glucose}][\Sigma PP_i]}$	= 4.55	[35]

Values are given at an ionic strength = 0.25, + = 38°C, free $[Mg^{2+}]$ = 1.0 mM and pH 7.0 $[CO_2]$ was taken to be 1.16 mM

AMP were assayed as described by Adam [20] except that Tris buffer was used instead of TEA. All other metabolites were assayed as described in [21].

Free cytosolic [PP_i], mitochondrial free [NAD^+]/[NADH], cytosolic free [$NADP^+$]/[NADPH] and cytosolic free [NAD^+]/[NADH] were calculated as in [21]. Free cytosolic [ΣATP]/[ΣADP][ΣP_i] ratios, free cytosolic [ΣADP] and myokinase ratios were calculated as described by Veech et al. [4]. Equilibrium constants of relevant reactions are given in table 1.

3. Results and discussion

There is no significant gradient of glucose concentration across the liver plasma membrane; therefore the concentration of blood glucose approximately reflects that of liver glucose. While increase of glucose 6-phosphate concentration is inevitably accompanied by increases in glucose 1-phosphate and fructose 6-phosphate, because of the near-equilibrium reactions catalysed by glucosephosphate isomerase and phosphoglucomutase, there is no constant relationship between glucose and glucose 6-phosphate. As the data in table 2 indicate, there is a general correlation between the content of glucose and that of glucose 6-phosphate, but there are notable exceptions to this. For example, 1 h after injection of bovine growth hormone there is a 2-fold rise in glucose 6-phosphate concentration and a fall in that of glucose [11]. Following induction of streptozotocin diabetes there is a 3-fold increase in liver glucose level accompanied by a 50% decrease in liver glucose 6-phosphate [21].

Many reactions contribute to the establishment of the steady state concentration of glucose 6-phosphate, which can be produced from glycogen via phosphoryl-

Table 2
Metabolite content of freeze-clamped rat liver

	Control	Phosphate (20 mmol/kg)	Pyrophosphate (1 mmol/kg)	Glucose (20 mmol/kg)	NaCl (20 mmol/kg)	Acetate (20 mmol/kg)	Butyrate (20 mmol/kg)	Oleate (3 mmol/kg)
n =	16	6	6	6	6	6	5	6
Glucose	7.30 ±0.16	11.1 ±0.3	15.7 ±0.7	75.9 ±1.4	7.93 ±0.37	12.4 ±0.2	15.68 ±0.54	11.0 ±0.4
Glucose 6-P	0.121 ±0.007	0.693 ±0.039	0.418 ±0.033	0.429 ±0.049	0.175 ±0.005	0.604 ±0.055	0.777 ±0.023	0.421 ±0.021
Glucose 1-P	0.010 ±0.0004	0.053 ±0.004	0.032 ±0.001	0.023 ±0.002	0.011 ±0.001	0.045 ±0.005	0.055 ±0.002	0.026 ±0.002
Dihydroxy-acetone-P	0.029 ±0.001	0.041 ±0.003	0.038 ±0.002	0.098 ±0.006	0.051 ±0.005	0.028 ±0.003	0.039 ±0.003	0.048 ±0.002
3-Phosphoglycerate	0.309 ±0.013	0.673 ±0.043	0.850 ±0.021	0.409 ±0.024	0.369 ±0.031	0.556 ±0.027	0.501 ±0.023	0.523 ±0.050
Pyruvate	0.086 ±0.009	0.219 ±0.013	0.095 ±0.008	0.372 ±0.034	0.165 ±0.025	0.069 ±0.013	0.067 ±0.005	0.133 ±0.011
Lactate	0.444 ±0.031	3.91 ±0.40	0.685 ±0.109	2.74 ±0.18	0.812 ±0.110	0.657 ±0.009	0.925 ±0.124	0.765 ±0.060
α-Ketoglutarate	0.279 ±0.019	0.124 ±0.018	0.095 ±0.013	0.169 ±0.006	0.276 ±0.021	0.134 ±0.026	0.072 ±0.008	0.179 ±0.022
Isocitrate	0.030 ±0.002	0.065 ±0.003	0.017 ±0.001	0.029 ±0.022	0.039 ±0.002	0.055 ±0.004	0.049 ±0.002	0.021 ±0.002
Glutamate	3.86 ±0.12	2.31 ±0.11	2.27 ±0.14	1.57 ±0.44	3.15 ±0.10	2.27 ±0.15	1.87 ±0.16	-- --
Malate	0.274 ±0.015	0.691 ±0.030	0.210 ±0.014	0.362 ±0.020	0.401 ±0.018	0.845 ±0.095	1.27 ±0.17	0.223 ±0.025
Acetoacetate	0.117 ±0.009	0.092 ±0.008	0.195 ±0.014	0.088 ±0.009	0.123 ±0.015	0.107 ±0.021	0.793 ±0.06	0.173 ±0.007
β-Hydroxy-butyrate	0.156 ±0.015	0.274 ±0.025	0.302 ±0.025	0.217 ±0.009	0.225 ±0.057	0.266 ±0.015	1.66 ±0.10	0.137 ±0.011

Values are in μmol/g wet wt liver ± SEM

ase and phosphoglucomutase, or from glucose via glucokinase. It can be dispersed via glycogen synthase, glucose 1-phosphate uridylyltransferase and phosphoglucomutase, or via glucose 6-phosphate dehydrogenase and the hexose monophosphate pathway, or via glucose phosphate isomerase, phosphofructokinase and the glycolytic pathway. Finally, glucose 6-phosphate may leave the liver as glucose, as a result of the action of glucose 6-phosphatase. With the exception of the reactions catalyzed by glucosephosphate isomerase and phosphoglucomutase, the products of which are in near-equilibrium with glucose 6-phosphate, all the other reactions accounting for the formation or utilization of glucose 6-phosphate have generally been thought to be controlled by regulation of enzyme activity, either by allosteric effectors or by covalent modification. Under this type of control, no constancy of the glucose/glucose 6-phosphate ratio would be expected. The data presented in tables 3 and 4, however, indicate that under most conditions studied, a state of near-equilibrium exists in the overall reaction represented as eq. (1). The value of the equilibrium constant for this reaction at ionic strength 0.25, 1 mM free Mg^{2+} and 38°C is [21]:

$$K_{eq.} = \frac{[\text{Glucose 6-phosphate}]\,[P_i]}{[\text{Glucose}]\,[\text{free cytopl. } PP_i]} = 45.9 \qquad (1)$$

In control animals, this ratio was 48.5. Fifteen minutes after injection of inorganic orthophosphate, the observed ratio was 173 indicating that near-equilibrium had not yet been re-established, but that the deviation was in the direction expected by increasing P_i. In a similar fashion, injection of glucose induced the expected disequilibrium where the metabolite ratio for eq. (1) was 4.6. Rather surprisingly, injection of PP_i resulted in only a 4-fold rise in free cytoplasmic pyrophosphate even though total PP_i content increased 100-fold. It is not possible to determine whether the measured PP_i, after PP_i injection, was in the mitochondria or whether a large portion of it remained in the extracellular space. In any case, the largest proportion of injected PP_i appeared to be separated from the glucose 1-phosphate uridylyltransferase activity. The value for eq. (1) of 38.2 suggested that the pyrophosphate–glucose–phosphotransferase reaction was maintained near-equilibrium. Near-equilibrium was re-established, after pyrophosphate injec-

Table 3
Phosphorylated nucleotides and phosphate content of freeze-clamped rat liver

	Control	Phosphate (20 mmol/kg)	Pyrophosphate (1 mmol/kg)	Glucose (20 mmol/kg)	NaCl (20 mmol/kg)	Acetate (20 mmol/kg)	Butyrate (20 mmol/kg)	Oleate (3 mmol/kg)
n =	6	6	6	6	6	6	5	6
ATP	2.72 ±0.07	2.35 ±0.08	2.82 ±0.06	2.50 ±0.05	2.78 ±0.08	2.47 ±0.08	1.58 ±0.07	2.68 ±0.06
Measured ADP	1.02 ±0.03	1.39 ±0.05	1.16 ±0.04	0.98 ±0.03	0.92 ±0.04	1.55 ±0.03	1.47 ±0.04	0.827 ±0.049
Calculated Free Cytoplasmic ADP	0.061 ±0.004	0.051 ±0.005	0.084 ±0.006	0.034 ±0.005	0.041 ±0.005	0.159 ±0.028	0.075 ±0.012	0.072 ±0.009
AMP	0.205 ±0.010	0.314 ±0.031	0.227 ±0.021	0.184 ±0.006	0.129 ±0.011	0.453 ±0.021	0.918 ±0.077	0.139 ±0.016
P_i	3.45 ±0.06	18.24 ±0.91	7.08 ±0.37	3.23 ±0.11	3.49 ±0.13	4.45 ±0.021	5.13 ±0.24	3.18 ±0.20
UTP	0.281 ±0.021	0.305 ±0.003	0.331 ±0.025	0.258 ±0.022	0.149 ±0.023	0.209 ±0.022	0.240 ±0.036	0.333 ±0.036
UDPG	0.509 ±0.022	0.517 ±0.024	0.471 ±0.023	0.326 ±0.009	0.474 ±0.011	0.495 ±0.016	0.526 ±0.016	0.555 ±0.030
Measured PP_i	0.0144 ±0.0014	0.0323 ±0.0022	1.58 ±0.14	0.0145 ±0.0004	0.0180 ±0.0007	0.142 ±0.060	0.119 ±0.067	0.0150 ±0.0006
Calculated Cytoplasmic PP_i	0.0012 ±0.00006	0.0070 ±0.0008	0.0050 ±0.0003	0.0039 ±0.0004	0.0009 ±0.0001	0.0042 ±0.0007	0.0055 ±0.0008	0.0033 ±0.002

Values are given in μmol/g wet wt ± SEM

Table 4
Free nucleotide ratios in freeze-clamped rat liver

	Control	Phosphate (20 mmol/kg)	Pyrophosphate (1 mol/kg)	Glucose (20 mmol/kg)	NaCl (20 mmol/kg)	Acetate (20 mmol/kg)	Butyrate (20 mmol/kg)	Oleate (3 mmol/kg)
n =	16	6	6	6	6	6	5	6
Cytoplasmic $[NAD^+]/[NADH]$ from K_{LDH}	1750 ±130	520 ±40	1360 ±180	1230 ±110	1790 ±160	950 ±100	700 ±90	1600 ±160
Mitochondrial $[NAD^+]/[NADH]$ from $K_{\beta HBDH}$	16 ±1	7 ±1	14 ±2	8 ±1	12 ±1	8 ±1	10 ±2	26 ±2
Cytoplasmic $[NADP^+]/[NADPH]$ from K_{ICDH}	0.010 ±0.001	0.0019 ±0.0003	0.0055 ±0.0005	0.0060 ±0.0005	0.0072 ±0.0008	0.0024 ±0.0004	0.0015 ±0.0002	0.0087 ±0.0009
Measured $[ATP]/[ADP][P_i]\ M^{-1}$	795 ±52	96 ±10	353 ±25	802 ±56	899 ±97	363 ±24	214 ±19	1076 ±125
Calculated $[ATP]/[ADP][P_i]\ M^{-1}$ from K_{G+G}	14000 ±1200	2700 ±310	5040 ±670	25300 ±3900	20900 ±2300	4800 ±1000	4600 ±700	13000 ±1800
Measured $\frac{[ATP][AMP]}{[ADP]^2}$	0.538 ±0.031	0.375 ±0.012	0.476 ±0.032	0.484 ±0.024	0.429 ±0.026	0.464 ±0.012	0.663 ±0.023	0.553 ±0.065
$\frac{[G6\text{-}P][P_i]}{[Glucose][PP_i]}$	48.5 ±2.9	173 ±20	38.2 ±3.3	4.6 ±0.3	96.3 ±19	55.7 ±6.3	50.8 ±8.4	37.3 ±3.2
$\frac{3\text{-Phosphoglycerate}}{\text{Pyruvate}}$	3.5	3.1	8.9	1.1	2.2	8.1	7.4	3.9

For method of calculation see text and table 1

tion by: (i) doubling the liver glucose; (ii) doubling the liver P_i; (iii) increasing glucose 6-phosphate by 4-fold; (iv) increasing the free cytoplasmic pyrophosphate 4-fold. The enzymic pathway catalyzing this near-equilibrium reaction cannot be defined at present; however, such a relationship does occur in vivo. Changes in free cytoplasmic PP_i concentration cannot, therefore, be ignored in considering the control of liver and blood glucose levels, nor in considering the mode of action of hormones which affect blood glucose concentrations.

Interest in control of liver glucose levels has generally focused on the control of either the glucokinase reaction [22] or the reactions of glycogen synthesis and breakdown [23]. The importance of free cytoplasmic $[\Sigma PP_i]$ in controlling liver glucose and, hence, blood glucose levels has not been generally recognized. Nevertheless, the effects of free cytoplasmic $[\Sigma PP_i]$ upon liver glucose levels can be demonstrated, without injecting PP_i, by taking advantage of the ability of cells metabolizing various chain length fatty acids to perturb PP_i levels in the cytoplasm, mitochondria or both.

Fatty acids are activated prior to oxidation by reactions of the following form:

$$\text{Fatty acid} + \text{ATP} + \text{CoA} \rightarrow \text{Fatty acyl CoA} + \text{AMP} + PP_i \quad (2)$$

Acetate is activated to acetyl CoA in cytoplasm and mitochondria, butyrate is activated to butyryl CoA solely in mitochondria [24] while oleate is activated to oleyl CoA solely in cytoplasm [25]. It follows that injection of acetate would be expected to produce pyrophosphate in both cytoplasm and mitochondria; butyrate solely in mitochondria and oleate solely in cytoplasm. Table 3 shows that butyrate or acetate raised total measured $[\Sigma PP_i]$ content about 10-times above control values; however, butyrate increased AMP content almost 5-times above control values while acetate raised AMP content only 2.5-times. In contrast, oleate increased free cytoplasmic $[PP_i]$ but did not affect total PP_i content or AMP levels. The difference between the effects of butyrate and oleate confirm that when oleate is activated, PP_i is produced solely in cytoplasm. The higher AMP levels with butyrate, in comparison with acetate, suggest either that

butyrate is activated more rapidly than acetate or that its activation is entirely mitochondrial while that of acetate occurs in both cytoplasm and mitochondria: this accords with the results of enzyme distribution studies.

If it is accepted that butyrate injection and activation leads to large production of AMP and PP_i in the mitochondrial matrix then several problems arise because AMP cannot be carried out of mitochondria on the adenine nucleotide translocator and myokinase is not present within mitochondria. The first question is: how does AMP get out of mitochondria during butyrate activation? The second is: why does PP_i not accumulate in stoichiometric amounts equivalent to the AMP produced during butyrate activation?

As to the question of how AMP leaves mitochondria during its formation after acetate or butyrate activation, the fall in [α-ketoglutarate] by 4-fold and the rise of [malate] by up to 4-fold suggests activation of α-ketoglutarate dehydrogenase and succinic thiokinase, with consequent GTP formation. This pathway provides the mechanism for the intramitochondrial conversion of AMP to ADP via the familiar pathway of substrate phosphorylation. Actual flux measurements will have to be made before this suggestion can be confirmed.

To turn to the second question: During butyrate metabolism, [AMP] increased from 0.2–0.9 μmol/g while [PP_i] increased only from 0.014–0.12 μmol/g. This could be taken as evidence for an active pyrophosphatase in mitochondrial matrix active in vivo. But the fact that butyrate, which should produce PP_i only in mitochondria, also raised the calculated free cytoplasmic [ΣPP_i] with all its attendant changes in the levels of glucose and of the entire sequence of glycolytic metabolites, strongly suggests that PP_i formed in the mitochondria can be exported to the cytoplasm. If this were not so, it would be difficult to explain how PP_i formed in mitochondria is able to affect cytoplasmic metabolites with which it is in apparent near-equilibrium.

Injection of NaCl solutions in molar concentrations equivalent to the sodium butyrate or sodium acetate injections caused no changes in glucose content. There was a 40% increase in [glucose 6-phosphate] but a decrease in the calculated free cytoplasmic [PP_i]. A comparison of tables 3 and 4 shows that any treatment which resulted in an increase in the concentration of liver glucose resulted in an increase in calculated free cytoplasmic [ΣPP_i]. That free cytoplasmic [ΣPP_i] is involved and not some other factor is indicated by the fact that oleate injection increased liver [glucose] and free [ΣPP_i] without changing the cytoplasmic [NAD^+]/[NADH] ratio as would be expected if oleate were a major liver substrate. In spite of all these metabolite changes, the value for $K_{eq.}$ obtained in eq. (1) remained close to the value of 45.9 expected at equilibrium.

These experiments demonstrate that alteration of free cytoplasmic pyrophosphate concentration, either by direct administration of PP_i or by administration of substrates which lead to pyrophosphate production, affects not only liver and blood glucose content, but also the content of the other substrates listed in eq. (1). While the nature of the enzyme or enzymes catalyzing this reaction remain unknown, possible candidates suggested have been glucose 6-phosphatase [26,27] or alkaline phosphatase [28]. These enzymes are thought, however, to exist in compartments other than cytoplasm and to have kinetic constants in vitro which would not be compatible with the apparent rates required in vivo.

An unexpected finding of this study was the apparent inverse relationship between an increase in total measured cellular pyrophosphate and a decrease in the cytoplasmic phosphorylation potential. In all cases studied where total measured [pyrophosphate] was >0.03 μmol/g wet wt (table 3), there was a decrease of 3–5-fold in the calculated free cytoplasmic phosphorylation potential or [ΣATP]/[ΣADP][ΣP_i] ratio (table 4). Associated with this elevation in total measured [ΣPP_i] there was a decrease in [α-ketoglutarate] and [glutamate] and an elevation of measured AMP content, and a decrease in the free cytoplasmic [$NADP^+$]/[NADPH] ratio. [Glutamate] and [α-ketoglutarate] are in apparent near-equilibrium with the free mitochondrial [NAD^+]/[NADH] [29], however, there appears to be no consistent change in the redox state of the pyridine nucleotides as a result of increasing mitochondrial pyrophosphate as judged by the [β-hydroxybutyrate]/[acetoacetate] ratio. The fall in α-ketoglutarate and glutamate, a decrease in the free cytoplasmic [$NADP^+$]/[NADPH] ratio and a rise in liver glucose is also seen after glucagon administration in vivo [11]. The similarity of effects of raising total PP_i or of injecting glucagon raises questions about the mode of glucagon action.

The free cytoplasmic [ΣATP]/[ΣADP][ΣP_i] ratio was calculated from the reactants of the glyceraldehyde 3-phosphate dehydrogenase and 3-phosphoglycerate

kinase reactions [4]. It is difficult to believe this system does not achieve near-equilibrium in liver, but why elevation of total liver PP_i content should cause a decrease in this parameter is unclear. It is worth noting, however, that both butyrate and acetate injection increase the total measured ADP 1.5-times in addition to increasing the measured AMP. In view of the insignificant change in calculated free cytoplasmic [ADP] (table 3), the increases in AMP and presumably in mitochondrial ADP suggest that a redistribution of adenine nucleotides occurs as a result of butyrate injection. The translocase cannot increase mitochondrial adenine nucleotide since it requires a cytoplasmic ADP. It is, of course, possible that mitochondria can undergo a net increase in adenine nucleotide by transporting ADP into mitochondria with Ca^{2+} [30].

This seems unlikely, however, under conditions of increased PP_i since there should exist a reciprocal relationship between free $[Ca^{2+}]$ and free $[\Sigma PP_i]$ since the solubility product of Ca_2PP_i is about 1.8×10^{-13} M [31]. In rats which have been starved for 72 h, butyrate injection can raise total measured PP_i to 2.5 μmol/g wet wt. Assuming all of this PP_i is in a mitochondrial matrix space of 10%, mitochondrial PP_i would approach 25 mM. Under these conditions mitochondrial $[Ca^{2+}] \gtrsim 8 \times 10^{-6}$ M may exceed the solubility product of Ca_2PP_i. Since the solubility product of $Mg_2P_2O_7$ is 1×10^{-13}–2×10^{-15} M, at pH 11, similar effects with mitochondrial Mg^{2+} are even more likely. Information on the mitochondrial matrix free $[Mg^{2+}]$ is however, unavailable at present.

Elevation of total or mitochondrial $[PP_i]$ to >0.04 μmol/g, in addition to altering cytoplasmic $[\Sigma ATP]/[\Sigma ADP][\Sigma P_i]$, also changes the [3-phosphoglycerate]/[pyruvate] ratio from the range of 1–3 which is characteristic of the fed animal to 7–11 (table 4) characteristic of the starved or streptozotocin diabetic or the glucagon-treated animal [21]. Thus while phosphate, pyrophosphate or glucose injection all raise the levels of glycolytic intermediates in the fed animal (table 2), pyrophosphate injection differed from the others in failing to increase [lactate] and [pyruvate] to the large extent seen after glucose or phosphate injection. The same effect is seen after acetate or butyrate injection, both of which raise total measured PP_i. Again oleate which produced only cytoplasmic PP_i had no effect on the [3-phosphoglycerate]/[pyruvate] ratio. Ca^{2+}-dependent protein kinases which affect the phosphorylation of pyruvate kinase have been defined [32] but unless the free cytosolic $[Ca^{2+}]$ is $<10^{-8}$ M, it is difficult to reconcile a simultaneous increase in both free $[PP_i]$ and free $[Ca^{2+}]$. It is known that Ca^{2+} is exported from isolated mitochondria in response to oxidation of the mitochondrial $[NAD^+]/[NADH]$ ratio [33]. There is, however, no correlation of this effect with oxidation of the mitochondrial $[NAD^+]/[NADH]$ as determined in vivo. There is a correlation of this effect with a decrease of cytosolic $[\Sigma ATP]/[\Sigma ADP][\Sigma P_i]$, but Ca^{2+} export from mitochondria in response to a decrease in cytoplasmic $[\Sigma ATP]/[\Sigma ADP][\Sigma P_i]$ has not been described.

A more likely possibility would seem to be that Ca^{2+} could leave mitochondria after butyrate or acetate injection as Ca_2PP_i and dissociate in cytosol where free $[PP_i]$ and free $[Ca^{2+}]$ are lower. Alternatively, a pyrophosphate:protein phosphotransferase [36] responsive to mitochondrial PP_i should be considered.

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