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ABBREVIATIONS

Bt_2-cAMP	dibutyryl adenosine 3',5'-monophosphoric acid
cAMP	adenosine 3',5'-monophosphoric acid
Fru 6-P	fructose 6-phosphate
Fru 1,6-P_2	fructose 1,6-bisphosphate
Glc 6-P	glucose 6-phosphate
FDPase	fructose 1,6-bisphosphatase (EC 3.1.3.11)
PK	pyruvate kinase (EC 2.7.1.40)
PFK	phosphofructokinase (EC 2.7.1.11)

I INTRODUCTION

For preservation of life mammals are dependent on a constant level of glucose in the blood. If glucose drops below a threshold value, the energy provision of the brain, erythrocytes and to a lesser extent muscle diminishes.

The maintenance of blood glucose is ensured by three main processes, namely food intake, glycogen utilization and gluconeogenesis. Gluconeogenesis is the process whereby glucose is synthesized from some precursors. The main precurors for gluconeogenesis are lactate and pyruvate, produced in exercising muscle and erythrocytes and glycerol, the end product of lipolysis in adipose tissue. Under low carbohydrate conditions aminoacids, released from muscle and other tissues or absorbed from the alimentary tract are also utilized. While many organs produce substrates for gluconeogensis, the process of gluconeogenesis itself is restricted to liver parenchymal cells and kidney cortex. Gluconeogenesis and glycolysis are both operational in these organs, although probably not simultaneously. The processes have common steps, but certain reactions that are displaced far from equilibrium are catalysed by different enzymes. These very enzymes possess unique regulatory properties, which allows a control of the flow in the direction of synthesis or degradation of glucose. Furthermore the concentration of these enzymes depends on the diet, whereby starvation leads to an increase in the gluconeogenic and a decrease in the glycolytic enzymes. A carbohydrate-rich diet has the opposite effect. These adaptive changes, the so-called "long term regulation", take place at the level of transcription and translation. Finally gluconeogenesis is under a minute to minute control by hormones, the so-called "short term regulation". This regulation can be exerted by covalent modification of enzymes and/or changes in their ionic environment.

The topic of this thesis is to point out which enzymes are affected by these hormones and to determine the molecular mechanism that underlies the activity changes. The involvement of cAMP and/or Ca^{2+} in the phosphorylation of particular enzymes was investigated. A method was developed that could also be applied on human liver biopsies. It is pointed out that a defect in the

regulatory mechanism might lead to lactic acidosis. Furthermore the properties of Fru 1,6-P_2, an important allosteric effector of which the concentration changes rapidly under different hormonal conditions, and of the enzyme that catalyses the hydrolysis of this metabolite, FDPase, were investigated more closely.

II THE PROCESS OF GLUCONEOGENESIS

1. General consideration

The sequence of reactions by which gluconeogenic substrates released from extrahepatic tissues are converted into glucose is given in Fig. 1. The process involves cytosolic and mitochondrial steps and furthermore carrier-mediated transport across plasma and mitochondrial membranes.
The conversion of 2 mol pyruvate into 1 mol glucose requires 6 mol ATP and 2 mol NADH. The demand for ATP must be fulfilled by mitochondrial catabolism. The transport of ATP from the mitochondrial matrix to the cytosol occurs through the adenine nucleotide translocator.

The balance of reducing equivalents is somewhat complicated and depends on the substrate considered. In the present case the reducing equivalents are provided by transport of malate and reconversion to oxaloacetate in the cytosol [1] (Fig. 2). If lactate is the substrate for gluconeogenesis, NADH is directly provided in the cytosolic compartment.

The balance of reducing equivalents does not only depend on the substrate used, but also on the species considered, i.e. on the location of phosphoenolpyruvate carboxykinase. In human liver this enzyme has a dual location both outside and inside the mitochondria [2,3]. Intramitochondrially generated phosphoenolpyruvate is readily transported by the tricarboxylate translocator.

The presence of both glycolytic and gluconeogenic enzymes in one compartment offers the possiblity that there is a constant "back flow" of gluconeogenic intermediates. This so-called "substrate cycling" can occur at the level of glucose/Glc 6-P, Fru 6-P/Fru 1,6-P_2 and phosphoenolpyruvate/pyruvate as visualized in Fig. 1.

Metabolic zonation of the liver [4], analogous to the heterogeneity in kidney cortex [5,6] should decrease cycling. It has been shown that the gluconeogenic enzymes phosphoenolpyruvate carboxykinase, glucose 6-phosphatase and FDPase are located predominantly around the terminal portal vessels and the glyco-

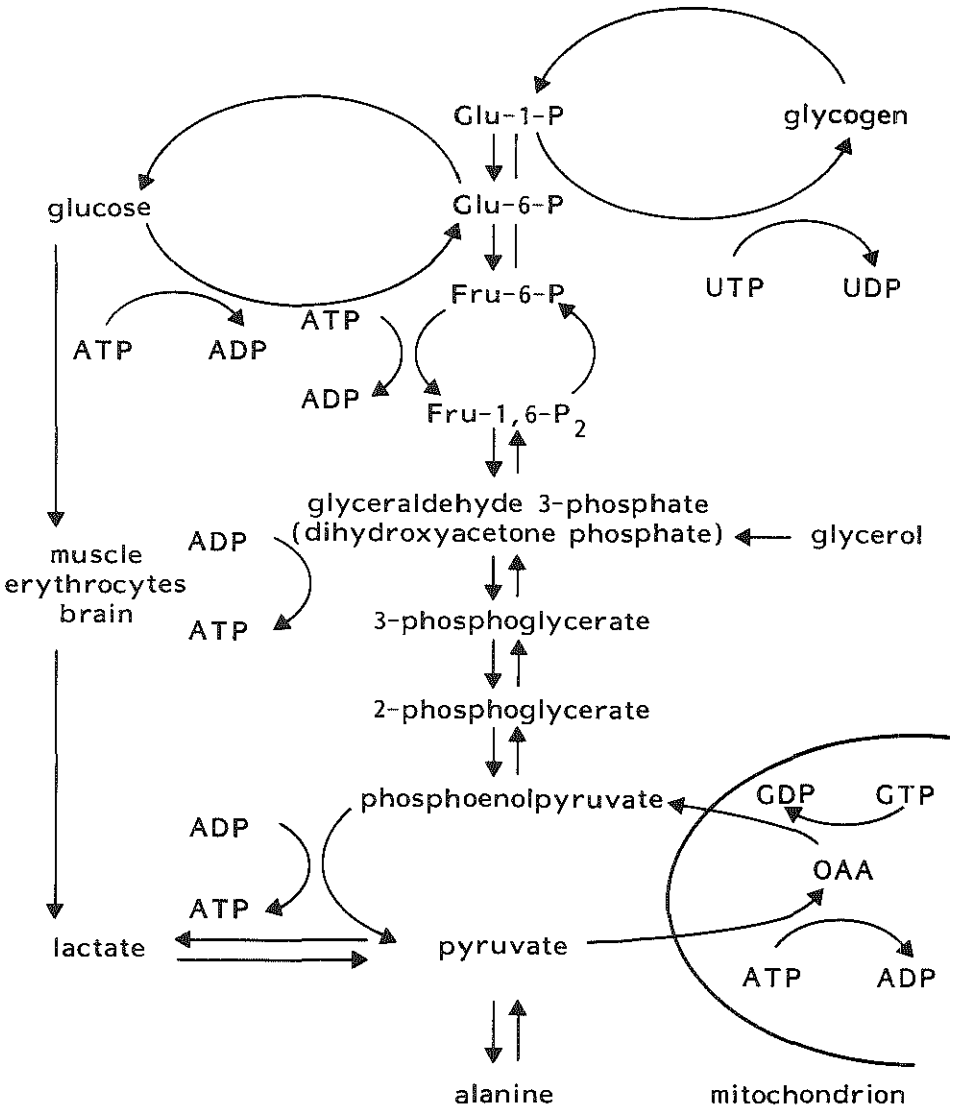
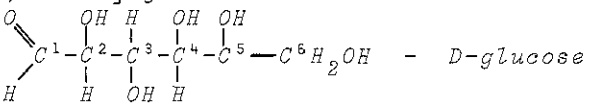


Fig. 1 Glucose metabolism in mammals.

lytic PK and glucokinase around the central vein [7-10]. The activity ratios of glycolytic and gluconeogenic enzymes are, however, only 2:1 to 5:1, which is small compared to the proximal to distal activity gradients in kidney tubules that approximate 10:1 [5,6]. Although the reciprocal localization of glycolytic and gluconeogenic enzymes in liver will lead to functionally significant metabolic zonation, the activity ratio is not so high to exclude cycling. Indeed it has been shown that glycolysis and gluconeogenesis occur simultaneously in isolated hepatocytes [11].

The methodology of the measurement of substrate cycles has been reviewed by Katz and Rognstad [12] and recently by Hue [13].

The glucose/Glc 6-P cycle can be measured from the release of ^{3}H from [2-^{3}H, U-^{14}C] glucose.



D-glucose

The calculated rates or recycling vary from 0.15-0.65 µmole min^{-1} per g of liver depending on the glucose concentration [13].

The recycling between Fru 6-P and triosephosphates has been estimated from the loss of ^{3}H from [5-^{3}H, U-^{14}C] glucose. This method has been subject to much criticism [14-17] since considerable release of ^{3}H can occur via the active transaldolase reaction or via the pentose phosphate pathway. The use of [3-^{3}H, U-^{14}C] glucose is recommended, although loss of ^{3}H during recycling in the pentose phosphate pathway still occurs [15]. An alternative method has been found in the measurement of randomization of ^{14}C from [1-^{14}C] galactose into C-6 of glucose [16]. During starvation very little randomization occurs. In fed rats the rate of recycling is 30% of the gluconeogenic rate [17,18].

The pyruvate /phosphoenolpyruvate cycle can be measured from the randomization of ^{14}C from [2-^{14}C] lactate into C-3 of lactate [19]. Under certain conditions the recycling is 50% of the gluconeogenic rate in the fed liver and about 10% in starved liver [16]. Higher values have been reported in the presence of pyruvate [19,20]. It must be realized that these experiments were performed with high substrate concentrations. It might well be that these values are quite different under more physiological conditions.

The utility of substrate cycles must be sought in the fact that the cycling enzymes can exert a high control on the net flux. This can be formulated in a quantitative way using a control theory [21]. Here the so-called control strength of an enzyme is defined as the relative change in pathway flux devided by the conjugated relative change in enzyme concentration. The sum of control strengths of all enzymes in a linear pathway is 1. A "rate limiting" enzyme has a control strength of 1; opposite a "non rate limiting" enzyme has a control strength of 0. A cycle in a pathway introduces an enzyme with negative control strength. This means that the other enzymes in the pathway have a high control strength; it is even possible that one enzyme has a control strength greater than 1. This principle has also been described in a quantitative way [154]. In addition the pyruvate/phosphoenolpyruvate cycle is also suggested to play a role in the transport of reducing equivalents from the mitochondria to the cytosol [22, 203]. This will be further discussed in connection with the hormonal regulation of PK (Chapter III.2).

In vivo the release of substrates from extrahepatic tissue regulates the rate of gluconeogenesis by a passive mechanism as the substrates reach the liver in a subsaturating concentration [23, 24]. Glucagon and adrenaline exert an active role by increasing the gluconeogenic flux. This has been demonstrated using a variety of substrates such as lactate, pyruvate, glycerol, dihydroxyacetone and fructose [17, 18, 25-31].

The site(s) of hormonal action could, according to Fig. 1, be summarized as changes in the activity of glycolytic and/or gluconeogenic enzymes, influences on the transport of substrates and/or ions across plasma and/or mitochondrial membrane and changes in mitochondrial metabolism, including alterations in phosphate potential and redox level. Many attempts have been performed to precisely localize the step(s) that are affected. Isolated hepatocytes particularly are very useful as a model system. But also the perfused liver and the intact animal are often used. The effects in the various systems are sometimes different.

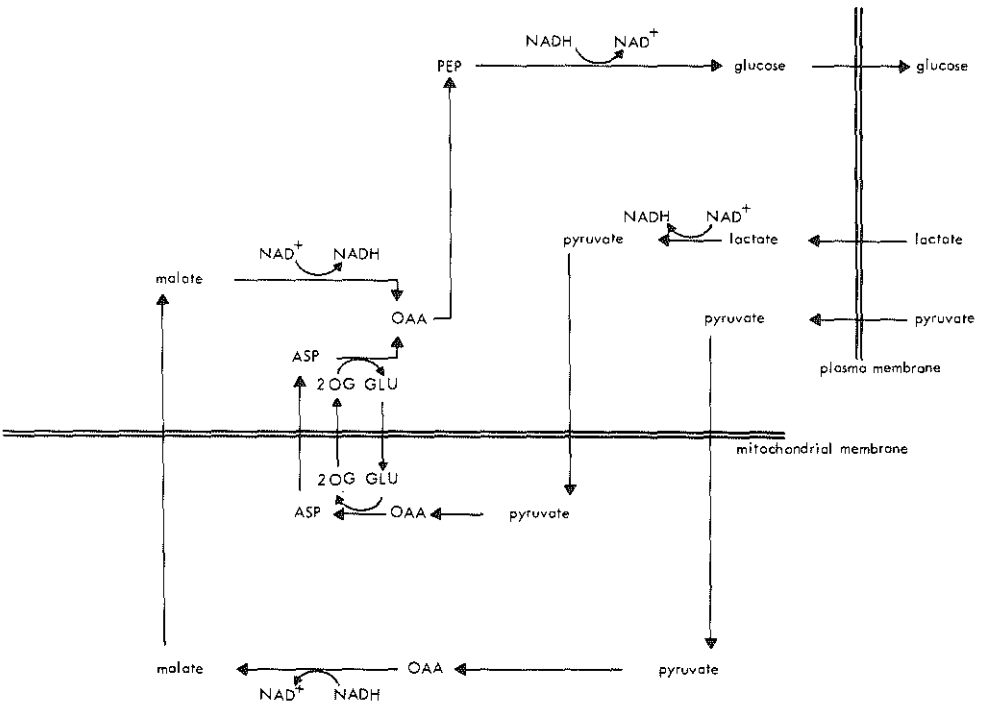


Fig. 2 Transport processes in gluconeogenesis.
Abbreviations: OAA, oxaloaceticacid; 2OG, 2-oxoglutarate; GLU, glutamate.

2. Control sites

It is assumed that only certain reactions are under the control of hormones. Three main experimental approaches have been used to find out which reactions these are.

1) The most obvious way to get insight in regulation is to compare the concentration of metabolic intermediates in control and hormone treated liver cells. A "cross-over" is suggested to indicate a control site [32].
Using lactate as a substrate and glucagon as an agonist, there is general agreement in the literature about a cross-over between pyruvate and triose phosphate either in fed [33, 34]

or starved liver [34-38] (however, see [31]). Some authors also report a cross-over between triosephosphate and hexose monophosphate [17, 33, 38] although others failed to observe such a change [34, 36]. When dihydroxyacetone was used as a gluconeogenic substrate, however, a cross-over was found at this point [29, 30]. Adrenaline provokes a cross-over between pyruvate and phosphoenolpyruvate [34], while the addition of Bt_2-cAMP leads to cross-overs at both substrate cycles [38].

This reasoning indicates that glucagon acts by altering the activities of at least one enzyme in the pyruvate phosphoenolpyruvate cycle and most likely also of an enzyme in the Fru 6-P/Fru 1,6-P_2 cycle, while adrenaline only seems to exert effect on an enzyme in the former cycle. Furthermore it is very likely that cAMP is the second messenger of glucagon action.

2) Several groups measured the flux through a specific enzyme using radioactive tracers, they found that glucagon depresses the PK flux by about 50% [39], just as cAMP does [20].
Glucagon also inhibits the flux through PFK [13, 16, 40, 41]. In the latter case a high dose of epinephrine is nearly as effective [16], but phenylephrine has no effect [13].
Unexpectedly even low concentrations of epinephrine increase PK flux [39], while vasopressin increases the flux through PFK [42].
Glucose phosphorylation is not affected by glucagon [13, 43] or phenylephrine [13].

Concerning glucagon the conclusion is the same as indicated above. Clearly the effect of catecholamines is quite different from that of glucagon; catecholamines have only a measurable effect in the pyruvate/phosphoenolpyruvate cycle.

3) Adaptive changes have also been used to indicate control sites of short term regulation. The rational here is that adaptive changes only occur at "rate limiting" steps.
Many groups have measured the activity of glycolytic and gluconeogenic enzymes under different nutritional conditions (see

TABLE I *The influence of dietary condition on the activity of glycolytic and gluconeogenic enzymes*

Enzyme	Dietary condition				Ref.
	fed	starved for 24 h	starved for 48 h	high carbohydrate fed	
Glucokinase	1.6	1.4	0.7	-	190
	-	-	0.7	1.8	191
Phosphofructokinase	1	0.5	0.5	0.8	192
Pyruvate kinase	37	16	14	145	193
	63	55	36	-	194
	52	-	52	-	195
	-	-	101	183	47
	12	-	7	-	182
Glucose 6-phosphatase	8	23	26	-	197
Fructose 1,6-bisphosphatase	2	-	-	4	198
	10	12	19	-	197
	15	30	38	-	199
	19	-	21	19	200
Phosphoenolpyruvate carboxykinase	5	-	13	-	49
	5	12	10	-	201
	5	14	19	-	197
	4	-	14	-	195
	3	21	26	-	199
Pyruvate carboxylase	4	10	8	-	197
	7	-	16	-	195
	7	16	15	-	199
Gluconeogenic flux	-	1.4	-	-	11
Glycolytic flux	3.4	-	-	-	193

Activities are calculated in μmol min^{-1} s^{-1} per 100 mg protein using the factors: liver wet weight per 100 g body weight equals 3.5 [204] and 1 g wet weight equals 73.1 mg protein [205].

Table I). Clearly no unambigeous conclusion is possible as the results vary. Regulatory changes seem to occur at the level of the pyruvate/phosphoenolpyruvate and the Fru 6-P/Fru 1,6-P_2 cycle (Table I). The activity of the glycolytic enzymes glucokinase (for a review see [44]), PFK and PK decrease upon starvation to about 50% of the activity in the fed state. The activity of the gluconeogenic enzymes FDPase and pyruvate carboxylase increases little if any at all, while phosphoenolpyruvate carboxykinase and glucose 6-phosphatase activity increase 2-3 fold.

The mechanisms that induce these adaptive changes are quite different from those involved in the short term regulation. The alteration in PK activity upon starvation results from a decrease in the amount of functional mRNA coding for the enzyme [45], while the half life of the enzyme is similar [46]. The activity of the purified enzyme also decreases some 30% [47, 48]. The increase in phosphoenolpyruvate carboxykinase activity *in vivo* can be blocked by actinomycin D or puromycin [49, 50]. In primary cultures of hepatocytes the enzyme can be induced by Bt_2-cAMP which can be blocked by inhibitors of transcription [51]. *In vivo* injection of Bt_2-cAMP results in a 10 fold increase in mRNA coding for phosphoenolpyruvate carboxykinase within 1 h [52]. These results indicate that the maximal activities of PK and phosphoenolpyruvate carboxykinase are regulated at the level of transcription.

The information gathered with the cross-over studies, flux determinations, dietary induced adaptations and the fact that gluconeogenesis from substrates entering the gluconeogenic pathwat at the level of pyruvate as well as triosephosphates can be stimulated by glucagon with a decrease in lactate/pyruvate [92] led to the supposition that the short term regulation is brought about by an alteration in one or more of the following enzymes: PFK, FDPase, PK, pyruvate carboxykinase or phosphoenolpyruvate carboxykinase. The regulation of glucose 6-phosphatase or glucokinase is sought to be secondary. The kinetic properties of glucokinase and glucose 6-phosphatase are such that a rapid control of the metabolic flow in response to the changing level

of glucose or Glc 6-P is ensured [13].
In terms of the control theory [21] this means that these enzymes have a high elasticity, i.e. there activity varies strongly with substrate concentration.

III HORMONAL ACTION IN LIVER

1. General consideration

Schimassek and Mitzkat [53] were the first to observe in the isolated perfused liver that glucagon stimulates gluconeogenesis. Since then many reports have confirmed this observation. Similar effects were reported using adrenaline (for a review see [54]); other hormones too, including vasopressin [55-57], angiotensin II [57], norepinephrine [25, 58, 59] and oxytocin [57] have the ability to stimulate gluconeogenesis. The above mentioned hormones act on their target cells through specific binding sites termed receptors located on the cell surface. The different sensitivities of the receptors to various catecholamines were used to make a distinction between so-called α- and β-receptors [60], which appear to be involved in different mechanisms of hormone action.

Stimulation of β-adrenergic receptors provokes a rise in cellular cAMP concentrations [61], while the intracellular message of α-adrenergic hormones is correlated with a rise in the Ca^{2+} concentration (for a review see [62-64]). The current hypothesis on the action of glucagon and catecholamines on the short term regulation of gluconeogenesis will be outlined in more detail below.

2. Targets for glucagon action

Already early in the seventies it became evident that cAMP was the second messenger in the glucagon-induced stimulation of gluconeogenesis in the liver [65]. Binding of cAMP to the so-called cAMP-dependent protein kinase promotes the kinase to dissociate into a regulatory and an active catalytic subunit (for a review see [66]).

The next step is to identify the substrate proteins for cAMP-dependent protein kinase and to show that indeed phosphorylation of that protein is directly related to the stimulation of gluconeogenesis. Section II discusses reasons why regulation of gluconeogenesis can be expected at the level of the Fru 6-P/

Fru 1,6-P_2 cycle and the pyruvate/phosphoenolpyruvate cycle. Following this reasoning particular attention has been given to enzymes which operate in these cycles. As the mitochondrial membrane is not permeable for cAMP, it is unlikely that pyruvate carboxylase will be a substrate protein for cAMP-dependent protein kinase. Using isolated hepatocytes negative evidence has even been obtained. Pyruvate carboxylase isolated from ^{32}P-injected rats does not contain covalently bound phosphate [67]. Also phosphorylation of cytosolic or mitochondrial phosphoenolpyruvate carboxykinase has not been reported till now. Phorphorylation of the other enzymes, i.e. FDPase, PFK and PK deserves special attention.

The criteria, originally proposed by Krebs [68], that must be met before an effect mediated by cAMP can be said to occur through phosphorylation of a specific protein, were recently evaluated by Nimmo and Cohen [69] (see also [70]). Based upon the progress made during the past few years they proposed the criteria cited in table II.

Below I shall provide an assessment of the extent to which these criteria have been met for the aforementioned enzymes. The case of PK is complete and therefore most illustrative to discuss first.

Pyruvate kinase

ad 1. Ljungström et al. [71] showed that purified L-type PK can be phosphorylated by a cAMP stimulated protein kinase *in vitro*. This observation has been confirmed by many others (for a review see [72]).
In appendix paper 2 I describe a system which allows the determination of phosphate incorporation into a specific protein of rat liver soluble fraction by the endogenous rat liver protein kinase. After *in vitro* phosphorylation, these proteins are separated in a polyacrylamide gel in the presence of SDS. The identification of L-type PK as a phosphoprotein in such a gel is reported in appendix paper 1. With the system described it is possible to investigate the kinetics of the phosphorylation and

TABLE II *Criteria for mediation of a hormonal effect through cyclic AMP-dependent phosphorylation according to Nimmo and Cohen [69].*

1.	A protein substrate for cyclic AMP-dependent protein kinase should exist which bears a functional relationship to the process mediated by cyclic AMP. The rate of phosphorylation of that protein, in its native state, should be adequate to account for the speed at which the process occurs *in vivo* in response to cyclic AMP.
2.	The function of the protein should be shown to undergo a reversible alteration *in vitro* by phosphorylation and dephosphorylation, catalysed by cyclic AMP-dependent protein kinase and a protein phosphatase.
3.	A reversible change in the function of the protein should occur *in vivo* in response to cyclic AMP.
4.	Phosphorylation of the protein should occur *in vivo* in response to a hormone at the same site(s) phosphorylated by cyclic AMP-dependent protein kinase *in vitro*.

dephosphorylation reaction of the native enzyme. In this way the phosphorylation rate of PK has been determined. Extrapolation of the data obtained *in vitro* to *in vivo* conditions leads to the conclusion that the phosphorylation of PK can be completed in 0.5 s. This high rate is adequate to account for the rapid stimulation of gluconeogenesis observed after the addition of glucagon.

ad 2. Engström and coworkers showed that the phosphorylated form of the enzyme has an increased $K_{0.5}$ for phosphoenolpyruvate when the enzyme is assayed at physiological pH in the absence of its allosteric effector Fru 1,6-P_2. At a saturating concentration

of Fru 1,6-P_2 the phosphorylated and dephosphorylated enzyme behave identically [73, 74].

The enzyme can be dephosphorylated by a rat liver protein phosphatase, which restores the activity of the enzyme [75-77]. The phosphatase can be separated from heat-stable inhibitor sensitive phosphorylase phosphatase [76, 77].

ad 3. Glucagon causes a rapid decrease in PK activity in perfused liver [78] or hepatocyte suspensions [79-83]. The inactivation can be reversed by addition of insulin [67, 78, 79] or after sustained incubation [79]. Incubation of the hormone treated cell sap in the presence of Mg^{2+} also leads to a reactivation of the enzyme [81].

In vivo inactivation of PK has been reported by relatively few authors. Taunton et al. [84, 85] observed a 50% decrease in activity 10 min after the injection of glucagon. The cAMP level was elevated already after 30s and maximal after 1-2 min. The lag phase observed in the inactivation of PK weakens the argument that there is a causal relationship between accumulation of cAMP and inactivation.

Riou et al. [86] observed a 40% decrease in activity ratio (defined as the ratio of activities measured with 0.1 mM phosphoenolpyruvate and with 4 mM) after *in vivo* injection of glucagon. In a similar experiment I found a decrease in activity ratio (defined as the ratio of activities as measured with 1.7 mM phosphoenolpyruvate in the absence and presence of Fru 1,6-P_2) from 0.58 ± 0.06 in control rats to 0.40 ± 0.03 in glucagon treated rats (n=4) within 5 min after the injection of glucagon.

ad 4. Phosphorylation of PK has been shown by incubation of hepatocytes in the presence of ^{32}P. Glucagon increases the phosphate content of the enzyme 3-5 fold [87-89]. *In vivo* a 2.5 fold increase in phosphate incorporation was found [86].

Finally also the phosphorylation site *in vivo* and *in vitro* were compared. Tryptic digests of the purified enzyme that was either labeled *in vivo* by injection of ^{32}P in the presence of glucagon or *in vitro* by cAMP-dependent protein kinase in the presence of [γ-^{32}P]-ATP revealed the same pattern in SDS poly-

acrylamide gelelectrophoresis [90].

Concluding we can say that all evidence indicates that PK is phosphorylated and consequently inactivated by a cAMP-dependent protein kinase after an *in vivo* injection of glucagon. In addition it can be said that also under physiological conditions an elevation of glucagon, i.e. starvation, leads to a decrease in PK activity [91, 92]. Yet we cannot conclude that the inactivation of PK is the sole event in the onset of gluconeogenesis. The allosteric activation of PK by Fru 1,6-P_2 must be prevented or even released. Furthermore Fru 1,6-P_2 inhibits the rate of phosphorylation of PK [196, 202, appendix paper 3]. The extent of phosphate incorporation is also deminished. In appendix paper 3 we conclude that in the presence of Fru 1,6-P_2 half of the phosphorylation sites is exposed to cAMP-dependent protein kinase. So a high concentration of Fru 1,6-P_2 will prevent in all respects the inactivation of PK in response to glucagon. This argues for a complementary regulatory site at the level of the Fru 6-P/ Fru 1,6-P_2 cycle which acts in concert with the effect on PK.

Fructose 1,6-bisphosphatase

Concerning FDPase there is little evidence for the direct participation of a phosphorylation mechanism in the onset of gluconeogenesis as will be outlined below.

Riou et al. [93] reported that purified FDPase can be phosphorylated by cAMP-dependent protein kinase. This was confirmed by Humble et al. [94] and Marcus and Hosey [95]. However, in an *in vitro* phosphorylation system of liver supernatant I found no evidence for phosphorylation of FDPase (see appendix paper 1). Such a system appears more closely related to the *in vivo* system than the totally purified system. One can argue that phosphorylation can only occur in the presence of some metabolites. We determined the effect of various metabolites on the phosphorylation of cytosolic enzymes (appendix paper 3). One can notice that also in the presence of these metabolites no phosphorylation band appears in the M_r 40,000 range. In addition we tested the effect of AMP, which is a well known inhibitor of

FDPase activity. No phosphoprotein could be detected with a similar mobility in SDS gels as FDPase.

Using *in vivo* concentrations of protein kinase and FDPase, Riou et al. found that 1 mol phosphate per mol enzyme can be incorporated during 20 min of incubation. From the results of Marcus and Hosey [95] I deduce that an incubation time of 7 min is sufficient and from the report of Humble et al. [94] a period of 17 min can be calculated. Cohen et al. [96] found that the rate of FDPase phosphorylation is only 0.6% of that of PK. Apart from these reports that leads to the conclusion that FDPase phosphorylation is slow, one report gives a much higher phosphorylation rate [97]. In this report it is claimed that the maximal phosphorylation rate of FDPase is 1/3 of that of PK, while the K_m of the protein kinase for FDPase is about 3 times greater than that for PK. This means that under *in vivo* conditions the phosphorylation rate of FDPase could be as high as 10% of the phosphorylation rate of PK and even so the rate is rather low. Furthermore it is not clear if phosphate incorporation is accompanied by an activity change.
Only Riou et al. [93] reported a 40% increase in activity at a maximal phosphate incorporation of 4 mol phosphate per mol enzyme.

The phosphorylated enzyme can also be dephosphorylated with a partially purified preparation of phosphoprotein phosphatase from rat liver [97]. If such a dephosphorylation is accompanied by an activity change has not been reported.

In vivo activation of FDPase in response to glucagon has been reported by Taunton et al. [84, 85] and recently by Mörikofer-Zwez [98] in rat liver and by Chatterjee and Datta [99] in mouse and rabbit liver. Concerning the latter report a phosphorylation mechanism is unlikely as it was found that rabbit and mouse liver FDPase cannot be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase [100]. It was concluded that these enzymes lack the peptide that contains the phosphorylation site. A similar situation is found in pig kidney [100]. However, more confusion raises as, at the same time, Mendicino et al. [101] report that pig kidney FDPase can incorporate 4 mol of phosphate, however, apart from that without activity change.

Also failures to measure increases in activity in response

to glucagon have been reported. In an attempt to reproduce the experiments of Taunton et al. [84, 85] I observed a peculiar non linear product formation in the FDPase reaction. As this phenomenon could mask or even be related to the expected kinetic changes a more systematic study was undertaken (appendix paper 5). It appeared that at least three parameters must be measured to standardize the activity of FDPase. These are the initial reaction rate, the final steady state reaction rate and the reaction constant related to the achievement of the steady state. The experiments of Taunton et al. were repeated exactly. However, none of the above described parameters were changed after glucagon injection (appendix paper 5). Hue [13] could not reproduce the experiments of Chatterjee and Datta [99] in which mouse liver is used. Veneziale et al. [83] used a hepatocyte incubation system and also found no change in FDPase activity after the addition of glucagon.
It is not clear which kinetic parameter might be changed: Riou et al. [86] indicated that the activity had to be measured in the absence of EDTA. In the absence of EDTA no maximal activity is measured (for a review see [102]). However, Chatterjee and Datta [99] used an assay in which EDTA is included. Taunton et al. used an unphysiological pH of 8.8 and inhibitory concentrations of Fru 1,6-P_2. The kinetic change must be established more profoundly to get more insight in its physiological significance. It is very well possible that the effects are due to changes of contaminating metabolites in the assay.

An increase in phosphate content in response to glucagon has only recently been reported [103]. On the other hand, Riou et al. [86] observed that in the presence of glucagon the phosphate content of FDPase is not changed. The same conclusion was reached by Garrison et al. [88] using hepatocyte incubations.

My present view concerning the involvement of cAMP-dependent phosphorylation of FDPase in the stimulation of gluconeogenesis by glucagon is as follows: FDPase contains a site that can be phosphorylated at least by cAMP-dependent protein kinase. (This site has been identified by two groups [94, 97]. Although the amino acid sequence around the serine (P) residue is not exactly

the same in these reports, most likely the same phosphopeptide was considered.) Phosphorylation of this site is too slow to be involved in the minute to minute regulation of gluconeogenesis. The diversity in the phosphorylation rates results most likely from partial denaturation of the enzyme near the phosphorylation site. Indeed the phosphorylation rate can be accelerated considerably when the enzyme is treated with urea [100]. Finally there is little evidence for *in vivo* phosphorylation or activity changes in response to glucagon particularly since the activity changes reported are poorly characterized. It must be realized that the recent discovery of Fru 2,6-P_2, which is a potent regulator of PFK activity, can change this picture. It is very well possible that this metabolite influences the phosphorylation of FDPase and/or the expression of its activity.

Concluding one can say that none of the criteria listed in Table II have been fully met in the case of FDPase.

Phosphofructokinase

The literature concerning hormonal regulation of PFK by a cAMP-dependent mechanism is limited, but active research is going on.

Brand and Söling [104] showed that rat liver contains a protein kinase and phosphatase which respectively activates and inactivates the enzyme. The protein kinase described, however, is cAMP-independent.

Kagimoto and Uyeda [105] reported that glucagon stimulates *in vivo* the phosphorylation of PFK approximately 3 to 5 fold. A similar result was obtained in the perfused rat liver [106] using physiological glucagon concentrations. In a hepatocyte incubation system a relatively high concentration of glucagon was necessary to obtain a 2 fold increase in ^{32}P incorporation [108]. On the other hand, Garrison [107] found no evidence that PFK is among the proteins that incorporate phosphate after treatment with glucagon. One can see that in an *in vitro* incubation system of liver supernatant (see appendix papers 1 and 2) no phosphoprotein that corresponds to the M_r of PFK (80,000) is present. The argument of Kagimoto and Uyeda [105] that PFK

escapes detection in SDS-gels can be rejected as its concentration is 10 times higher than they assume.

Considerably less controversy exists about *in vivo* inactivation after injection of glucagon [105, 109] or after addition of the hormone to hepatocyte incubations [83, 108, 110, 111]. The kinetic change most likely is an increase in $K_{0.5}$ for Fru 6-P [108, 110, 111] although also a higher sensitivity to inhibition by ATP has been observed [105]. The inactivation does not persist after ammoniumsulphate precipitation [108] or gel filtration [112] (however, see [110]).

It was attractive to think that these activity changes are caused by cAMP-dependent phosphorylation of the enzyme. No clear evidence for such a causal relationship was presented. Recent investigations indicate that the regulation is excerted by a newly identified metabolite.

Concluding: some experiments point in the direction of cAMP-dependent phosphorylation of PFK, however, the prerequisite that the enzyme is a substrate for cAMP-dependent protein kinase *in vitro* (1 in Table II) is not fulfilled. Furthermore Claus et al. [108] pointed out that there is no correlation between inactivation and glucagon concentration, which indicates that the inactivation is probably a secondary event.

Fructose 2,6-bisphosphate

First it was shown that rat liver cytosolic fraction contains a factor that activates PFK [108, 112, 113]. This factor was tentatively identified as Fru 2,6-P_2 by Van Schaftingen et al. [114] which was confirmed later [115, 116]. The concentration of Fru 2,6-P_2 increases with increasing glucose concentration and rapidly decreases after the addition of glucagon [44, 116, 117]. Half maximal effects are already observed with a glucagon concentration of 10^{-13} M [118] or 10^{-9} M [119]. A decrease in the Fru 2,6-P_2 concentration leads to the above described increase in $K_{0.5}$ for Fru 6-P [120-123] and it relieves the inhibition by ATP [109, 122, 123]. These effects are observed with Fru 2,6-P_2 concentrations well below 1 µM [115, 120, 123-125]. Several groups concluded that the activity of PFK is most likely regu-

lated by this factor rather than Fru 1,6-P_2 [122, 123, 126]. Furuya and Uyeda [109] describe a model in which phosphorylation of PFK still is the primary event; phospho-PFK should posses a decreased affinity for the activating factor. Söling et al. [126] indicate that the phosphatase catalyzed inactivation of PFK is inhibited by Fru 2,6-P_2.
The inactivation of PFK in response to subsaturating concentrations of glucagon is spontaneously reversible upon prolonged incubation, while insulin accelerates the reactivation [106].

The discovery of Fru 2,6-P_2 as a regulatory compound is so important that it deserves some more attention here. After the recognition that it strongly influences the activity of PFK it was investigated if FDPase and PK are also regulated by Fru 2,6-P_2. Indeed, inhibition of FDPase might occur as a result of the increased $K_{0.5}$ for its substrate in the presence of Fru 2,6-P_2 [124, 125]. The inhibition is synergistic with the inhibition by AMP [124, 125]. It was also observed that PK is slightly stimulated by Fru 2,6-P_2 [12]. It must be stressed that in the case of FDPase and PK relatively high concentrations are necessary so that these effects are most likely of little physiological importance.

The enzyme responsible for the synthesis of Fru 2,6-P_2 has been recently partially purified [123, 127, 128]. It catalyses the transfer of phosphate from ATP to Fru 6-P. The apparent molecular weight of the enzyme is 85,000-90,000 D [129]. The enzyme is stimulated by P_i and AMP and inhibited by phosphoenolpyruvate and citrate [128]. It has been shown that the enzyme can be separated from PFK [127, 128].

After addition of phenylephrine the level of Fru 2,6-P_2 increases [119]. Vasopressin also causes the accumulation of the factor and stimulates glycolysis [119, 130]. However, vasopressin stimulates gluconeogenesis from ^{14}C labeled lactate or pyruvate [33, 55]. This could mean that indeed the inhibitory effect of Fru 2,6-P_2 on FDPase activity is of minor importance.

The problem remains of understanding the sense of the regulatory properties of Fru 2,6-P_2 as Fru 1,6-P_2 has essentially the same effect. If we assume that *in vivo* only PFK activity is affected by the compound, then it is striking that Fru 2,6-P_2 is

one of the few compounds which only regulates PFK and not PK activity. This makes it possible that gluconeogenesis is stimulated by inhibition of PFK while the pyruvate-phosphoenolpyruvate cycle still operates to transport reducing equivalents to the cytosol.

In appendix paper 3 it is shown that hexose phosphates inhibit the cAMP-independent phosphorylation of a M_r-68,000 protein. The phosphorylation of PK can only be inhibited by hexose-diphosphates. Two explanations can be given for the differential effect of hexosephosphates on PK and the M_r-68,000 protein. In the first place the effect of hexosephosphates on the phosphorylation of the M_r-68,000 protein could be non-specific. The second explanation is that a compound that is quickly synthesized from the added hexose-phosphates, is responsible for the inactivation. In this respect it would be interesting to investigate a possible regulatory role of Fru 2,6-P_2 in this phosphorylation reaction. It appears that in human liver also a M_r-68,000 phosphoprotein is present which has similar regulatory properties (appendix paper 7). It is remarkable that in all human liver samples tested this protein is the main phosphoprotein detectable.

So far our conclusion must be that only in the case of PK convincing evidence exists that the enzyme is phosphorylated in response to glucagon. Below I shall discuss the possibility and the evidence that subcellular compartments, as mitochondria and endoplasmic reticulum, are affected after glucagon treatment of the intact cell.

Mitochondria and endoplasmic reticulum

Numerous effects in mitochondria and endoplasmic reticulum in response to glucagon have been reported. One of the early events observed in mitochondria isolated from glucagon-treated hepatocytes is the increase in state 3 and uncoupler stimulated respiration (see Table III). A similar stimulation is observed in submitochondrial particles [139], however, when ascorbate is the substrate no change in respiratory rate is observed [119, 131,

TABLE III *Effects of glucagon observed in rat liver mitochondria*

Mitochondrial function/property	effect	ref.
State 3 respiration	↑	83, 131-138
Uncoupler stimulated respiration	↑	83, 131-138
Transmembrane pH gradient	↑	138, 141, 142
Membrane potential	↑	142 (however 138)
Succinate dehydrogenase activity	↑	134, 136
Uncoupler dependent ATPase activity	↑	131, 143
Adenine nucleotide translocator act.	↑	131, 143
ATP/ADP ratio	↑	35, 133, 135, 143, 202, 203
Adenine nucleotide content	↑	144, 145
Citrulline synthesis	↑	133, 135, 144, 146, 147
Glutamate synthesis	↑	67
Pyruvate carboxylation	↑	83, 143, 146-148, 203
Pyruvate decarobylation	↑	146, 148, 203
Pyruvate transport	↑	138, 141, 142, 149
Phosphate transport	↑	150
Ca^{2+} retention	↑	137, 151
Mg^{2+} content	↑	142, 152
N-acetyl glutamate content	↑	144 (however 135)

132, 138]. An explanation of these changes is based on the observation of Zahlten et al. [140] that glucagon stimulates the phosphorylation of mitochondrial membranes. Together with the fact that a cross-over was found in the respiratory chain between cytochrome b and c [132], it was postulated that the activity of cytochrome c was stimulated by a glucagon-induced phosphorylation mechanism [132]. However, experimentally no change in the phosphoprotein pattern of mitochondria was observed [134]. Furthermore the cross-over between cytochrome b and c was not observed by others [137, 139]. Other changes that have been reported to occur in mitochondria from glucagon treated animals or cells are listed in Tabel III. An intriguing question in the understanding of these effects is how the extracellular derived signal, carried by glucagon, reaches the mitochondria and what the final target is.

Recently it was described that glucagon stabilizes rather than activates mitochondrial functions [153]. It appears that only small effects are observed on state 3 respiration and the activities of succinate dehydrogenase and pyruvate carboxylase when mannitol instead of sucrose is used for isolation or when quinoline derivates are added to the isolation medium. This means that most of the effects reported in Table III are suspect to doubt. The increase in the mitochondrial concentrations of acetyl-CoA, ATP and phosphoenolpyruvate reported by Siess et al. [35] are derived from mitochondria obtained with a digitonin fractionation procedure. Consequently this result cannot be explained by a stabilization of mitochondria in sucrose medium. Similarly the increase in N-acetyl-glutamate is observed in liver extracts rather than isolated mitochondria [144] and must be related to other effects of glucagon.

Endoplasmic reticulum-rich fractions show glucagon-sensitive Ca^{2+} uptake. This uptake is ruthenium red-insensitive and can be inhibited by insulin [155-157]. Glucagon acts, however, relatively slowly [156, 157] so that this is probably a more trophic effect of glucagon.

A high concentration of glucagon stimulates Ca^{2+} efflux from hepatocytes [158-163] and does not increase the uptake [158]. This might imply that the cytosolic Ca^{2+} concentration drops in

response to glucagon. An important implication could be that FDPase will become more active as Ca^{2+} is a potent inhibitor of the enzyme [164] (see also Fig. 4 in [13]).

3. The pyruvate kinase hypothesis

According to the so-called second messenger hypothesis glucagon provokes a rise in the intracellular cAMP concentration. As outlined in the previous section this leads to the phosphorylation of PK. It is not clear at the moment if other enzymes are also phosphorylated or that intracellular organelles are affected. The question is: Can we explain the increased gluconeogenesis in response to glucagon solely by assuming that PK is phosphorylated by cAMP-dependent protein kinase?

Phosphorylation of PK, which decreases its activity, leads to a diminished cycling between phosphoenolpyruvate and pyruvate. The phosphoenolpyruvate concentration will increase and so stimulate gluconeogenesis by a feed-forward mechanism. In the latter statement lies an argument that already enfeebles the possibility of a sole role of PK: The increased substrate concentration will saturate the enzyme and nullify the effect of phosphorylation. Otherwise a feed-forward mechanism is also unlikely, as this will cause an increase in the subsequent gluconeogenic intermediates including Fru 1,6-P_2. By this, PK becomes fully activated in spite of the fact that the enzyme is phosphorylated.

Yet inactivation of PK offers an elegant explanation for the inhibition of gluconeogenesis observed in particular cases. This phenomenon has been reported in starved rat liver using pyruvate, at a high concentration, as a substrate [20, 165-168] (however, see [25-27]). At first this inhibition was attributed to hormonally induced inhibition of pyruvate dehydrogenase [17] which would prevent alleviation of the deficiency in NADH. This hypothesis could be rejected as dichloroacetate, an activator of this enzyme, did not overcome the inhibition [165]. It was also proposed that inhibition of PK would prevent the energy-dependent translocation of reducing equivalents from the mitochondria to the cytosol [20, 168]. This implies that there is a considerable recycling in the presence of pyruvate at the level of phospho-

enolpyruvate/pyruvate in fasted rats. This probably stems from the high phosphoenolpyruvate concentration under this condition [34]. The high PK flux can be inhibited by cAMP [20, 168] or glucagon [34] which will reduce the generation of cytosolic reducing equivalents and consequently inhibit gluconeogenesis. This hypothesis is further strengthened by experiments with chicken hepatocytes. The PK isomer of these cells is not inactivated in response to glucagon and concomitantly no decrease in gluconeogenesis from pyruvate is observed [166].
A quite different explanation has been put forward by Clark and Jarret [169]. They showed that at a high NAD^+/NADH ratio, as in the presence of pyruvate, the increase in cAMP content due to glucagon is diminished. It was proposed that the molecular basis of this observation is a lack of inhibition of cAMP phosphodiesterase by NADH. This hypothesis gives, however, no explanation how glucagon causes the inhibition of gluconeogenesis from pyruvate and why this is restricted to starved livers.

Concluding one can say that the cAMP-dependent phosphorylation of PK alone cannot account for the stimulation of gluconeogenesis in fed or starved liver. Other factors must act in concert with the phosphorylation of PK. The cytosolic Ca^{2+} concentration might change as well and affect protein phosphatases by which other enzymes are phosphorylated or dephosphorylated. The diminishment in the Fru 2,6-P_2 concentration might inhibit PFK activity, by which a control of the Fru 1,6-P_2 concentration is possible. The mechanism by which the concentration of the former metabolite is regulated needs to be elucidated before its importance in the regulation of gluconeogenesis can be fully recognized.

4. Targets for catecholamine action

As stated in chapter II.2. adrenaline stimulates gluconeogenesis from substrates that enter the pathway at the level of pyruvate and lactate and also, although not so commonly reported, from substrates that enter at triosephosphate [14, 16, 27, 30]. Concerning phenylephrine only gluconeogenesis from pyruvate and lactate has been studied [25, 33, 55, 58]. The mechanism involved

in the stimulation of gluconeogenesis by catecholamines does not necessarily involve an increase in cAMP. Adrenaline for instance gives rise to an elevation of the cAMP concentration [58, 170] which can, however, be blocked by the β-blocker propranolol with preservation of its stimulatory effect [58]. Dehydroergotamine, an α-blocker, effectively blocks adrenaline stimulation of gluconeogenesis, while other α-blockers, such as phentolamine and phenoxybenzamine, are less effective [58]. Phenylephrine does not cause the cAMP concentration to rise [54].
These observations have led to the statement that the effects of catecholamines on liver are caused by an α-adrenergic mechanism (for reviews see [62-64]). In rat liver such a mechanism involves the mobilization of Ca^{2+} ions from mitochondria resulting in an increase in cytosolic Ca^{2+} concentration. It is not clear how mitochondria receive the extracellularly derived information. A primary response in α-adrenergic stimulation is an increase of phosphatidylinositol breakdown (for a review see [171]). Michell proposed that the product formed, myoinositol 1,2 cyclic phosphate, could act as an intracellular second messenger. However, up till now no experimental evidence has been given for this hypothesis.

The mechanism whereby an increased Ca^{2+} concentration leads to a physiological response is largely unknown. In the case of glycogen breakdown the explanation is straightforward. Muscle phosphorylase kinase (for review see [172]) is directly activated by Ca^{2+}. A similar activation has been reported for liver phosphorylase kinase [173, 174]. This leads to the phosphorylation and concomittant activation of phosphorylase . Also because of the discovery of Ca^{2+}-stimulated phosphorylation in membrane preparations from various tissues [175, 176] it was thought that Ca^{2+}-activated protein kinases stimulate the phosphorylation of specific cytosolic proteins. The effect of Ca^{2+} could be exerted through a calcium binding protein particularly calmodulin (for the most recent review see [177]).

Garrison [197] found that phenylephrine increases the phosphorylation of cytosolic proteins and a comparison with glucagon treated liver cells revealed that in both cases the same proteins become phosphorylated. He reported that phosphory-

lation of these proteins in the presence of phenylephrine, vasopressin, angiotensin II and A23187 was Ca^{2+}-dependent [80]. We investigated this topic further as described in appendix paper 2. The relative role of cAMP-dependent, cAMP-independent and Ca^{2+}-dependent phosphorylation was examined. It appeared that the substrate specificity of Ca^{2+}-dependent protein kinases is quite different from that of cAMP-dependent protein kinase. Only the phosphorylation of a M_r-100,000 protein, which was tentatively identified as phosphorylase, was cAMP- and Ca^{2+}-dependent. So in the cytosolic phosphorylation system no evidence for a similar Ca^{2+}- and cAMP-dependent phosphorylation system has been obtained. In contrast specific Ca^{2+} effects were noticed and it was also found that Ca^{2+} most likely stimulates the dephosphorylation of a M_r-115,000 protein.

It is interesting to note that according to the interpretation of Garrison [107] glucagon and adrenaline exert the same physiological effect through phosphorylation of the same proteins. We conclude (appendix paper 2) that the mechanism by which these hormones could provoke this phosphorylation must be quite different. The most intriguing question here is why there should be different mechanisms to exert the same effect.

Pyruvate kinase

In chapter III.2 I have already discussed the fact that PK becomes phosphorylated and inactivated by a cAMP-dependent mechanism. As in some cases cAMP rises after addition of an α-adrenergic agonist, we have to be very careful to conclude that there is an α-adrenergic, cAMP-independent mechanism by which PK becomes phosphorylated or inactivated. So, the inactivation in response to adrenaline reported by Kemp and Clark [178] can be explained by a cAMP-dependent mechanism as they found a rise in cAMP and an increase in cAMP-dependent protein kinase activity. The phenylephrine induced inactivation observed by Blackmore et al. [163] can also be attributed to a cAMP-dependent mechanism. Other reports give no decisive about a possible involvement of a cAMP-dependent mechanism in the inactivation of PK by phenylephrine [179] or adrenaline in the absence [79] or

presence of propranolol [90, 180, 181]. Chan and Exton [33] showed that addition of phenylephrine leads to a small inactivation of PK without a rise in the cAMP concentration. Using adrenaline + propranolol they found no rise in cAMP and no inhibition of PK which was also reported by Hue et al. [55] using phenylephrine as an agonist. So at this time no clear proof exists that PK can be phosphorylated as a consequence of an α-adrenergic mechanism. It must be stressed that the measurement of activity changes in PK can be misleading. If even small amounts of Fru 1,6-P_2 are bound to the enzyme, the inactivation escapes detection. For this reason special precautions have to be taken to remove Fru 1,6-P_2 [182].

Fructose 1,6-bisphosphatase

The consequence of an increase in cytosolic Ca^{2+} will be an inhibition of FDPase activity [164]. This results in an increased Fru 1,6-P_2 level, which will cause the allosteric activation of PK. A stimulation of PK flux in the fed liver has indeed been observed [39]. It is not understood how these effects on PK will increase gluconeogenesis. Probably the main regulatory site is still at the level of Fru 1,6-P_2. In this respect it is interesting to discuss some aspects of FDPase that are described in appendix papers 4 and 5. In appendix paper 5 it is shown that FDPase is a hysteretic enzyme. After addition of substrate the enzyme slowly changes from a high activity form with low affinity for its substrate to a low activity form characterized by an extremely high affinity. Evidence is presented that the transition is introduced by a Fru 1,6-P_2-divalent cation complex, the divalent cation being most likely Zn^{2+}. The phenomenon cannot be masked by preincubation with Zn^{2+}, Fru 1,6-P_2 or Fr 6-P+P_i. An explanation must be given how metal ions, that bind relatively weak to Fru 1,6-P_2, introduce the low K_m form of the enzyme. A simple scheme of the hydrolysis of Fru 1,6-P_2 by FBPase is given by:

$$E + \text{Fru } 1,6\text{-}P_2 \rightleftarrows E\ \text{Fru } 1,6\text{-}P_2 \rightarrow E + \text{Fru } 6\text{-}P + P_i$$

Suppose that the enzyme also binds the Zn-Fru 1,6-P_2 complex. Then the hystereses could be related to the transfer of the metal ion from the substrate to the enzyme:

$$E + \text{Zn-Fru 1,6-}P_2 \rightleftarrows \overset{\text{Zn}}{\underset{|}{\text{E-Fru 1,6-}P_2}} \rightarrow \overset{\text{Zn}}{\underset{|}{\text{E-Fru 1,6-}P_2}}$$

The enzyme-zinc complex corresponds to the high K_m form of the enzyme.
After hydrolysis of the substrate or after addition of EDTA the dissociation reaction predominates

$$Zn - E \rightarrow Zn^{2+} + E$$

and the enzyme is in the low K_m form. This rate of dissociation is slow compared to the rate of catalyses.

The change in K_m is remarkable (from 2 µM to 0.3 µM) and offers a possibility to decrease the Fru 1,6-P_2 concentration well below 1 µM. Another aspect of the FDPase reaction is its regulation of the distribution of α- and β-anomeric conformers of Fru 1,6-P_2. This is caused by the fact that FDPase hydrolyses the α-conformer and furthermore that the rate constant of the β → α-anomerization is relatively low.

In appendix paper 4 it is described how this rate constant can be determined using ^{31}P-NMR. We found that $k_{\beta\alpha}$ is $4s^{-1}$, however, in the presence of excess Mg^{2+} this rate constant is rather $1s^{-1}$. If we assume that the specific activity of the purified enzyme is 50 U/mg [93] and that the intracellular concentration of the enzyme is of the order of 5 µM, then one can calculate that at a Fru 1,6-P_2 concentration of 10 µM the enzyme activity is 3.5 times the anomerization rate. At a Fru 1,6-P_2 concentration of 2 µM this value is 15 times. This means that the fraction of α-anomer drops from 0.20 at infinite Fru 1,6-P_2 concentration to 0.12 at 10 µM and as low as 0.07 at 2 µM. This might have important regulatory consequences as proposed by Koerner et al. [183].

Returning to the adrenergic induced rise in the Ca^{2+} concentration; it might be possible that this event introduces the low

K_m form of FDPase. The concomitant decrease in the activity of FDPase is still sufficient to account for the total gluconeogenic flux (compare Table I).

Mitochondria

At least some of the changes in mitochondrial metabolism observed after the addition of glucagon are also observed with catecholamines. Adrenaline and phenylephrine stimulate citrulline synthesis [135] and uncoupler-dependent ATPase activity [135, 143]. Adrenaline gives rise to an increased ATP/ADP ratio [135]. These effects are mediated by an α-adrenergic mechanism as they can be blocked by dihydroergotamine [135].
Adrenaline also causes an increase in the rate of pyruvate (de)carboxylation, pyruvate uptake [138, 146] and state 3 respiration [138]. It has been reported that the mitochondrial transmembrane proton gradient is not affected in contrast to the effect with glucagon [138]. However, the matrix volume decreases [138]. As already discussed in section III.2 it might be possible that these effects are a consequence of a stabilizing effect of hormones on the mitochondria during the isolation procedure.

IV GLUCONEOGENESIS AND LACTIC ACIDOSIS

Gluconeogenesis is important in the regulation of the blood glucose level in situations with a limited supply of food or where there is a sudden excessive need for energy. On the other hand conversion of lactic acid to glucose is a way to metabolize hydrogen ions and so to prevent lactic acidosis [186]. Indeed it has been reported that a deficiency in one of the gluconeogenic enzymes, such as pyruvate carboxylase (see [184] and references therein) or FDPase (see [185] and references therein) leads to a lactic acidosis. However, several cases are described, in which no underlying deficiency could be detected (see cases in [186]). As discussed in the previous chapter, the process of gluconeogenesis is under stringent control of hormones. In consequence not only a deficiency in one of the gluconeogenic enzymes, but also an aberration in the hormonal regulatory mechanism might cause lactic acidosis. Some support for this hypothesis can be found considering the effects of biguanides. Phenformin (N-phenylethyl biguanide) is a hypoglycemic agent and it increases blood lactate levels (for an extensive discussion see [186, 187]). Phenformin together with other factors (shock, diabetes) can be a causative factor in the development of lactic acidosis. The theory that phenformin acts through an inhibition of tissue oxidation has been criticized [188]. More likely the rate of removal of lactic acid is decreased [189]. Tolbert and Fain [143] showed that biguanides depress the hormonal stimulation, but not the basal level of gluconeogenesis in hepatocytes. This suggests that the impairment of the hormonal control of gluconeogenesis after administration of phenformin could be the cause of lactic acidosis. A similar diminished hormonal response is observed at low pH. In the experiment shown in Fig. 3 it appears that glucagon does not stimulate gluconeogenesis at a medium pH of 6.9. This observation might be related to the clinical experience that the patient hardly recovers, i.e. gluconeogenesis cannot be stimulated by hormones. Prolonged alkalinization with bicarbonate is mostly necessary for full recovery of the patient.

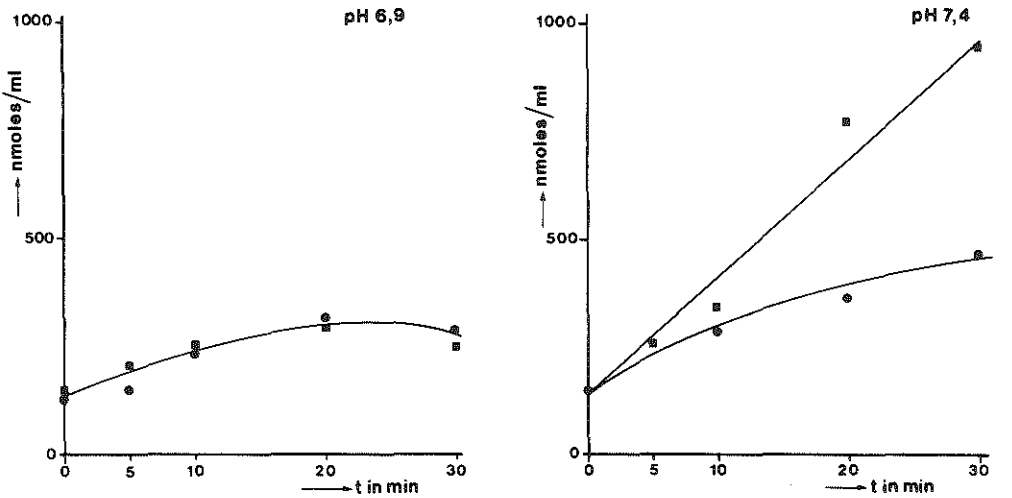


Fig. 3 Stimulation of gluconeogenesis in rat-liver cells by glucagon. Gluconeogenesis was measured as the production of [^{14}C]-glucose from [2-^{14}C]-lactate. Hepatocytes were incubated during 10 min at 37°C in Krebs-Ringer bicarbonate buffer. Cells were continuously gassed with carbogen and continuously shaked. pH was set by varying the amount of bicarbonate in the buffer. Gluconeogenesis was initiated by the addition of 10 mM lactate at t=o.

Clinical relevance of the investigations

The investigations presented in this thesis were undertaken to develop a procedure for the detection of aberrations in the regulating mechanism of gluconeogenesis. With such a procedure it is possible to test the hypothesis that some cases of lactic acidosis are related to deficiencies in the control mechanism of metabolism rather than in metabolism itself. The work hypothesis was that hormonal stimulation is affected as consequent on the covalent modification of gluconeogenic and/or glycolytic enzymes

by cAMP-dependent and/or Ca^{2+}-dependent phosphorylation. Special interest was given to develop a screening procedure that could be applied to biopsy material.

In appendix paper 7 the main characteristics of the phosphoprotein pattern of human liver samples are described. It appeared that some striking resemblances with rat liver exist. The phosphorylation of PK is stimulated by cAMP. Furthermore the phosphorylation of PK and a M_r-68,000 protein can be inhibited by Fru 1,6-P_2. The effects of phosphorylation on the kinetics of PK activity are reported in appendix paper 6. Similar to the results with rat liver an increase in $K_{0.5}$ for phosphoenolpyruvate is observed after phosphorylation. The study in appendix paper 6 also shows that the phosphorylation reaction is very rapid and that stimulation of the phosphorylation is obtained with physiological concentrations of cAMP.
On the other hand it must be clarified why a relatively small number of phosphoproteins is detected in human liver samples. The samples tested were post mortem and it might be possible that the use of fresh material changes the picture.

It must be realized that a study with human liver on the hormonal regulation of gluconeogenesis is still premature as long as the process is not fully understood in the laboratory animal. Further studies on the molecular basis of the hormonal regulation of gluconeogenesis are needed for the understanding of the occurrence of lactic acidosis in patients.

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APPENDIX PAPERS

IDENTIFICATION OF L-TYPE PYRUVATE KINASE AS A MAJOR PHOSPHORYLATION SITE OF ENDOGENOUS CYCLIC AMP-DEPENDENT PROTEIN KINASE IN RAT LIVER SOLUBLE FRACTION

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1. Introduction

The mechanism by which hormones such as glucagon and catecholamines stimulate gluconeogenesis in liver has as yet not been elucidated. Injection of glucagon or epinephrine has been reported to lead to a lowered activity of the glycolytic enzymes phosphofructokinase and pyruvate kinase, whereas the gluconeogenic enzyme fructose-1,6-diphosphatase shows an increased activity; all enzymes being measured under one particular condition [1,2]. The mechanism of these changes in enzyme activity has not been investigated. The most well-known action of glucagon on liver cells is the stimulation of cyclic AMP-dependent protein kinases. It is obvious to explain the action of glucagon on gluconeogensis by phosphorylation of the above-mentioned enzymes which results in a change of enzyme activity. This seems indeed to be the case for pyruvate kinase, as judged from in vitro studies (reviewed in [3]) and from in vivo experiments [4]. Phosphorylation of purified phosphofructokinase [5] and fructose-1,6-diphosphatase [6] with concurrent change in enzyme activity has been reported. Experimental evidence for in vivo regulation of these enzymes by phosphorylation–dephosphorylation reaction is up till now absent.

Phosphorylation of rat liver cell sap [7–9] or rat hepatocyte soluble proteins [10] has been studied in the presence of added exogenous protein kinase. The effect of glucagon and catecholamines on the phosphorylation of supernatant proteins has been studied [10] in intact hepatocytes where endogenous protein kinase was used. Although it was not proven, pyruvate kinase was indicated as one of the proteins of which the phosphorylation is greatly stimulated by these hormones. No evidence for a stimulated phosphorylation of fructose-1,6-diphosphatase was found. We investigated the cyclic AMP-dependent phosphorylation of rat liver soluble proteins by endogenous protein kinase(s). Phosphorylated proteins were analysed by SDS–polyacrylamide gel electrophoresis and subsequent autoradiography. With this system we found that L-type pyruvate kinase is the major protein which incorporates ^{32}P from [γ-^{32}P]ATP in a cyclic AMP-stimulated reaction. The phosphorylation of pyruvate kinase is greatly diminished in the presence of the substrate phosphoenolpyruvate and not influenced by the allosteric activator fructose-1,6-diphosphate. Phosphorylation of fructose-1,6-diphosphatase was not detectable in this system.

2. Materials and methods

2.1. *Preparation of soluble fractions*

Minced rat liver was homogenized (20% w/v) with a Potter Elvehjem homogenizer in 250 mM sucrose, 25 mM Tris–HCl (pH 7.5), 2 mM mercaptoethanol and centrifuged at 105 000 × *g* for 60 min at 4°C. A human liver sample was obtained from a 7 month old baby. The sample was frozen in liquid N_2 within 4 h after death. The sample was treated as above.

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2.2. *Phosphorylation experiments*

Soluble fraction (10 μl) was preincubated for 1 min with 10 μl from a mixture containing 25 mM Tris–HCl (pH 7.5), 40 mM phosphate, 200 mM KCl and 10 mM theophylline at 37°C. Phosphorylation was started by adding 10 μl of a mixture of [γ-^{32}P]ATP, ATP and $MgCl_2$. The final concentration of $MgCl_2$ was 5 mM. ATP concentrations ranged from 10–100 μM (see figure legends). The specific activity of [γ-^{32}P]ATP was 3000 Ci/mmol (The Radiochemical Centre, Amersham). About 5 μCi/incubation was used. The reaction was stopped with 15 μl of a solution containing 0.3 M Tris–H_3PO_4 (pH 6.8), 3% SDS, 50 mM 2-mercaptoethanol and 20% glycerol. In a pilot study it was established that the inactivation of pyruvate kinase activity, measured at suboptimal phosphoenolpyruvate concentrations, was complete ⩽ 30 s with 10 μM ATP. At 100 μM ATP this inactivation occurred ⩽ 10 s. Samples were heated to 95°C for 5 min before electrophoresis.

2.3. *Electrophoresis*

SDS–polyacrylamide slab gel electrophoresis was performed as in [11] in 10% gels. Gels were 0.75 mm thick. Marker proteins were (mol. wt): phosphorylase (92.5 × 10^3), bovine serum albumin (67 × 10^3), catalase (60 × 10^3), ovalbumin (45 × 10^3), chymotrypsinogen (25 × 10^3). Marker proteins were mixed with an equal amount of the soluble fraction prior to electrophoresis. The gels were stained in a solution of 0.2% Coomassie brilliant blue R 250 in 50% methanol 3.5% acetic acid and destained in a mixture of 5% methanol and 7.5% acetic acid and subsequently vacuum dried. Autoradiographs were made by exposing the dried gel to Kodak XR-1 film. Scanning of the autoradiographs was performed with a Vitatron TLD 100 densitometer at 510 nm. Peak area was determined by weighing the cut out densitogram. Different exposure times were used to check linearity of amounts of radioactivity with optical density.

2.4. *Specific removal of proteins from a soluble fraction*

Antiserum against human liver L-type pyruvate kinase was raised in rabbit. The antiserum is monospecific against human liver L-type pyruvate kinase, but shows crossreactivity with L- and M-types pyruvate kinase of rat liver. In a concentration range of this antiserum and rat liver soluble fraction we detected 1 weak precipitation line in an Ouchterlony test. Antiserum against rat liver fructose-1,6-diphosphatase was raised in cavia. This antiserum showed 4 precipitation lines in an Ouchterlony test. Antiserum

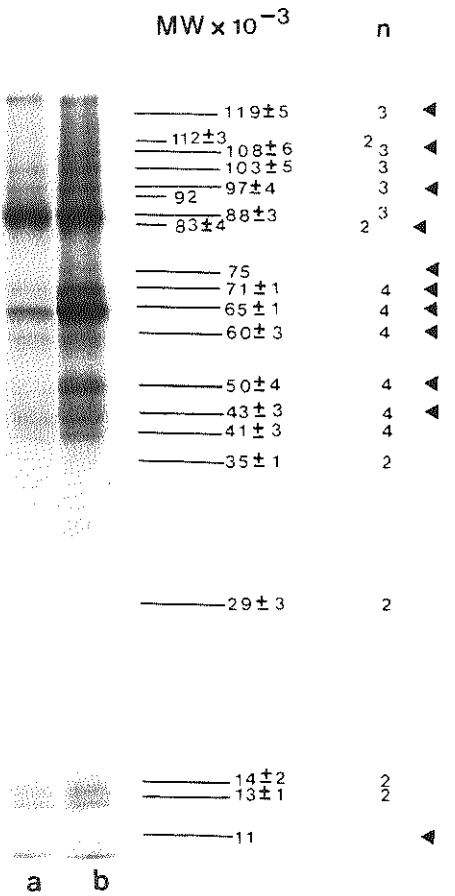

Fig.1. Cyclic AMP-dependent phosphorylation of rat liver soluble proteins as detected in an autoradiograph of a SDS–slab gel. Rat liver soluble fraction was phosphorylated as in section 2 with 10 μM ATP, for 30 s in the absence (a) or presence of 10 μM cyclic AMP (b). *n* denotes number of determinations; cyclic AMP-dependent increase in phosphorylation is indicated by (▲).

and control serum proteins were precipitated by 50% ammonium sulfate and after centrifugation dissolved in and subsequently dialysed against 0.1 M $NaHCO_3$, 0.5 M NaCl. The proteins were coupled to CNBr-activated Sepharose 4 B as described by the manufacturer (Pharmacia, Sweden). Soluble fractions were incubated with control serum- or antiserum-coupled Sepharose for 20 min at room temperature. Removal of enzyme was tested by measuring the remaining enzyme activity in the supernatant.

3. Results and discussion

3.1. *Phosphorylation pattern*

Rat liver soluble proteins separated by SDS–polyacrylamide gel electrophoresis contains 20 clear phosphorylation bands. The phosphorylation of at least 11 bands is stimulated by cyclic AMP as judged by scanning of the autoradiographs and visual interpretation, with the most pronounced effect on a protein with mol. wt ($65 \pm 1 \times 10^3$) (figs.1,2). A similar pattern was found with rat hepatocyte supernatant fraction upon incubation with protein kinase catalytic subunit [10].

3.2. *Identification of phosphorylation bands*

To detect pyruvate kinase in this pattern, we prepared a soluble fraction, from which pyruvate kinase was removed (see section 2). The major phosphorylation band disappeared specifically upon this treatment (fig.3). The possibility exists that M-type pyruvate kinase is responsible for this observation, because antiserum against human pyruvate kinase shows crossreactivity with L- and M-types pyruvate kinase of rat liver [12]. However, the same result was obtained with a soluble fraction from parenchymal cells which contains only L-type pyruvate kinase [16]. Furthermore, in human liver, this band with approximately the same molecular weight also disappeared after treatment with Sepharose-bound antiserum against L-type pyruvate kinase. In this latter case the antiserum is monospecific for L-type pyruvate kinase. From these experiments it must be concluded that the major phosphorylation band originates from the subunits of L-type pyruvate kinase.

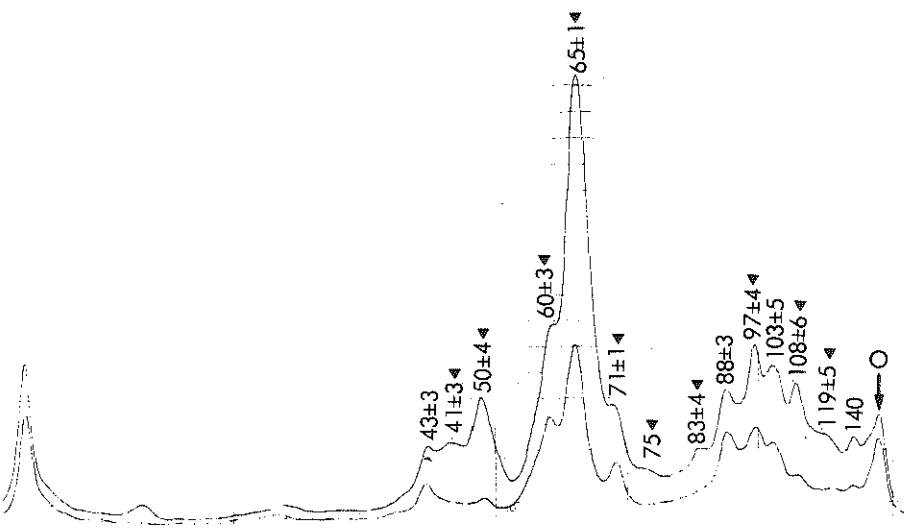

Fig.2. Cyclic AMP-dependent phosphorylation of rat liver soluble proteins. A typical example of a scanning of an autoradiograph is shown. For conditions see fig.1. The mol. wt $\times 10^{-3}$ is indicated. Cyclic AMP-stimulated bands are indicated by (▲).

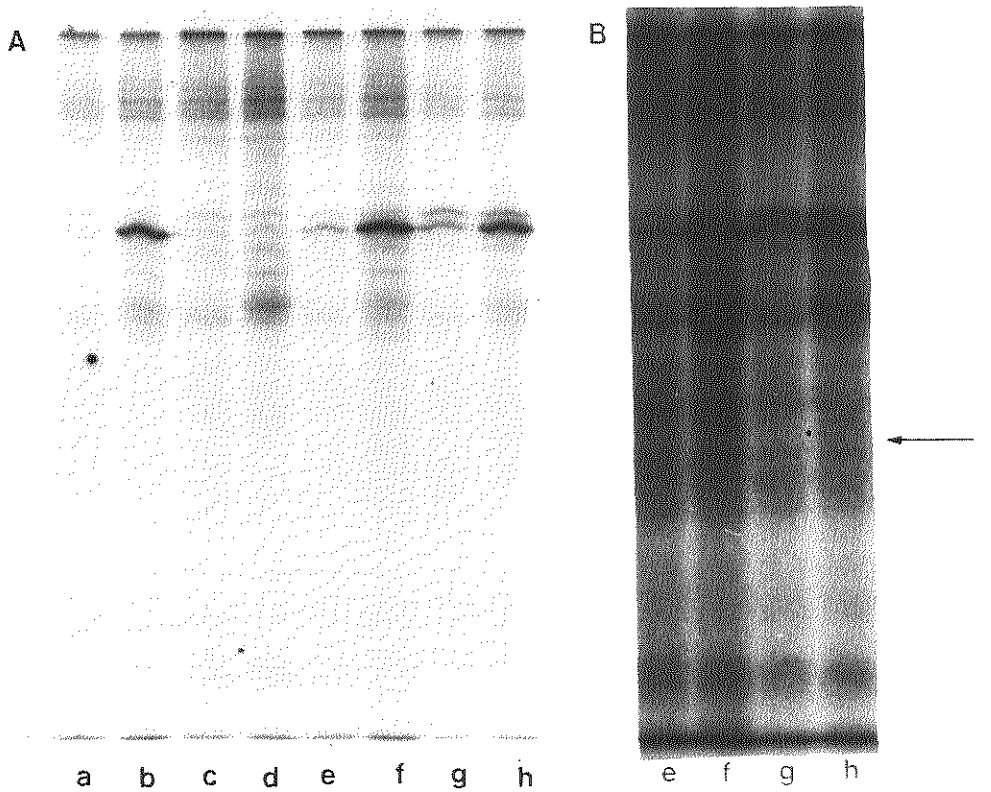

Fig.3.(A) Detection of pyruvate kinase and fructose-1,6-diphosphatase in SDS–slab gel electrophoresis of rat liver soluble proteins. Soluble fractions were incubated with sera bound to Sepharose and afterwards phosphorylated at 1 μM ATP for 10 s. Rabbit control serum-treated sample: (a) – cyclic AMP; (b) + 10 μM cyclic AMP. Rabbit antiserum against pyruvate kinase treated sample: (c) – cyclic AMP; (d) + 10 μM cyclic AMP. Cavia control serum treated sample: (e) – cyclic AMP; (f) + 10 μM cyclic AMP. Cavia antiserum against fructose-1,6-diphosphatase-treated sample: (g) – cyclic AMP; (h) + 10 μM cyclic AMP. (B) e–h were as in (A) but a longer exposure time was used. This experiment was also performed with 100 μM ATP for 2 min. The phosphorylation pattern was almost identical as shown here. Arrow indicates approximate mobility of fructose-1,6-diphosphatase subunits.

From this, direct evidence is obtained that the reported changes in kinetic parameters of pyruvate kinase in liver homogenates [13] or homogenates of parenchymal cells [14–16] upon incubation with MgATP and cyclic AMP are indeed parallelled by an increased phosphorylation state of the enzyme. The fact that pyruvate kinase is identified as a major phosphorylated protein is not necessarily surprising as the concentration of this enzyme in liver cells is high (± 2 μM, calculated from data in [17]). The concentration of fructose-1,6-disphosphatase is similar (calculated from data in [6]). However, no intensive phosphorylation bands are visible in the region of the subunit molecular weight of this enzyme (38×10^3

[6]) in SDS–gel electrophoresis (fig.1). Removal of low molecular weight components which could act as inhibitors of phosphate incorporation in this system, by gel filtration, did not influence the phosphorylation pattern. Phosphorylation of a fructose-1,6-diphosphatase-free soluble fraction did not lead to the disappearance of a phosphorylation band as compared to control serum treated soluble fraction (fig.3). It is not very likely that the non-monospecificity of the antiserum used impaired this result. The absence of a detectable phosphorylation band of fructose-1,6-diphosphatase makes it rather doubtful whether the reported in vitro phosphorylation of the purified enzyme [6] is involved in the hormonal regulation of gluconeogenesis.

3.3. *Effects of phosphoenolpyruvate and fructose-1,6-diphosphate on phosphorylation pattern*

It has been argued that the incubation conditions applied here in a crude cellular preparation is representative of native conditions [14]. We agree with this view because in these systems, probably because endogenous protein kinase is involved, the kinetic changes develop very quickly after addition of cyclic AMP [13,14] in contrast to experiments in a purified system [3] and the inactivation occurs at physiological cyclic AMP concentrations [13–15]. Great care has to be taken, however, when changes in kinetic parameters in these experiments are interpreted as changes in phosphorylation state. Especially the binding of very small amounts of the allosteric activator fructose-1,6-diphosphate to pyruvate kinase can influence the determination of changes in enzyme activity upon incubation, unless special precautions are taken [18]. The described system makes it possible to discriminate between effects upon the phosphorylation state of pyruvate kinase and changes in pyruvate kinase activity. As shown in table 1, phosphoenolpyruvate caused a large decrease in phosphate incorporation in pyruvate kinase. This agrees with experiments in which enzyme activity was used as a parameter for cyclic AMP-induced phosphorylation of pyruvate kinase [14,15]. Fructose-1,6-diphosphate at 1–50 μM, did not have any influence on the phosphorylation state of pyruvate kinase (table 1), in contrast to the effect upon enzyme activity, as reported [14,15]. The absence of an effect of fructose-1,6-diphosphate on the phosphorylation of pyruvate kinase agrees with experiments performed in a purified enzyme system [19]. It seems most likely that the reported inhibition by fructose-1,6-diphosphate of the cyclic AMP-dependent inactivation of pyruvate kinase activity is brought about by binding of the allosteric activator of pyruvate kinase, fructose-1,6-diphosphate. It is important to note the detection of a somewhat higher molecular weight of pyruvate kinase ($65 \pm 1 \times 10^3$) in SDS–gel electrophoresis of a rat liver soluble fraction compared with purified enzyme [3,17]. This difference may be explained by partial proteolysis of pyruvate kinase upon purification. The identification of pyruvate kinase as a major substrate for cyclic AMP-dependent protein kinase in vitro opens the possibility to study the relation between the phosphorylation state of pyruvate kinase and the reported inactivation of the enzyme upon starvation [18,20].

Table 1
Effect of phosphoenolpyruvate (PEP) and fructose-1,6-diphosphate (FDP) on phosphate incorporation in pyruvate kinase

Addition	– cyclic AMP	+ cyclic AMP (10 μM)
none	28	100
0.1 mM PEP	25	88
1.0 mM PEP	8	37
none	26	100
1 μM FDP	28	92
10 μM FDP	32	101
50 μM FDP	23	94

Results are expressed as % of phosphate incorporation without addition of metabolities in the presence of 10 μM cyclic AMP. Soluble fraction was pretreated by Sephadex G-25 filtration

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The Role of Ca^{2+} and Cyclic AMP in the Phosphorylation of Rat-Liver Soluble Proteins by Endogenous Protein Kinases

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Rat liver soluble proteins were phosphorylated by endogenous protein kinase with [γ-^{32}P]ATP. Proteins were separated in dodecyl sulphate slab gels and detected with the aid of autoradiography. The relative role of cAMP-dependent, cAMP-independent and Ca^{2+}-activated protein kinases in the phosphorylation of soluble proteins was investigated. Heat-stable inhibitor of cAMP-dependent protein kinase inhibits nearly completely the phosphorylation of seven proteins, including L-type pyruvate kinase. The phosphorylation of eight proteins is not influenced by protein kinase inhibitor. The phosphorylation of six proteins, including phosphorylase, is partially inhibited by protein kinase inhibitor. These results indicate that phosphoproteins of rat liver can be subdivided into three groups: phosphoproteins that are phosphorylated by (a) cAMP-dependent protein kinase or (b) cAMP-independent protein kinase; (c) phosphoproteins in which both cAMP-dependent and cAMP-independent protein kinase play a role in the phosphorylation.

The relative phosphorylation rate of substrates for cAMP-dependent protein kinase is about 15-fold the phosphorylation rate of substrates for cAMP-independent protein kinase. The K_m for ATP of cAMP-dependent protein kinase and phosphorylase kinase is 8 μM and 38 μM, respectively.

Ca^{2+} in the micromolare range stimulates the phosphorylation of (a) phosphorylase, (b) a protein with molecular weight of 130000 and (c) a protein with molecular weight of 15000. The phosphate incorporation into a protein with molecular weight of 115000 is inhibited by Ca^{2+}. Phosphorylation of phosphorylase and the 15000-M_r protein in the presence of 100 μM Ca^{2+} could be completely inhibited by trifluoperazine. It can be concluded that calmodulin is involved in the phosphorylation of at least two soluble proteins. No evidence for Ca^{2+}-stimulated phosphorylation of subunits of glycolytic or gluconeogenic enzymes, including pyruvate kinase, was found. This indicates that it is unlikely that direct phosphorylation by Ca^{2+}-dependent protein kinases is involved in the stimulation of gluconeogenesis by hormones that act through a cAMP-independent, Ca^{2+}-dependent mechanism.

The processes of gluconeogenesis and glycogenolysis in liver are under stringent control of hormones. Originally it was postulated that the effects of hormones in liver are mediated by cAMP (for a review see [1]). According to this concept glucagon stimulates gluconeogenesis and glycogenolysis by activating adenyl cyclase. This elevates the intracellular cAMP level and by this means the cAMP- dependent protein kinase activity increases. Subsequently, specific enzymes were shown to be phosphorylated by cAMP-dependent protein kinase, which alters their activity. Evidence exists that phosphorylase kinase [2], glycogen synthase [3, 4] and L-type pyruvate kinase are phosphorylated in this way. Of these enzymes only pyruvate kinase has been convincingly identified as an enzyme of which the activity is regulated by cAMP-dependent protein kinase in response to glucagon (for a review see [5]).

Recently, we described the phosphorylation of rat liver soluble fraction by endogenous protein kinases [6]. After dodecyl sulphate polyacrylamide gel electrophoresis and autoradiography, 20 phosphorylated bands could be distinguished. The phosphorylation of 11 of these proteins appeared to be stimulated by cAMP. The question remains if these proteins are solely phosphorylated by a cAMP-dependent protein kinase. In the experiments that we describe here we investigated the substrate specificity of cAMP-dependent protein kinases in rat liver.

Abbreviation. cAMP, adenosine 3',5'-monophosphoric acid.

Enzymes. Pyruvate kinase (EC 2.7.1.40); phosphorylase (EC 2.4.1.1).

The intracellular response that mediates the effects of catecholamines is not clearly identified. It appears that in the action of phenylephrine or vasopressin a rise in the cAMP level is not a prerequisite for the stimulation of gluconeogenesis and glycogenolysis, as these effects are also observed without a concomitant increase in cAMP [7–9] or cAMP-dependent protein kinase activity [9]. Furthermore, a stable activity change (phosphorylation) in phosphorylase kinase [10] and pyruvate kinase [11] does not take place upon addition of phenylephrine to hepatocytes.

Recently it has been suggested that the effects of phenylephrine and vasopressin are mediated by alterations in Ca^{2+} flux [12–14], resulting in an increased intracellular Ca^{2+} level. This can explain the stimulation of glycogenolysis by phenylephrine and vasopressin without a rise in cAMP, as phosphorylase kinase activity is known to be stimulated by micromolar Ca^{2+} concentrations [15,16]. A possible site of action of Ca^{2+} on hepatic gluconeogenesis is unknown. Whether phosphorylase kinase or some other Ca^{2+}-dependent protein kinase mediates the effects of phenylephrine and vasopressin on gluconeogenesis is unclear. Inhibition of a phosphatase by Ca^{2+} could also increase the phosphorylation state of specific proteins. An investigation of the role of Ca^{2+} on the phosphorylation of rat liver soluble proteins is therefore also reported in this paper.

MATERIALS AND METHODS

Preparation of Soluble Fraction

Wistar rats, fed *ad libitum*, were killed between 9.00 and 10.00 a.m. The liver was excized, minced and washed with ice-cold 0.9% NaCl solution. The minced liver was then homogenized (20%, w/v) with a Potter-Elvehjem homogenizer in 250 mM sucrose, 25 mM Tris/HCl (pH 7.5), 2 mM 2-mercaptoethanol and centrifuged at $20000 \times g$ for 20 min at 4 °C. The supernatant was centrifuged at $105000 \times g$ for 60 min at 4 °C. Low-molecular-weight components were removed by applying 5 ml of the soluble fraction to a column (2 × 15 cm) of Sephadex G-25 (medium) equilibrated with 25 mM Tris/HCl (pH 7.5), 1 mM 2-mercaptoethanol. A fraction of the eluant was used in the experiments.

Phosphorylation Experiments

Soluble fraction (10 µl) was preincubated for 2 min with 10 µl of a mixture containing 25 mM Tris/HCl (pH 7.5), 200 mM KCl, 10 mM theophylline, 40 mM phosphate and all additions as indicated in the legends to the figures. The final volume was 30 µl. Protein concentration, if not indicated, was 0.1 mg/assay. The reaction was started by adding [γ-^{32}P]ATP (0.10 mM) + $MgCl_2$ (5.0 mM). The specific activity of [γ-^{32}P]-ATP was about 1 Ci/mmol. The reaction was stopped with 15 µl of a solution containing 0.3 M Tris/H_3PO_4 (pH 6.8), 3% sodium dodecyl sulphate, 50 mM 2-mercaptoethanol and 20% glycerol. Before electrophoresis samples were heated at 95 °C for 5 min.

Electrophoresis

Dodecyl sulphate/polyacrylamide slab gel electrophoresis was performed as described earlier [6] in 10% or 7.5% gels. Usually 15 µl of the incubation mixture was applied per gel slot.

Marker proteins were subunits of RNA polymerase (M_r 165000, 155000, 39000) β-galactosidase (116000) [17] skeletal muscle phosphorylase (97500) [18], bovine serum albumin (68000), catalase (58000), ovalbumin (43000), chymotrypsinogen (25000).

We think that a numerical order of the phosphoproteins, as used by Garrison [19], is premature because the number of bands detected depends on the specific activity of [γ-^{32}P]ATP and on biological variations in the amounts of phosphoproteins present.

Quantification of Phosphorylation Bands

Quantification of the amount of P_i incorporated was done either by scanning the autoradiograph or counting the radioactivity incorporated into the protein bands.

Scanning the Autoradiograph. The vacuum-dried gels were exposed to Kodak XR-1 film for different periods of time. Autoradiographs were scanned with a Vitatron TLD 100 densitometer at 510 nm. Relative amounts of ^{32}P incorporated were determined by integration of the peak with a Vitatron integrator. Different exposure times were applied to check linearity of the amount of radioactivity with absorbance. It appeared that resolution of the peaks by scanning methods is always inferior to visual interpretation of the autoradiographs.

Incorporation of Radioactivity. The absolute amount of ^{32}P incorporated was determined by cutting out the radioactive band, which was then treated with 1 ml 37% H_2O_2 for 2 h at 70 °C. Samples were mixed with Instagel (Packard) and counted in a Packard model 3380 liquid scintillation spectrometer.

The methods described here were discussed earlier [20]. In our hands the autoradiography-densitometry method was the most reliable method.

The influence of proteolytic inhibitors on the phosphorylation pattern was checked by including either 0.2 mg/ml phenylmethylsulfonyl fluoride or 0.1 mM *p*-mercuribenzoate or 1.0 mM iodoacetate or a mixture of these inhibitors of proteolytic activity in all buffers used. No change of the phosphorylation pattern or protein pattern was observed.

Protein was estimated by the biuret method [21] with bovine serum albumin as a standard.

Chemicals and Enzymes

[γ-^{32}P]ATP was obtained from the Radiochemical Centre, Amersham. ATP was determined enzymatically [22]. Protein kinase inhibitor, purified essentially according to Walsh [23] and calmodulin, purified according to Lin [24], were gifts from Dr H. R. de Jonge.

Phosphorylase activity was measured in the presence of 5'AMP according to [25]. Glycogen synthase activity was measured in the presence of glucose 6-phosphate according to [26].

Trifluoperazine was supplied by Smith, Kline & French Laboratories. The powder was dissolved in 20% propylene glycol pH 6.

RESULTS

Earlier we reported that 20 proteins in rat liver soluble fraction incorporate phosphate from ATP. Phosphate incorporation of 11 of these proteins appeared to be stimulated by cAMP [6]. In the present study we paid particular attention to specific major phosphorylation bands in the dodecyl sulphate slab gels, namely L-type pyruvate kinase (molecular weight 65000 [6]) and proteins with molecular weights of 100000, 68000 and 50000 as detected in 7.5% sodium dodecyl sulphate gels.

Garrison et al. [27] identified a phosphoprotein with a molecular weight of 93000 as phosphorylase. As no other clear phosphoproteins appear in this region, we assume that the 100000-M_r protein observed in our system is phosphorylase. An accurate molecular weight determination in a dodecyl sulphate gel is given in Fig. 1. The molecular weight of 100000 is the same as the molecular weight of purified rabbit liver phosphorylase determined in dodecyl sulphate gel [28]. The experiments that we present below further confirm the supposition that the 100000-M_r protein is phosphorylase.

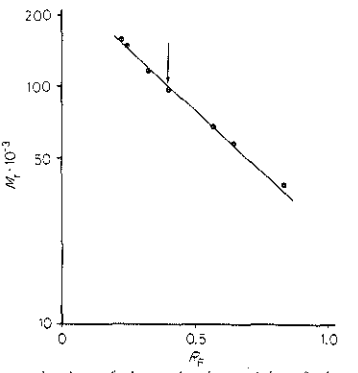

Fig. 1. *Determination of the molecular weight of phosphorylase.* Marker proteins (0.1 mg/ml) were mixed 1:1 with soluble fraction and run in a 7.5% sodium dodecyl sulphate gel. M_r was plotted on a logarithmic scale. Arrow indicates the position of rat liver phosphorylase

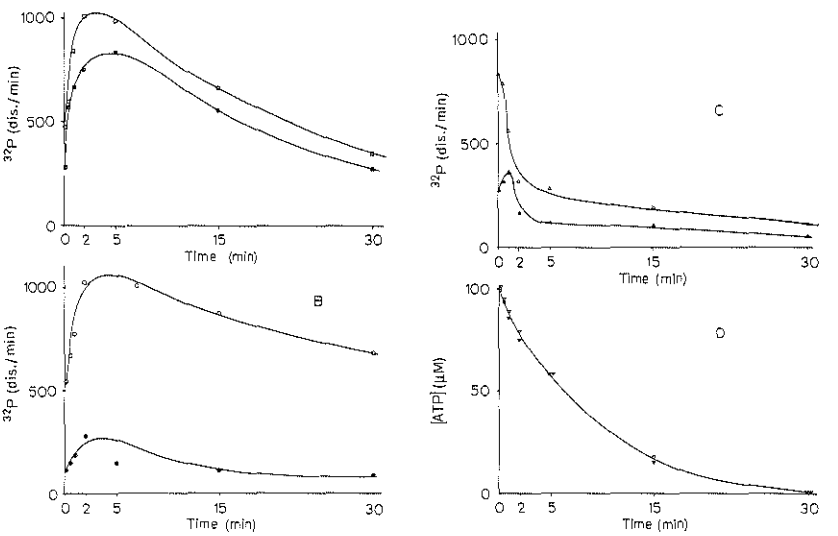

Fig. 2. *Time course of the phosphorylation of rat liver soluble proteins by endogenous protein kinases.* Rat liver soluble fraction was incubated in the absence (closed symbols) or presence (open symbols) of 10 μM cAMP at 37°C. (A) Phosphorylase, (B) L-type pyruvate kinase, (C) protein with M_r 50000, (D) concentration of ATP in the incubation mixture. The shortest sampling time was 10 s after the addition of [γ-^{32}P]ATP

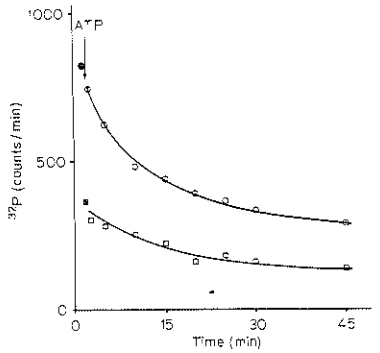

Fig. 3. *Time course of the dephosphorylation of rat liver soluble proteins by endogenous phosphatases.* Rat liver soluble fraction was incubated in the presence of 10 µM cAMP at 37 °C. After 1 min the incorporation of ^{32}P was stopped by isotope dilution with 12.5 mM ATP. (□) Phosphorylase; (○) L-type pyruvate kinase. Closed symbols: amount of ^{32}P incorporated 10 s before the addition of unlabeled ATP. Shortest sampling time was 10 s after the addition of unlabeled ATP

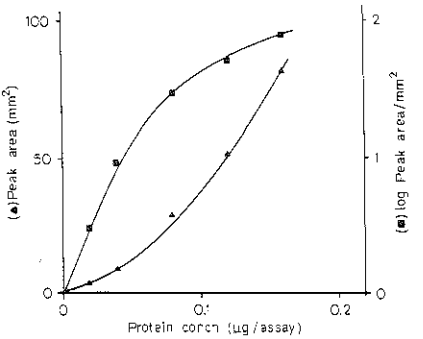

Fig. 4. *Dependency of the phosphorylation rate of L-type pyruvate kinase on protein concentration.* Rat liver soluble fraction was incubated in the presence of 10 µM cAMP at 25 °C during 10 s

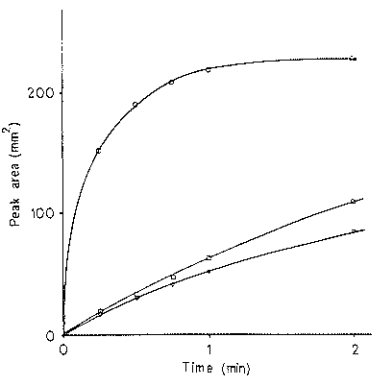

Fig. 5. *Initial phosphorylation rates of rat liver soluble proteins.* Phosphorylation was performed in the presence of 10 µM cAMP at 6 °C. The protein concentration was 0.05 mg/assay. (○) L-type pyruvate kinase; (□) phosphorylase; (▽) protein with M_r 68000

Time Course of the Phosphorylation Reaction

The time dependency of the phosphorylation of pyruvate kinase and phosphorylase in the presence and absence of cAMP is shown in Fig. 2. The phosphorylation of these proteins is very rapid, reaching a maximum after 2–3 min, whereafter a slow dephosphorylation occurs. This time course is, under the conditions used, virtually equal for all phosphoproteins in the soluble fraction with one exception: a protein with a molecular weight of 50000. The phosphorylation of this protein is greatly enhanced by cAMP and reaches a maximum already within 10–30 s, whereafter a rapid dephosphorylation is noticed (Fig. 2 C).

Fig. 2 D illustrates the decline in ATP concentration under the applied conditions. Within 15 min the ATP concentration drops to 15% of the initial value, while the extent of phosphorylation of the proteins, the 50000-M_r protein excepted, is still more than 70% of the maximal amount. This suggests that the endogenous phosphatase activity under the applied condition is rather low compared to the protein kinase activity. If the phosphatase inhibitor phosphate is replaced by fluoride, the time course is not changed, although the maximal amount of radioactivity incorporated is slightly elevated (maximal 20%). If a phosphatase inhibitor is omitted, the maximal amount of radioactivity incorporated is reduced by approximately 10%.

The extent of dephosphorylation under the applied conditions is further illustrated in Fig. 3. It appears that phosphatase activity is indeed low compared to protein kinase activity. After a 30-min incubation in the presence of a more than 125 times excess of unlabeled ATP, 50% of the initial ^{32}P is still present in the protein bands. If the protein kinase reaction is stopped by complexing Mg^{2+} with 10 mM EDTA, a similar dephosphorylation pattern is obtained (not shown).

Protein Dependency of the Phosphorylation Reaction

The amount of ^{32}P incorporated into pyruvate kinase increases exponentially with increasing protein concentration (Fig. 4). Replotting of the data on a logarithmic scale delivers a linear relationship at 25 °C up to protein concentrations of 0.05 mg/assay (Fig. 4). This indicates a second-order reaction which can be expected as the protein kinase as well as its substrate, pyruvate kinase, increase with increasing protein concentrations. At high protein concentrations it is not

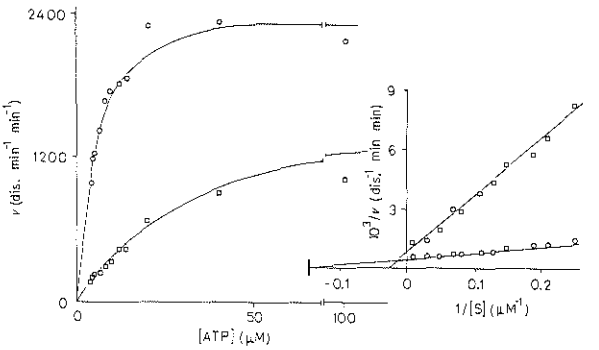

Fig. 6. *The initial phosphorylation rate as a function of [ATP].* Rat liver soluble fraction was phosphorylated in the presence of 10 μM cAMP during 20 s at 25 °C. (○) L-type pyruvate kinase; (□) phosphorylase. Insert: Lineweaver-Burk plot

possible to measure the initial velocity of the phosphorylation reaction, so that the linear relationship is lost.

Kinetics of the Phosphorylation Reaction

The conditions used so far are suitable to detect as many phosphorylation bands as possible. To obtain reliable kinetic data about the phosphorylation of the major bands it is, however, necessary to lower the temperature of incubation or the protein concentration in order to obtain initial phosphorylation rates. Under such conditions a great difference in phosphorylation rate of the various proteins becomes evident (Fig. 5). The phosphorylation of pyruvate kinase is complete within 2 min, whereas the phosphorylation of phosphorylase and a 68000-M_r protein (not cAMP-stimulated) is about 15-fold slower. As a general feature we observed that cAMP-dependent phosphorylation is much more rapid than cAMP-independent phosphorylation. Eventual activators of cAMP-independent protein kinase can change this picture. Extrapolation of these data to protein concentrations *in vivo* indicates that *in vivo* there is a capacity to phosphorylate pyruvate kinase within 0.1 s once the cAMP-dependent protein kinase is activated.

The affinity of the protein kinases for ATP is shown for pyruvate kinase and phosphorylase. Normal Michaelis-Menten kinetics are observed (Fig. 6) and the K_m for ATP of the cAMP-dependent protein kinase involved in the phosphorylation of pyruvate kinase is low (8 μM) compared to the K_m for ATP of phosphorylase kinase involved in the phosphorylation of phosphorylase (38 μM). The K_m determined for cAMP-dependent protein kinase agrees well with the value (5 μM) obtained with the purified rat liver cAMP-dependent protein kinase with calf thymus whole histone as a substrate [29]. Vandenheede et al. [30] found for purified rat liver phosphorylase kinase with skeletal muscle phosphorylase as a substrate a K_m for ATP of 70 μM at pH 8.0 which agrees well with the value reported here.

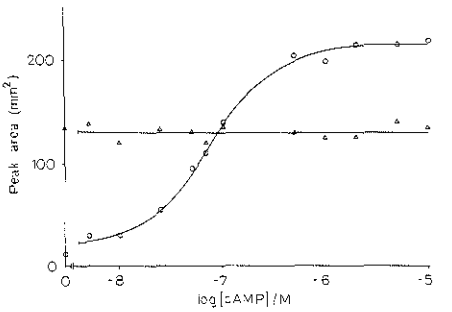

Fig. 7. *cAMP dependency of the phosphorylation of rat liver soluble proteins.* Rat liver soluble fraction was phosphorylated during 2 min at 37 °C. (○) L-type pyruvate kinase; (△) 68000-M_r protein

cAMP-Dependent and cAMP-Independent Phosphorylation

The cAMP-dependency of the phosphorylation of pyruvate kinase and the 68000-M_r protein is shown in Fig. 7. The phosphorylation of pyruvate kinase is maximal at 1 μM cAMP. Half-maximal stimulation is reached with 0.07 μM cAMP. This value makes a phosphorylation-dephosphorylation process *in vivo* likely. The phosphorylation of the 68000-M_r protein is clearly cAMP-independent.

Whether the phosphorylation of a protein is cAMP-dependent or not is often difficult to establish, because in the presence of cAMP the background of the autoradiograph increases. Furthermore, even in the absence of cAMP, some free catalytic subunits of cAMP-dependent protein kinase may be present. To establish the role of cAMP-dependent protein kinase in the phosphorylation of the different proteins,

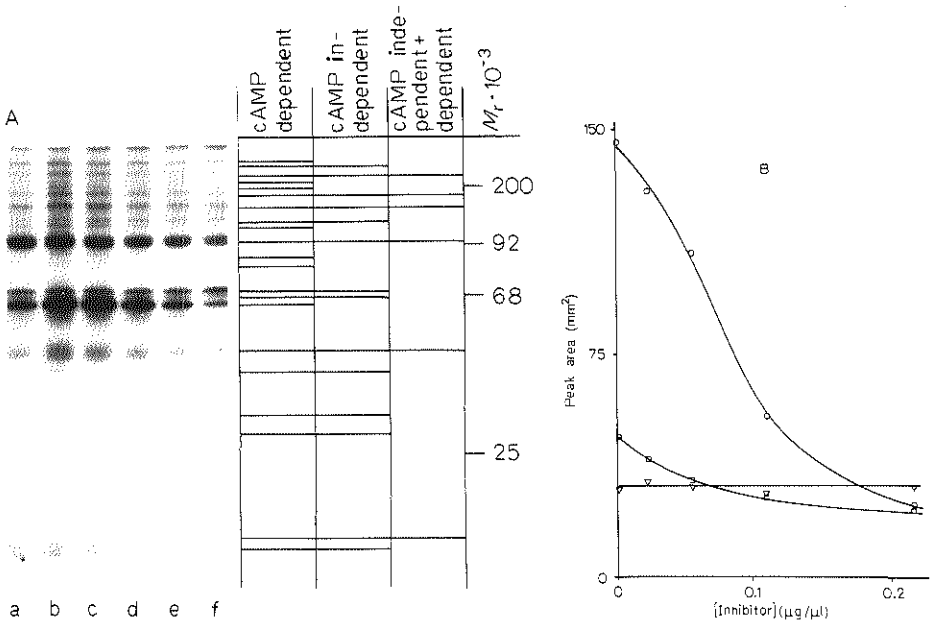

Fig. 8. *The effect of protein kinase inhibitor on the phosphorylation of rat liver soluble proteins.* Rat liver soluble fraction was incubated in the presence of 10 µM cAMP during 20 s at 37 °C. (A) Autoradiograph of the phosphorylation pattern in the presence of increasing concentrations of protein kinase inhibitor. Protein kinase inhibitor concentration in µg/µl: (c) 0.02, (d) 0.05, (e) 0.11, (f) 0.22, (a, b) without protein kinase inhibitor in the presence (b) and absence (a) of 10 µM cAMP. The two most intensive phosphoproteins represent phosphorylase and pyruvate kinase. (B) Quantification of the data presented in (A). (○) L-type pyruvate kinase; (□) phosphorylase; (▽) 68000-M_r protein

protein kinase inhibitor, which inhibits specifically the catalytic subunit of cAMP-dependent protein kinase [31], was added to the assay medium in different concentrations. Fig. 8A shows the effect of increasing protein kinase inhibitor concentration on the phosphorylation pattern. Careful inspection of the autoradiograph shows that seven phosphorylation bands vanish at high protein kinase inhibitor concentration. A typical example of this group of phosphoproteins is pyruvate kinase. The phosphate incorporation of six phosphoproteins is partially inhibited by protein kinase inhibitor. In Fig. 8B it can be noticed, for example, that the phosphorylation of phosphorylase is decreased by 50% in the presence of the highest amount of inhibitor used. In the phosphorylation of the proteins that are partially inhibited by protein kinase inhibitor both cAMP-dependent and cAMP-independent protein kinases seem to play a role.

The phosphorylation of eight proteins is not influenced by protein kinase inhibitor and can therefore be identified as phosphorylated by cAMP-independent protein kinase. The 68000-M_r protein is a representative of this group of proteins (Fig. 8B).

Ca^{2+}-Dependent Phosphorylation

The effect of Ca^{2+} on the phosphorylation pattern is studied in an assay containing a Ca^{2+}/EGTA buffer. Under these conditions the Mg · ATP concentration is kept constant, whereas the buffer has the capacity to absorb 1 µM endogenous Ca^{2+} [32].

In the absence of cAMP, micromolar concentrations of Ca^{2+} stimulate the ^{32}P incorporation into phosphorylase and into proteins with molecular weights of approximately 15000 and 130000 (Fig. 9A). The 15000-M_r protein appears as a relatively weak band so that a longer exposure time of the gel is necessary (Fig. 9C). In the presence of cAMP the phosphorylation of the 130000-M_r protein is no longer dependent on the presence of Ca^{2+} (Fig. 9B). Half-maximal stimulation of the ^{32}P incorporated into phosphorylase is obtained with 0.8 µM Ca^{2+}, while maximal stimulation is observed with 4 µM Ca^{2+}. The presence of cAMP does not alter these data (Fig. 9B and 10). Glycogen synthase (M_r 85000) [3, 33] was not detected as a substrate for phosphorylase kinase. It appeared that glycogen synthase activity was absent

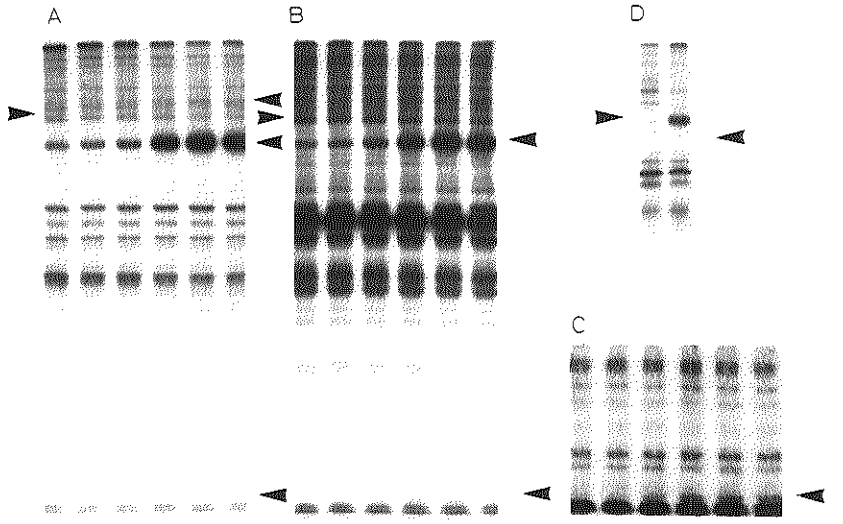

Fig. 9. *Ca^{2+}-dependency of the phosphorylation of rat liver soluble proteins.* Rat liver soluble fraction was incubated in the presence of 100 μM EGTA and increasing amounts of $CaCl_2$ at 25 °C during 1 min. The protein concentration was 0.05 mg/assay. Phosphatase inhibitor was omitted. Equilibrium concentrations of Ca^{2+} were taken from the tables of Bartfai [32]. (A) Autoradiograph of the effect of Ca^{2+} on phosphorylation pattern in the absence of cAMP and (B) in the presence of 10 μM cAMP. Concentration of Ca^{2+} from left to right: 0; 0.13; 0.25; 0.50; 1.0 and 2.0 μM. (C) Detail of the autoradiograph presented in (B) showing the phosphorylation of a protein with M_r 15000. (D) Detail of the autoradiograph showing the disappearance of a phosphoprotein with M_r 115000 upon addition of Ca^{2+}. Left, absence of Ca^{2+}; right, presence of 10 μM Ca^{2+}. 10 μM cAMP was present. Arrows placed at the left-hand side of the figure indicate the disappearance of a phosphorylation band. Arrows placed at the right-hand side indicate the appearance of a phosphorylation band

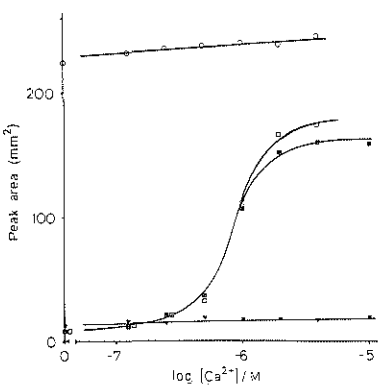

Fig. 10. *Ca^{2+}-dependency of the phosphorylation of rat liver soluble proteins.* For details, see Fig. 9. Closed symbol: absence of cAMP; open symbols: presence of 10 μM cAMP. (○) L-type pyruvate kinase; (□) phosphorylase; (▼) 68000-M_r protein

Table 1. *Total activity of glycogen synthase and glycogen phosphorylase in rat liver fractions*

Fraction	Activity of	
	glycogen synthase	glycogen phosphorylase
	nmol min^{-1} mg^{-1}	
20000 × *g* supernatant	7	65
105000 × *g* supernatant	0	32
105000 × *g* pellet	21	68

in the 105000 × *g* supernatant in contrast to phosphorylase activity (see Table 1).

In the high-molecular-weight region, Ca^{2+} inhibits the ^{32}P incorporation into a protein with a molecular weight of 115000 (Fig. 9 D). This inhibition and also the phosphorylation of the 130000-M_r protein is not always reproducible. Furthermore, sometimes other high-molecular-weiht proteins are visible of which the phosphorylation is stimulated or inhibited by Ca^{2+}. A prolonged preincubation (30 min at 25 °C) with 15 mM $MgCl_2$ to dephosphorylate the proteins did not improve the reproducibility. The observations reported here are made with at least three different preparations. At higher Ca^{2+} concentrations (100 and 500 μM) no other phosphoproteins appeared.

Calmodulin potentiates the effect of Ca^{2+} on the phosphorylation of membrane proteins of various tissues [34]. For this reason we added partially purified calmodulin in the presence of 100 μM EGTA or

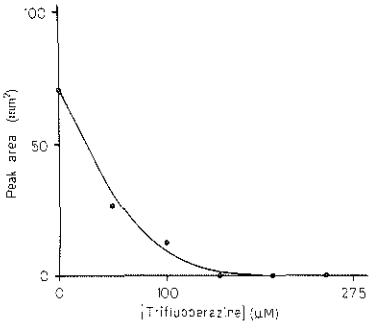

Fig. 11. *Effect of trifluoperazine on the phosphorylation of phosphorylase.* Rat liver soluble fraction was preincubated in the presence of 100 μM $CaCl_2$ and trifluoperazine during 15 min at 6 °C. Phosphorylation time was 1 min. Protein concentration was 0.1 mg/assay

100 μM Ca^{2+} to the phosphorylation assay. The amount used was sufficient to stimulate a rat brain phosphodiesterase preparation purified according to [35] fourfold (J. M. J. Lamers, unpublished result). We did not observe a stimulation of the phosphorylation of the Ca^{2+}-dependent phosphoproteins by added calmodulin. A possible effect of added calmodulin is most likely masked by the presence of endogenous calmodulin. This was tested by using trifluoperazine which specifically binds to calmodulin in the presence of Ca^{2+} [36]. If the soluble fraction was incubated with trifluoperazine in the presence of 100 μM Ca^{2+}, phosphorylase kinase was inhibited in a dose-dependent way (Fig. 11). The phosphorylation of the 15000-M_r protein was also inhibited by trifluoperazine. Unfortunately the phosphorylation pattern in the high-molecular-weight region was always rather vague in the presence of trifluoperazine, so that we cannot conclude anything about an inhibition of the phosphorylation of the 130000-M_r protein. The inhibition of the phosphorylation of the 115000-M_r protein in the presence of Ca^{2+} remained in the presence of trifluoperazine and Ca^{2-}, so that a possible indirect involvement of calmodulin in the inhibition of the phosphorylation of this protein can be excluded.

DISCUSSION

The system as described above allows the study of the mechanism of an altered phosphorylation state of rat liver proteins. The value of this system for studies concerned with the effects of hormones on physiological processes must be regarded as an extension of studies with intact hepatocytes because the mechanism by which possible second messengers influence the phosphorylation state of certain proteins can be investigated in a direct way. The application of the system is restricted by the amount of protein in the incubation mixture, because high amounts of protein increase endogenous ATPase activity (Fig. 1 D).

For rat liver soluble fraction three types of phosphoproteins can be distinguished: proteins whose phosphorylation can be (a) fully, (b) partially or (c) not at all inhibited by heat-stable protein kinase inhibitor. This indicates that rat liver soluble fraction contains (a) proteins which are solely phosphorylated by the cAMP-dependent protein kinase, (b) proteins in which both cAMP-dependent and cAMP-independent kinases play a role in the phosphorylation and (c) proteins which are solely phosphorylated by cAMP-independent protein kinase. The finding that for the phosphorylation of six proteins both cAMP-dependent and cAMP-independent protein kinase are involved can be explained in two ways. A cAMP-dependent protein kinase increases the activity of cAMP-independent protein kinase leading to phosphorylation of only one site of the substrate protein or both protein kinases phosphorylate different sites in one protein.

It could be suggested that the proteins whose phosphorylation depends on cAMP-independent as well as cAMP-dependent protein kinase are phosphorylated by the same protein kinase, i.e. phosphorylase kinase. As the phosphorylation of only one protein of this group (phosphorylase) depends on Ca^{2+}, it can be concluded that phosphorylase kinase is not involved in the phosphorylation of the other five proteins.

The results that we present here on phosphorylation of phosphorylase (effect of cAMP, Ca^{2-}-dependency, effect of protein kinase inhibitor and K_m for ATP) are in good agreement with earlier studies in rat liver soluble fraction in which changes in phosphorylase activity were used to determine phosphorylase kinase activity [2, 10, 15, 16, 30]. So these results substantiate the supposition that the 100000-M_r protein represents phosphorylase.

Garrison [19] showed that when hepatocytes are incubated with $^{32}PO_4^{3-}$, glucagon and phenylephrine increase the phosphorylation state of 11 proteins. In similar experiments Garrison et al. [27] showed that vasopressin and angiotensin II stimulate the phosphorylation of these proteins through a Ca^{2-}-requiring, cAMP-independent mechanism, while glucagon acts through a Ca^{2+}-independent mechanism. From these experiments the question emerged how different mechanisms exert the same effects on the phosphorylation pattern. A Ca^{2+}-dependent mechanism could act through Ca^{2-}-stimulated protein kinases or through Ca^{2+}-inhibited phosphatases. From our study the first possibility seems unlikely, because we obtained no evidence for a Ca^{2-}-activated protein kinase with a similar substrate specificity as cAMP-dependent protein kinase. Actually, Ca^{2+} has a very specific effect on the phosphorylation pattern. The phosphorylation

of three proteins is stimulated by Ca^{2+}. One of these proteins is phosphorylase. The Ca^{2+}-dependency of the other proteins (M_r 15000 and 130000) parallels the Ca^{2+}-dependency of phosphorylase (Fig. 9). The phosphorylation of the 15000-M_r protein and phosphorylase appeared to be calmodulin-dependent. This conclusion can not as yet be made for the 130000-M_r protein because of the poor resolution of this protein in the autoradiographs in the presence of trifluoroperazine. The inhibition of the phosphorylation of the 115000-M_r protein in the presence of Ca^{2+} can be most easily explained by assuming an inhibition of the kinase reaction by making the phosphorylation site less susceptible. The activation of a phosphatase by Ca^{2+} can not, however, be excluded. The observed Ca^{2+}-dependent phosphorylation of the 115000-M_r and the 130000-M_r proteins was subject to variations in different experiments. A possible explanation of these variations lies in the initial phosphorylation state of the proteins. The assumption that all proteins are dephosphorylated after a preincubation in the presence of high Mg^{2+} concentrations is probably not valid. It is quite possible that Ca^{2+}, metabolites or phosphoproteins regulate phosphatase activity. Examples of each of these suggestions can be given. Pyruvate dehydrogenase phosphatase activity is stimulated by Ca^{2+} [37]. The inactivation of liver phosphorylase by its specific phosphatase is enhanced by glucose [38]. The loss of phosphate from the β subunits of skeletal muscle phosphorylase kinase is 50-fold more rapid in the absence of divalent cations if the α subunit is also phosphorylated [39]. To clarify this problem more knowledge about phosphatase specificity and its regulation in rat liver has to be gathered.

The rapidly phosphorylated band with M_r 50000 probably represents the self-phosphorylation of the regulatory subunit of type II cAMP-dependent protein kinase. This reaction is known to be very fast [40]. Furthermore, the observed molecular weight is in agreement with published values [41]. The question arises about the mechanism by which the rapid phosphorylation of the protein is followed by a rapid dephosphorylation while in the meantime the ATP concentration is hardly changed (Fig. 1). It appears that an activation of a specific highly active dephosphorylation reaction occurs. Two explanations can be given. (a) A specific highly active phosphatase is phosphorylated by the cAMP-dependent protein kinase which activates the enzyme or, alternatively, a phosphatase inhibitor is phosphorylated which release the inhibition. (b) The dephosphorylation is caused by the reverse protein kinase reaction. The delay is explained then because the substrate for this reaction, ADP, gradually increases in concentration with time. Indeed, the concentration of ADP required for half-maximal activation of the reverse reaction in the presence of cAMP is very low (15 μM [42]). Further experiments are necessary, however, to explore the nature of this interesting observation.

It is not possible to speculate about the nature of the Ca^{2+}-dependent phosphoproteins. One important conclusion can be drawn, however: no enzymes of the glycolytic or gluconeogenic pathways are phosphorylated by Ca^{2+}-dependent protein kinases in rat liver, as none of these enzymes has a subunit molecular weight that agrees with the high and low molecular weights of the Ca^{2+}-dependent phosphoproteins. The observed stimulation of gluconeogenesis by phenylephrine and vasopressin and in particular the observed inhibition of pyruvate kinase (for a review see [43]) cannot be explained by an increased phosphorylation state of this enzyme by the action of a Ca^{2+}-stimulated protein kinase. The possibility that specific Ca^{2+}-inhibited phosphatases are involved must therefore be further investigated.

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REGULATION OF THE CYCLIC AMP-DEPENDENT PHOSPHORYLATION OF PYRUVATE KINASE AND THE CYCLIC AMP-INDEPENDENT PHOSPHORYLATION OF A 68 K MOLECULAR WEIGHT PROTEIN BY PHOSPHORYLATED HEXOSES

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Eur. J. Biochem., submitted for publication

SUMMARY

Rat liver soluble proteins were phosphorylated by endogenous protein kinase with [γ-^{32}P]ATP.
The maximal amount of phosphate that can be incorporated into pyruvate kinase type L is 4 mol per mol enzyme. Fru 1,6-P_2 inhibits the c AMP-dependent phosphorylation of pyruvate kinase type L and the initial phosphorylation rate is decreased by 68%, while maximally 2 mol P/mol of enzyme can be incorporated.
The inhibition of the pyruvate kinase phosphorylation is specific for Fru 1,6-P_2 and Glu 1,6-P_2. We conclude that Fru 1,6-P_2 and Glu 1,6-P_2 induce such a conformational change in the molecule that this results in half of the sites phosphorylation. The correlation between phosphorylation and inactivation of the enzyme is determined. Almost complete inactivation is obtained if two sites are phosphorylated.

Fru 1,6-P_2 inhibits the c AMP-independent phosphorylation of a protein with M_r 68,000 completely. The K_i for Fru 1,6-P_2 is 1.1 µM. The inhibition is not specific for Fru 1,6-P_2. Fru 6-P and Glu 6-P are evenly effective. Glu 1,6-P_2, however, is less effective. The M_r 68,000 protein does not represent pyruvate kinase type M_2. The data indicate a role of phorphorylated hexoses as possible effectors of cyclic AMP-dependent and cyclic AMP-independent phosphorylation.

INTRODUCTION

The activity of pyruvate kinase type L in human [1] or rat liver [2, 3] can be inhibited by incubation of homogenates in the presence of MgATP and c AMP. This inactivation is accompanied by an increase in ^{32}P content of the enzyme [4]. Both phenomena, i.e. inactivation (Th.J.C. van Berkel, unpublished result) and phosphorylation of the enzyme are inhibited by protein kinase inhibitor [5]. These facts prove that the c AMP induced inactivation of the enzyme in these systems is caused by phosphorylation of the enzyme.

The effect of the allosteric activator Fru 1,6-P_2 on the

c AMP-dependent inactivation and phosphorylation is under discussion. We [4] and Berglund et al. [6] found no effect of Fru 1,6-P_2 on the phosphate incorporation, whereas others reported a diminished phosphorylation rate [7] or inactivation rate [2, 3] in the presence of Fru 1,6-P_2. To clarify this discrepancy we further investigated the phosphorylation of pyruvate kinase in the presence of Fru 1,6-P_2. During the course of this study we discovered another soluble protein of which the phosphorylation is influenced by Fru 1,6-P_2. These findings will also be presented here.

Fru 1,6-P_2 in the micromolar range is a potent activator of pyruvate kinase [8] and phosphofructokinase activity [9]. Evidence exists that Fru 1,6-P_2 is a compound of which the concentration is strongly influenced by hormonal and dietary conditions [10]. Several studies consider Fru 1,6-P_2 as a key metabolite in the regulation of gluconeogenesis-glycolysis [11]. In this paper we report an investigation on the role of Fru 1,6-P_2 as a modulator of the phosphorylation state of rat liver soluble proteins.

MATERIALS AND METHODS

Preparation of soluble fraction

Male Wistar rats, fed ad libitum, were anesthetised with ether. After decapitation the liver was excized. Soluble fraction was prepared and separated from low molecular weight components as described [5]. If indicated this fraction was preincubated in the presence of 5 mM $MgCl_2$ during 30 min at 20°C, at pH 7.5 with a protein concentration of 10 mg/ml.

Phosphorylation experiments

Soluble fraction (10 µl) was preincubated at 6°C for 1 min with 10 µl of a mixture containing 25 mM Tris-HCl (pH 7.5), 200 mM KCl, 5.0 mM $MgCl_2$, 10 mM theophylline, 40 mM phosphate and additions as indicated in the legends to the figures. The final

volume was 30 µl. The protein concentration used depended on the phosphorylation reaction that was studied. As c AMP-dependent phosphorylation in this system is much faster than c AMP-independent phosphorylation [5], the protein concentrations used were 0.05 mg per assay in the former and 0.1 mg per assay in the latter. The reaction was started by adding [γ-^{32}P]ATP (0.10 mM). The final specific activity of [γ-^{32}P]ATP was about 1 Ci/mmole. The reaction was stopped with 15 µl of a solution containing 0.3 M Tris-H_3PO_4 (pH 6.8), 3% sodium dodecyl sulphate, 50 mM 2-mercaptoethanol and 20% glycerol. Before electrophoresis samples were heated at 95°C for 5 min. The activity change of pyruvate kinase was measured after an incubation as described above in a 20 times larger incubation volume except for fig. 5. The incubation was stopped by adding saturated $(NH_4)_2SO_4$ (0°C) to 45% saturation. After centrifugation for 15 min 20,000 x g at 4°C the pellet was dissolved in 25 mM Tris-HCl pH 7.5, 10 mM 2-mercaptoethanol and immediately assayed for pyruvate kinase activity at 30°C. The assay medium contained 75 mM Tris-HCl pH 7.5, 200 mM KCl, 2 mM ADP, 2 mM PEP, 0.13 mM NADH and 4 U LDH per ml.

Electrophoresis

Dodecyl sulphate polyacrylamide slab gel electrophoresis was performed as described in [12] in 10% or 7.5% gels. Per gel slot 45 µg of protein was applied.

Marker proteins were (M_r) skeletal muscle phosphorylase (97,000). bovine serum albumin (68,000), catalase (60,000), ovalbumine (43,000), chymotrypsinogen (25,000).

Quantification of phosphorylation bands

Quantification of the amount of P_i incorporated was done either by: a) scanning of the autoradiograph or b) by counting radioactivity incorporated into the protein bands.

a. The vacuum dried gels were exposed to Kodak XR-1 film for different periods of time. Autoradiographs were scanned with a Vitatron TLD 100 densitometer at 510 nm. Relative amounts of ^{32}P

incorporated were determined by integration of the peak with a Vitatron integrator. Different exposure times were applied to check linearity of the amount of radioactivity with optical density.

b. The absolute amount of ^{32}P incorporated was determined by cutting out the radioactive band, which was first made visible by autoradiography. The piece of gel was treated with 1 ml 37% H_2O_2 for 2 h at 70°C. These extracts were mixed with Insta-Gel (Packard) and counted in a Packard model 3380 liquid scintillation spectrometer. Background radioactivity was determined in a piece of gel cut out in a region close to the phosphoprotein of interest, where no other phosphoproteins were visible.

The number of mol phosphate incorporated per mol pyruvate kinase was estimated as follows: M_r of a subunit is 62,000 [13]; specific activity of the purified enzyme is 520 U/mg [13].

Pyruvate kinase activity was in these experiments measured as described in [13].

The specific radioactivity of [γ-^{32}P]ATP was corrected for hydrolysed ATP as follows:

3 µl of the [γ-^{32}P]ATP solution, as it was used for the incubations, was added to 200 µl 10 mM H_3PO_4 followed by 20 µl ammonium molybdate (30%, w/v) in 5 mM H_2SO_4 and 0.6 ml isobutanol saturated with H_2O.

The mixture was vigorously mixed. The organic phase was discarded and the extraction was repeated once. An aliquot of the water phase was mixed with Insta-Gel and counted. ATP concentration was determined enzymatically [14].

Specific removal of pyruvate kinase type M_2 from the soluble fraction

Antiserum against rat pyruvate kinase type M_2 raised in rabbits was a generous gift from Dr. E. Eigenbrodt (Institut für Biochemie und Endokrinologie, Justus-Liebig-Universität Giessen, Giessen, F.R.G.). Immunoglobulins of 0.5 ml antiserum were precipitated by 50% ammonium sulphate saturation. After centrifugation the pellet was dissolved in and subsequently dialyzed against 0.1 M $NaHCO_3$, 0.5 mM NaCl. The antibodies were

then coupled to 0.2 g CNBr-activated Sepharose 4B as described by the manufacturer (Pharmacia, Sweden). Preincubated soluble fraction was incubated with control or antiserum coupled to Sepharose during 45 min at 20°C in an end over end mixer. Addition of 0.4 ml of Sepharose coupled antibody to 0.8 ml homogenate results in a complete removal of M_2-type pyruvate kinase.

RESULTS AND DISCUSSION

Effect of preincubation and sampling

In earlier experiments we observed no effect of Fru 1,6-P_2 on the phosphorylation of pyruvate kinase type L in an *in vitro* incubation system of rat liver soluble fraction [4]. Also Berglund et al. [6] detected no effect of Fru 1,6-P_2 on the phosphorylation rate of the purifed enzyme. In our study we determined the maximal amount of radioactivity which could be incorporated into the enzyme starting from a not-preincubated fresh liver supernatant. Now it appears that the state of the sample must be more strictly defined. Immediately after gel filtration of the soluble rat liver fraction the maximal amount of phosphate that

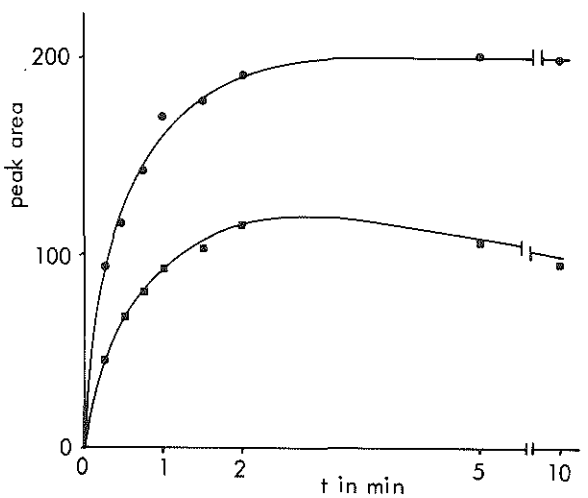


Fig. 1. The influence of preincubation of pyruvate kinase type L in rat liver soluble fraction. Phosphorylation was performed in the presence of 10 µM c AMP at a protein concentration of 0.05 mg/assay (■) without preincubation (●) preincubation during 30 min at 20°C in the presence of 5 mM $MgCl_2$

can be incorporated into pyruvate kinase is less than 50% of the amount that can be incorporated after a 30 min preincubation in the presence of 5 mM $MgCl_2$ at 20°C (fig. 1).
During this preincubation the activity of pyruvate kinase measured at a subsaturating PEP concentration increases, while the maximal activity is not changed (fig. 2). These results can be explained as a reactivation of the enzyme by the action of a Mg^{2+}-stimulated protein phosphatase [15].

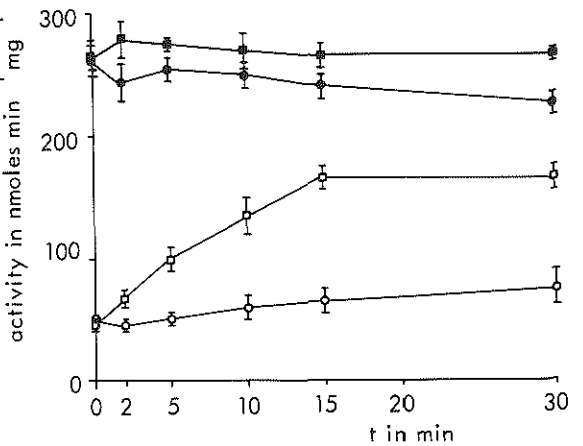


Fig. 2 The reactivation of pyruvate kinase in the absence and presence of 5 mM $MgCl_2$. The soluble fraction was incubated at 20°C and the reaction was stopped at the indicated times by 50% $(NH_4)_2SO_4$. Pyruvate kinase activity of the precipitate was assayed at 2 mM PEP in the absence (open symbols) or presence of 50 µM Fru 1,6-P_2 (closed symbols). □,■ 5 mM $MgCL_2$ present; ○,● no further addition.

It appears that the initial phosphorylation state of the enzyme depends on the method by which the rats are killed. Ether anesthetic results in a largely phosphorylated enzyme in contrast to decapitation. Preincubation of the soluble fraction under the described condition leads independent of the sampling procedure, to a reproducible phosphorylation pattern.

Effect of Fru 1,6-P_2 on the phosphorylation

The effect of Fru 1,6-P_2 on the phosphorylation of pyruvate kinase was reexamined in a preincubated sample. Fig. 3 shows an autoradiograph of rat liver soluble proteins phosphorylated in the presence or absence of Fru 1,6-P_2 and subsequently separated in a dodecyl sulphate gel. The identification of pyruvate kinase type L in these gels was described earlier [4].

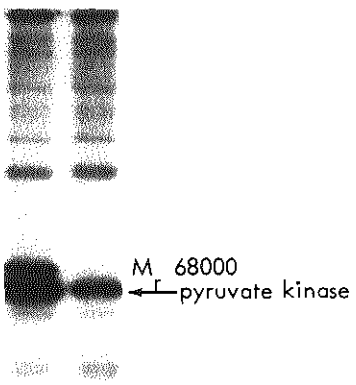


Fig. 3. The effect of Fru 1,6-P_2 on the phosphorylation of rat liver soluble proteins. Rat liver soluble fraction was phosphorylated in the presence of 10 µM c AMP without (a) or with (b) 50 µM Fru 1,6-P_2. Protein concentration was 0.1 mg/assay.

Fru 1,6-P_2 has an effect on two phosphoproteins: a) the phosphorylation of a protein with M_r of 68,000 is completely abolished and b) the phosphorylation of pyruvate kinase is diminished. The first observation is most clear if the cytosolic fraction is phosphorylated in the absence of c AMP, as this protein runs shortly behind pyruvate kinase in a 10% gel and is phosphorylated

by a c AMP-independent protein kinase. The effect of Fru 1,6-P_2 on the phosphorylation of the protein is not dependent on preincubation of the sample. Low molecular weight components (Fru 1,6-P_2) must be removed completely from the cytosolic fraction by gel filtration before incorporation of phosphate into this protein can be detected. Some phosphorylation characteristics of this protein, with a molecular weight of 68,000, were described earlier [5].

A comparison of the effect of Fru 1,6-P_2 on the kinetics of the phosphorylation of pyruvate kinase in a preincubated sample is given in Fig. 4. In a not-preincubated sample Fru 1,6-P_2 has no effect on the phosphorylation (Fig. 4A). In a preincubated sample, however, the rate of the phosphorylation is decreased by 68% and the maximal amount of phosphate that can be incorporated into the enzyme is reduced by 51% (2 mol instead of 4 mol P/tetramer). This suggests that in the presence of Fru 1,6-P_2 half of the phosphorylation sites cannot be occupied.

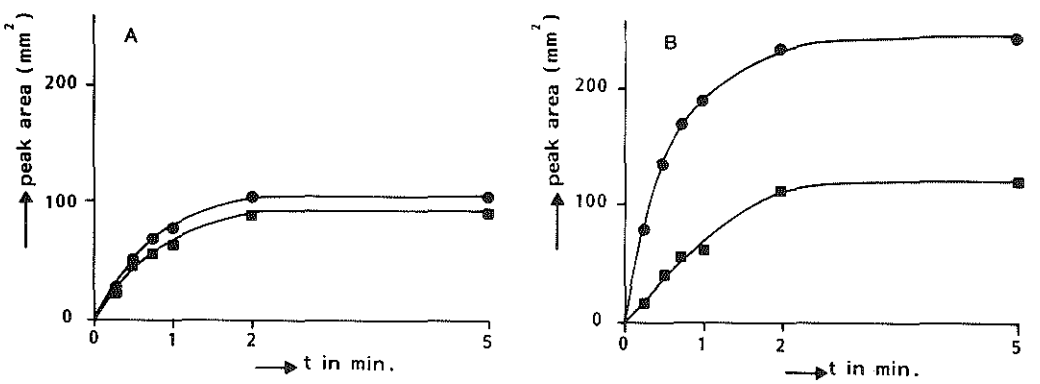


Fig. 4. The influence of preincubation on the Fru 1,6-P_2 dependent phosphorylation of pyruvate kinase. Preincubated (B) or not preincubated (A) soluble fraction was phosphorylated with 10 µM c AMP in the absence (●) or presence (■) of 50 µM Fru 1,6-P_2. Protein concentration was 0.05 mg/assay.

El Maghrabi et al. [16] also reported that the effect of Fru 1,6-P_2 was only observed starting with a fully dephosphorylated

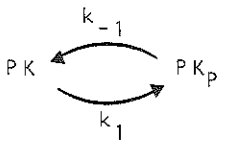
enzyme. These experiments were performed after preincubation of purified pyruvate kinase and catalytic subunit of c AMP-dependent protein kinase, taking advantage of the reverse phosphorylation reaction to fully dephosphorylate the enzyme. The endogenous phosphatase that is responsible for the here described dephosphorylation cannot be the reverse protein kinase reaction as the necessary substrate for this reaction, ADP, is absent during the preincubation. So we conclude that pyruvate kinase can be fully dephosphorylated by an endogenous Mg^{2+}-stimulated soluble protein phosphatase under the described condition.

El Maghrabi et al. [16] did not reach the conclusion that Fru 1,6-P_2 diminishes the maximal phosphate content of the enzyme. This can be explained as evidently these authors did not reach a plateau value of phosphate incorporation in the relevant experiments.

The initial phosphorylation rate in the presence of Fru 1,6-P_2 is 35 ± 5% (n=4) of control values. This value is in good agreement with the one reported earlier [16]. To explain this result we have to assume that Fru 1,6-P_2 not only blocks half of the phosphorylation sites but also makes the two others less susceptible to phosphorylation.

The diminished phosphorylation of pyruvate kinase in the presence of Fru 1,6-P_2 is accompanied by a diminished inactivation of the enzyme. The relative activity of pyruvate kinase ($^{v}/V$) decreases from 0.64 to 0.10 upon full phosphorylation, while phosphorylation in the presence of 50 µM Fru 1,6-P_2 leads to an enzyme form with a relative activity of 0.25 (Table I).

An intriguing question is what the physiological meaning of this half of the sites phosphoenzyme is. The activities presented in Table I suggest that we have to do with an intermediate activity form. One function could be as suggested earlier [2] that Fru 1,6-P_2 protects pyruvate kinase against full inactivation both by diminishing the phosphorylation rate and even more importantly by creating a half of the sites phosphoenzyme. It can be argued that a reduction of the phosphorylation rate by 70% will not effectively reduce the total amount of phosphoenzyme. This can be seen if we write a simple scheme of the phosphorylation-dephosphorylation reaction:



The maximal amount of phosphate incorporated is given by

$$PK_P \text{ max} = \frac{k_1}{k_1 + k_{-1}} \; PK_{tot.}$$

Assuming a $k_1 = 100$ and $k_{-1} = 2$ [5], the fraction of phosphorylated enzyme is maximal 0.98. A reduction of k_1 by 70% results in a fraction of 0.94. This makes only a small difference in the final activity of the enzyme. Of course the time to reach the maximal phosphate incorporation is considerably diminished by a factor $\frac{30-2}{100-2} = \sim 0.3$. As the phosphorylation reaction is very fast (we calculated that *in vivo* pyruvate kinase can be fully phosphorylated within 0.1 s [5]) it is difficult to see how an extension of this period by a factor 3-4 could form an effective protection of the enzyme against full phosphorylation. A half of the sites phosphoenzyme with intermediate activity will, however, be a more effective mechanism to protect the enzyme against full inactivation.

Phosphorylation versus inactivation of pyruvate kinase

The data shown in Table I, together with those from fig. 4, already indicate that phosphorylation of half of the sites does not correspond with a half maximal inactivation. To investigate the relation between phosphorylation and inactivation a determination of the stoichiometry of the reaction was made. The maximal phosphorylated enzyme contains 3.9 ± 0.7 mol phosphate per mol enzyme (n=4). From this result it is obvious to conclude that each of the four identical subunits possesses one phosphorylation site.

Fig. 5 shows the relation between phosphorylation and inactivation. The curve suggests that the greatest activity change is obtained with the incorporation of the second mol of

Table I *The influence of Fru 1,6-P_2 on the c AMP-dependent inactivation of pyruvate kinase type L.*

Phosphorylation time was 5 min. Activities are expressed in nmoles.min^{-1}.mg^{-1} at 2 mM phosphoenolpyruvate (v) or at 2 mM PEP + 0.5 mM Fru 1,6-P_2 (V). All values are mean ± SD of four different preparations. Activity was measured in a 0-45% $(NH_4)_2SO_4$ precipitate.

	Not preincubated			Preincubated		
Addition	v	V	$^v/_V$ x 100	v	V	$^v/_V$ x 100
No	61 ± 21	261 ± 42	23 ± 6	182 ± 55	281 ± 76	64 ± 8
50 µM Fru 1,6-P_2	58 ± 22	244 ± 46	23 ± 6	192 ± 69	275 ± 69	69 ± 14
10 µM c AMP	29 ± 12	262 ± 34	11 ± 4	30 ± 8	290 ± 89	10 ± 3
10 µM c AMP + 50 µM Fru 1,6-P_2	29 ± 7	240 ± 29	12 ± 3	70 ± 20	276 ± 71	25 ± 5

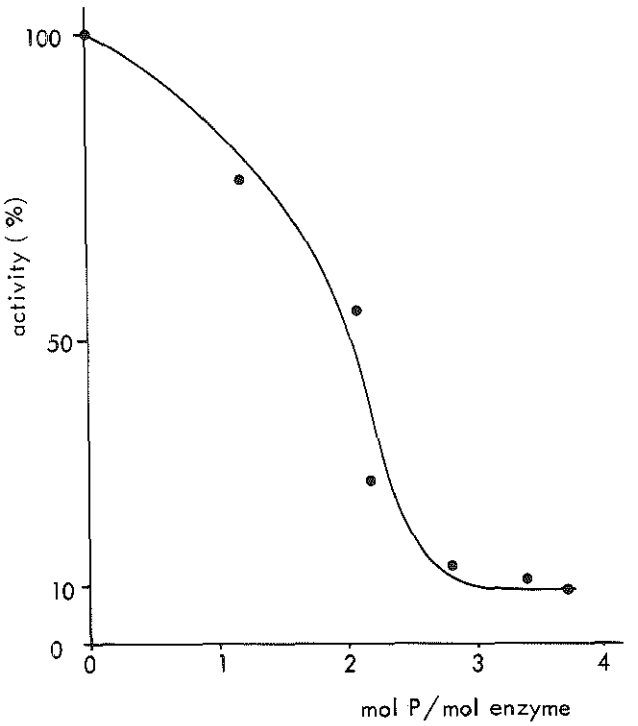


Fig. 5. Relation between phosphorylation and inactivation of pyruvate kinase. Activity of dephospho pyruvate kinase was set to 100%. Experimental points were obtained from a time course. The reaction was stopped by 50% $(NH_4)_2SO_4$ saturation. Activity and ^{32}P content were determined in the same samples as described in the Materials and Methods section.

phosphate, while no activity change occurs with the incorporation of the last mol of phosphate. El Maghrabi et al. [16] obtained essentially the same curve with phosphorylation of purified pyruvate kinase and came to a similar conclusion.

Comparison of the effect of hexose phosphates on pyruvate kinase and M_r 68,000 protein

For reason that the phosphorylation of both pyruvate kinase and the M_r 68,000 protein is influenced by Fru 1,6-P_2 it was investigated whether other hexose phosphates also have similar effects upon both phosphorylatable proteins. Half-maximal inhibition of the phosphorylation occurs at 1.1 µM and 1.4 µM Fru 1,6-P_2 for the 68,000 M_r protein and pyruvate kinase respectively (Fig. 6). These concentrations are in the range at which Fru 1,6-P_2 shows other regulatory properties ($K_{0.5a}$ for pyruvate

kinase is 0.5 µM [8] and $K_{0.5}$ for Fru 1,6-P_2 in the FDPase reaction is 1.2 µM [17].

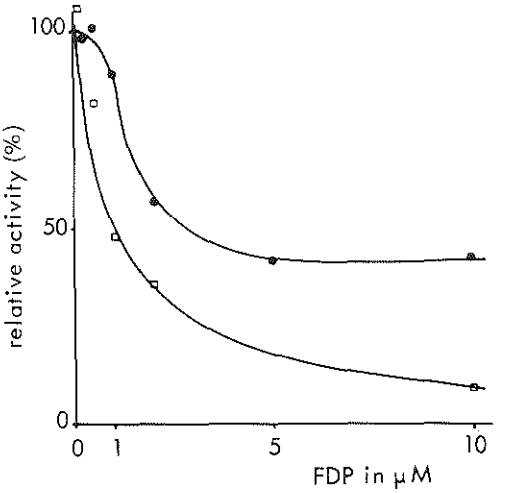


Fig. 6. Inhibition of initial phosphorylation rate of pyruvate kinase and M_r 68,000 protein by Fru 1,6-P_2. Phosphorylation was performed during 1 min at a protein concentration of 0.05 mg/assay for pyruvate kinase in the presence of 10 µM c AMP (●) and 0.1 mg/assay for the M_r 68,000 protein in the absence of c AMP (□). v was calculated as percentage of peak area in the absence of Fru 1,6-P_2. 100% value for pyruvate kinase is 205 mm^2 and for the M_r 68,000 protein 115 mm^2. Similar curves were obtained in two other experiments.

The effect of other hexose mono- and diphosphates on both proteins is shown in Fig. 7. It appears that the inhibition of the phosphorylation of pyruvate kinase is specific for the hexose diphosphates Fru 1,6-P_2 and Glu 1,6-P_2. These are both allosteric activators of pyruvate kinase [18, 19]. The effectiveness of Fru 1,6-P_2 and Glu 1,6-P_2 agrees with the effectiveness as allosteric activators of pyruvate kinase i.e. Fru 1,6-P_2 is a more potent inhibitor of the phosphorylation reaction than Glu 1,6-P_2 [18, 19].

The phosphorylation of the 68,000 M_r protein is not specifically inhibited by Fru 1,6-P_2. The hexose monophosphates Fru 6-P en Glu 6-P are also effective inhibitors of the phosphorylation (Fig. 7). Only Glu 1,6-P_2 is less effective. As added hexose monophosphates are quickly converted to an equilibrium mixture of

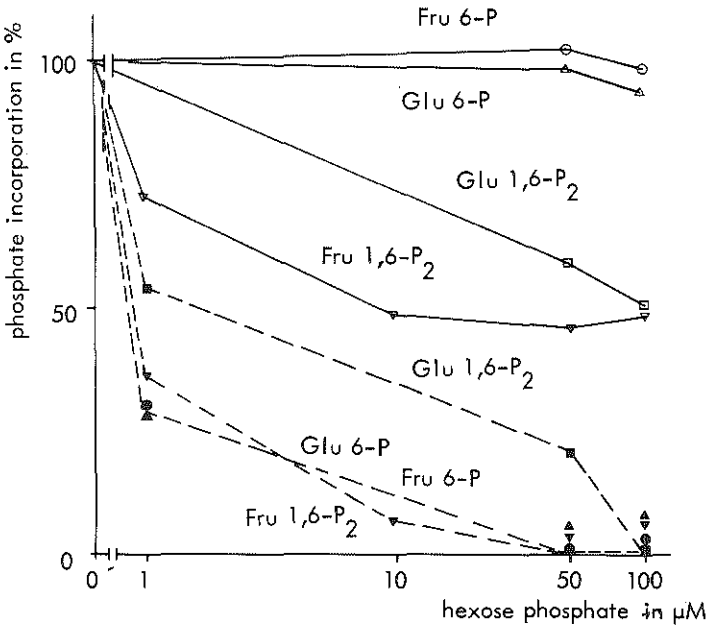


Fig. 7. The effect of hexose phosphates on the phosphorylation of pyruvate kinase and M_r 68,000 protein.
Additions: Fru 6-P (○,●), Glu 6-P (▲,△), Glu 1,6-P_2 (□, ■) and Fru 1,6-P_2 (▽, ▼). Open symbols phosphorylation of pyruvate kinase in the presence of 10 µM c AMP at a protein concentration of 0.05 mg/assay. Closed symbols phosphorylation of M_r 68,000 protein in the absence of c AMP at a protein concentration of 0.1 mg/assay.

Glu 6-P, Glu 1-P and Fru 6-P by the active phosphoglucose isomerase and phosphoglucomutase reactions, it is unlikely that a discrimination between the effects of hexose monophosphates can be made in this system.

It is possible that Fru 1,6-P_2 is not the inhibitor, but a product that is derived from Fru 1,6-P_2. In this respect it is interesting to note that Van Schaftingen et al. [20] reported recently that Fru 2,6-P_2 can regulate phosphofructokinase activity. Because apparently the Fru 2,6-P_2 concentration can be hormonally regulated [21] the role of this compound in the regulation of the phosphorylation of the M_r 68,000 protein deserves special attention.

Lack of identification of M_r 68,000 protein as pyruvate kinase type M_2

Phosphofructokinase [22] and pyruvate kinase type M_2 [23] have been reported as substrates for c AMP-independent protein kinase. Regulation of the phosphorylation of these enzymes by Fru 1,6-P_2 seems quite possible and has even been reported for pyruvate kinase type M_2 in chicken liver [24]. Eigenbrodt et al. [24] reported that Fru 1,6-P_2 lowers and L-alanine increases the rate of phosphorylation of this enzyme. We checked the possibility that the 68,000 M_r protein represents the subunits of pyruvate kinase type M_2. We observed that L-alanine is not able to increase the phosphorylation rate of the 68,000 M_r protein. This already suggests that the protein does not represent type M_2. Definite evidence was obtained in the following experiment: Type M_2 was specifically removed from the soluble fraction by antibody against type M_2 coupled to CNBr activated Sepharose. It was checked that no M_2 type remained in the soluble fraction by separating M_2 and L type activity by ammonium sulphate fractionation. However, the 68,000 M_r phosphoprotein remains in the anti-M_2 type treated sample. So we conclude that the 68,000 M_r protein is not pyruvate kinase type M_2.
Phosphofructokinase is not very likely as a condidate, as the M_r of the subunits of this enzyme is much higher (80,000) [25, 26].

We now attempt to characterize the 68,000 M_r protein. Preliminary results indicate that the protein also in a SDS free medium has a molecular weight of 68,000. Furthermore its isoelectric point is 5.8, while the protein can be collected in a 55-80% $(NH_4)_2SO_4$ fraction.

CONCLUSION

This study strengthens the evidence that changes in the Fru 1,6-P_2 concentration has a very important regulatory role in liver metabolism [19, 27]: Fru 1,6-P_2 stimulates pyruvate kinase and phosphofructokinase activity, Fru 1,6-P_2 inhibits the phosphorylation of pyruvate kinase by c AMP-dependent protein kinase

and Fru 1,6-P_2 or a related component inhibits the c AMP-independent phosphorylation of a M_r 68,000 protein. As the Fru 1,6-P_2 concentration changes rapidly under different hormonal conditions [11], we suggest that Fru 1,6-P_2 or a related phosphorylated hexose [20, 21] can be considered for the liver cells as a "third messenger" to translate extracellular hormonal changes into intracellular phosphorylation changes.

A further study on the phosphorylation state of the 68,000 M_r protein in intact hepatocytes under different metabolic conditions is necessary to determine the significance of the regulation of this newly identified protein by hexose phosphates.

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Zn^{2+}, Mg^{2+}, and H^+ Binding to D-Fructose 1,6-Bisphosphate Studied by ^{31}P and ^1H NMR

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The anomeric composition and mutarotation rates of fructose 1,6-bisphosphate were determined in the presence of 100 mM KCl at pH 7.0 by ^{31}P NMR. At 23 and 37°C the solution contains (15 ± 1)% of the α anomer. The anomeric rate constants at 37°C are (4.2 ± 0.4) s^{-1} for the $\beta \rightarrow \alpha$ anomerization and (14.9 ± 0.5) s^{-1} for the reverse reaction. A D_2O effect between 2.1 and 2.6 was found. From acid base titration curves it appeared that the pK values of the phosphate groups range from 5.8 to 6.0. Mg^{2+} and Zn^{2+} bind preferentially to the 1-phosphate in the α-anomeric position. Zn^{2+} has a higher affinity for this phosphate group than Mg^{2+} has. At increasing pH the fraction α anomer decreases slightly. At increasing Mg^{2+}/fructose 1,6-bisphosphate ratios the fraction α anomer increases till 19% at a ratio of 20. Proton and probably Mg^{2+} binding decreases the anomerization rate. The time-averaged preferred orientation of the 1-phosphate along the C_1–O_1 bond of the α conformer is strongly pH dependent, gauche rotamers being predominant at pH 9.4. In the presence of divalent cations the orientation is biased toward trans. A mechanistic model is proposed to explain the Zn^{2+}, Mg^{2+}, and pH-dependent behavior of the gluconeogenic enzyme fructose 1,6-bisphosphatase.

D-Fructose-1,6-bisphosphate has been shown by ^{13}C NMR spectroscopy to be an equilibrated mixture in aqueous solution composed of approximately 20% α, 80% β, and 2–4% keto anomer (1–3) (see Fig. 1). The gluconeogenic enzyme fructose 1,6-bisphosphatase (fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) specifically uses α-Fru 1,6-P_2[2] as a substrate (4). Based on *in vitro* data it was calculated that in rat liver the rate of spontaneous anomerization is about 10–15 times slower than the maximal activity of FBPase (5, 6). This led Koerner *et al.* (5) to propose a model for the regulation of the Fru 6-P, Fru 1,6-P_2 cycle based on the anomeric specificities of phosphofructokinase and FBPase. Earlier it has been proposed that divalent cations might shift the equilibrium of the anomerization to the α form by the formation of a chelate between the cation and the *cis*-oriented phosphate groups (7). This would mean that *in vivo* the formation of the α anomer is more rapid than assumed. Experiments, however, contradicted this proposal (7). It is also possible that divalent cations increase both anomeric rate constants. Therefore we reinvestigated the influence of cations on the anomerization of Fru 1,6-P_2. It appeared that binding of Mg^{2+} will have little effect *in vivo*.

In the course of this study it appeared that Zn^{2+} and Mg^{2+} bind preferentially to the 1-phosphate in the α position. This phenomenon might have implications for

[1] To whom all correspondence should be addressed.

[2] Abbreviations used: Fru 1,6-P_2, fructose 1,6-bisphosphate; UMP, uridine 5′-monophosphate; Fru 6-P, fructose 6-phosphate; EDTA, ethylenediaminetetraacetic acid; FBPase, fructose 1,6-bisphosphatase.

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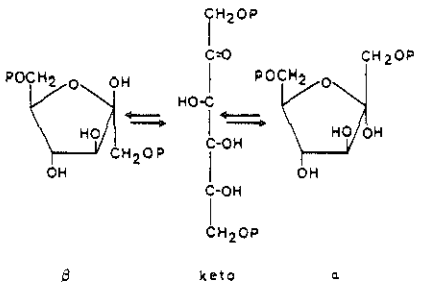

FIG. 1. Schematic representation of anomeric structures of Fru 1,6-P_2.

the inhibition of rat liver FBPase by Zn^{2+} (8–10) and Mg^{2+} (11).

MATERIALS AND METHODS

Chemicals. Fru 1,6-P_2 was purchased from Boehringer, Mannheim. KCl and $Mg(NO_3)_2$ (suprapure) were from E. Merck, Darmstadt. $ZnCl_2$ (ultrapure) was obtained from Ventron, Karlsruhe. Fru 1,6-P_2 was prepared in a 200 mM stock solution. If indicated this solution was chromatographed over a column loaded with Dowex 50W. The acidic fractions were collected and set to pH 7.0 with KOH. Thereafter the Fru 1,6-P_2 was lyophilized and stored at 4°C until use. The powder was redissolved in a concentration as indicated in the legends of the figures in the presence of 100 mM KCl in 50% D_2O for field frequency locking and other additions as indicated. The pH was set to the desired value with KOH or HCl.

NMR spectroscopy. The ^{31}P NMR spectra were recorded on a Varian XL-100 spectrometer operating in the Fourier transform mode at 40.5 MHz. Heteronuclear proton noise decoupling was used to remove the J coupling induced by fructose protons. A pulse width of 20 μs was employed corresponding to a flip angle of 45°. Usually 200–1000 scans were accumulated with an aquisition time of 5 s, a computer delay of 0.5 s, and a digital resolution of 0.06 Hz/pt. Temperature was set with an accuracy of ±1°C by a Varian temperature controller.

Chemical shifts are given relative to 20% H_3PO_4 as an external reference with downfield shifts defined as positive.

^1H NMR spectra were recorded on a Bruker WM 90. Chemical shifts are referred to 4,4-dimethyl-4-silapentane-1-sulfonate.

RESULTS

Figure 2 shows a ^{31}P NMR spectrum of Fru 1,6-P_2 at 23°C in the presence of EDTA. On the basis of their relative chemical shifts, intensities, and ^{31}P–^1H coupling constants the resonances at 5.2 and 4.0 ppm can be assigned to the 1- and 6-phosphates of α-Fru 1,6-P_2, respectively, while those at 4.4 and 4.1 ppm are the corre-

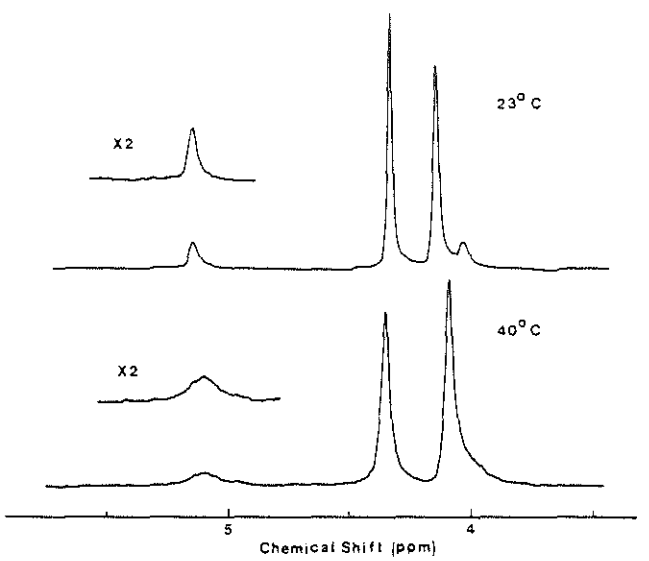

FIG. 2. ^{31}P NMR spectrum of Fru 1,6-P_2 at 23 and 37°C. Spectrum of a solution containing 50 mM Fru 1,6-P_2, 100 mM KCl, and 0.5 mM EDTA at pH 7.0.

sponding resonances of the β conformer (12). No resonances could be detected for the keto conformation, even in spectra measured at −10°C in the presence of dimethyl sulfoxide. It appeared that the resonance of the analog of keto-Fru 1,6-P_2, the keto conformer of dihydroxyacetone phosphate, measured under identical conditions, has the same chemical shift as the 1-phosphate of β-Fru 1,6-P_2. So most likely the resonance of the keto conformer of Fru 1,6-P_2 coincides with those of the β conformation.

In the absence of EDTA considerable line broadening is observed, most likely due to contaminating paramagnetic ions. It is also observed that in the absence of EDTA the resonances are shifted upfield. This could be due to binding of divalent cations present in the commercial preparation of Fru 1,6-P_2. Indeed, when the Fru 1,6-P_2 solution was pretreated with a cation exchanger (see Materials and Methods) a spectrum was obtained comparable to the spectrum in the presence of EDTA (not shown).

Kinetics of the Mutarotation

When the temperature is increased, the resonance of the α and β conformers belonging to the 1-phosphate as well as the 6-phosphate broaden and move to each other (Fig. 2). This is typical for an exchange situation. Kinetic information can be obtained from the excess linewidth due to this exchange. We shall restrict ourselves to the resonances of the 1-phosphate, since these are best resolved. According to the reaction sequence given in Fig. 1, the exchange of this phosphate should be treated as a three-sites problem.

From the reaction rates given by Midelfort *et al.* (3) and the position of the keto conformer as outlined above, it follows that the lifetime of the keto conformation is small compared to its chemical shift differences (in Hz) with the α and β conformations. Furthermore, the keto conformation constitutes only 2% of the total amount of Fru 1,6-P_2 (3). With these considerations it can be shown that the three-sites exchange situation can be simplified to a two-sites problem (13); in this case between the α and β conformations.

TABLE I

LINEWIDTH OF UMP ^{31}P NMR RESONANCE

T (°C)	pH	Addition	$\Delta\nu^0$
23	5.1	—	0.45 ± 0.07
23	7.0	—	0.45 ± 0.06
23	9.8	—	0.44 ± 0.05
37	7.0	—	0.45 ± 0.06
23	7.0	300 mM $Mg(NO_3)_2$	0.67 ± 0.04

Note. Spectra were recorded from a solution containing 100 mM UMP and 100 mM KCl. EDTA was present in a concentration of 0.5–10 mM and the D_2O concentration was 30–100%. The concentration of EDTA and D_2O did not influence the linewidth. Values are given ±S.D. (n = 3).

Because, in the present case, the limit of slow exchange applies, the rate constants of the $\alpha \rightarrow \beta$ anomerization ($k_{\alpha\beta}$) and the $\beta \rightarrow \alpha$ anomerization ($k_{\beta\alpha}$) can be determined from the linewidth of the two individual resonances by

$$k_{\alpha\beta} = \Pi(\Delta\nu_\alpha - \Delta\nu_\alpha^0),$$

$$k_{\beta\alpha} = \Pi(\Delta\nu_\beta - \Delta\nu_\beta^0),$$

in which $\Delta\nu$ stands for the width at half-height of the resonance (14). The value $\Delta\nu^0$ is the width in the absence of exchange. This value was determined by measuring the linewidth of UMP. This is a good approximation since UMP has almost the same molecular weight as Fru 1,6-P_2. Relevant values of $\Delta\nu^0$ are given in Table I. Only Mg^{2+} has a significant effect on $\Delta\nu^0$.

It appeared that the linewidths of the resonances of Fru 1,6-P_2 depend on the D_2O concentration (Fig. 3). Because such a D_2O effect could not be measured on the linewidth of the ^{31}P resonance of UMP and dimethylhydroxyacetone phosphate, which do not exhibit conversions (not shown), it is concluded that this is due to a change in mutarotation rate of Fru 1,6-P_2. The rate constants were evaluated from the extrapolated linewidth at zero D_2O concentration (Fig. 3). At 23°C this gives a $k_{\alpha\beta}$ of (5.1 ± 0.2) s^{-1} and a $k_{\beta\alpha}$ of (1.2 ± 0.3) s^{-1}. These values were also evaluated at 37°C resulting in a $k_{\alpha\beta}$ of (14.9 ± 0.5) s^{-1} and a $k_{\beta\alpha}$ of (4.2 ± 0.4) s^{-1}. The D_2O effect

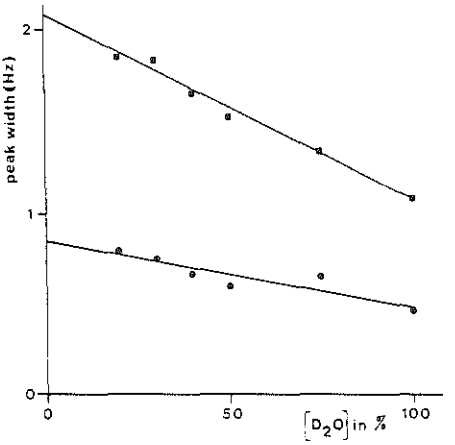

FIG. 3. The effect of D_2O on the linewidth of Fru 1,6-P_2 resonances. Spectra were recorded from a solution containing 120 mM Fru 1,6-P_2, 10 mM EDTA, 100 mM KCl at pH 7.0 and 23°C. Linewidth of α anomeric (■) and β-anomeric (●) resonances of the 1-phosphate group.

(defined as $k_{(H_2O)}/k_{(D_2O)}$ was found to be between 2.1 and 2.6.

The anomeric composition of Fru 1,6-P_2 was determined by integration of the α and β resonances of the 1-phosphate. At 23 and 37°C the fraction α anomer is (15 ± 1)%. This value is not dependent on the D_2O concentration. Because nuclear Overhauser effects, due to the decoupling of the sugar protons, could be different for α and β resonances and hence affect the calculated ratio α/β-anomer, spectra were also recorded without decoupling. In this way we found the fraction α anomer to be 16% which is in good agreement with the above established value.

Effect of Mg^{2+}, Zn^{2+}, and H^+

The effect of divalent cations on the ^{31}P resonances was studied with a Dowex-treated Fru 1,6-P_2 solution without EDTA.

Figure 4 shows that in the presence of $Mg(NO_3)_2$ all resonances are shifted upfield. Up to a $Mg(NO_3)_2$/Fru 1,6-P_2 ratio of about 1, however, the resonances corresponding to the 6-phosphates are not affected which indicates that Mg^{2+} binds preferentially to the 1-phosphate. Nonlinear regression of the titration curves yields

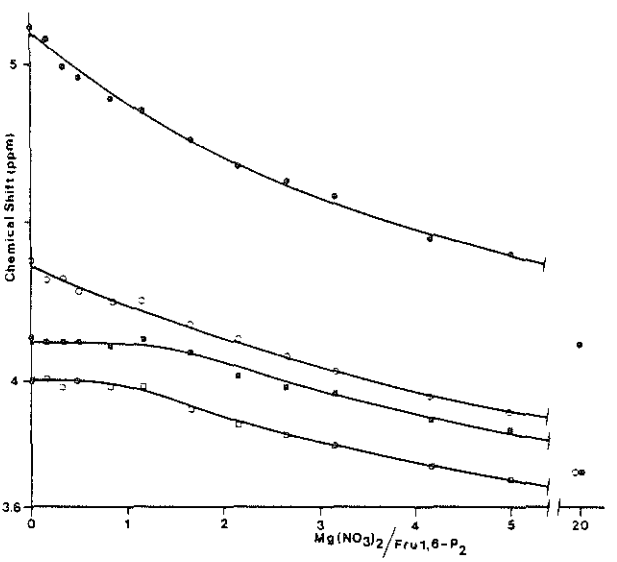

FIG. 4. Chemical shifts of Fru 1,6-P_2 resonances as a function of the $MgCl_2$ concentration. Spectra were recorded from a Dowex-treated Fru 1,6-P_2 sample. The final solution contained 60 mM Fru 1,6-P_2, 100 mM KCl at pH 7.0. Temperature was 23°C. Circles, resonances of 1-phosphates; squares, resonances of 6-phosphates. ● □, α Resonances; ○ ■, β resonances.

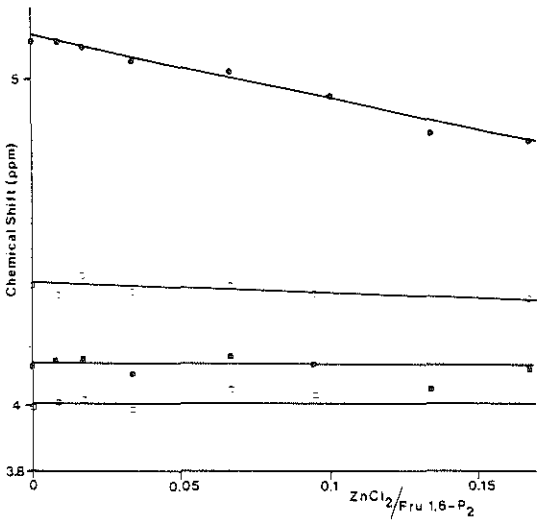

FIG. 5. Chemical shifts of Fru 1,6-P_2 resonances as a function of the $ZnCl_2$ concentration. For details see Fig. 4.

a maximum shift for the 1-phosphate group of the α anomer of 1.05 ppm and for the β anomer of 0.80 ppm. The titration points up to a Mg^{2+}/Fru 1,6-P_2 ratio of 1.2 were used for a Scatchard analysis. If one Mg^{2+} cation binds to a phosphate, we calculate an apparent binding constant for the 1-phosphate of the α anomer of 13 and 6 M^{-1} for the β anomer. This indicates that Mg^{2+} binds preferentially to the 1-phosphate of the α anomer.

When we consider the binding of Zn^{2+} (Fig. 5) we may also conclude that Zn^{2+} has a relatively high affinity for the 1-phosphate group of the α anomer. However, a quantitative evaluation is hampered by the fact that at high concentrations of $ZnCl_2$ a precipitate is formed of $Zn(OH)_2$. If we assume that the maximal shift is the same as with $Mg(NO_3)_2$, then we calculate that at a cation/Fru 1,6-P_2 ratio of 0.17, the affinity for Zn^{2+} is four times higher than for Mg^{2+}.

The effect of divalent cations on the linewidth and anomeric composition were also evaluated. To avoid linebroadening from contaminating paramagnetic ions, a small amount of EDTA was added. Because an effect on the T_2 relaxation time of resonances of phosphate groups in the presence of Mg^{2+} has been reported (15), linebroadening can be expected. As can be seen in Table I, linebroadening is observed upon addition of Mg^{2+} to UMP.

Considering Fru 1,6-P_2 we observed a

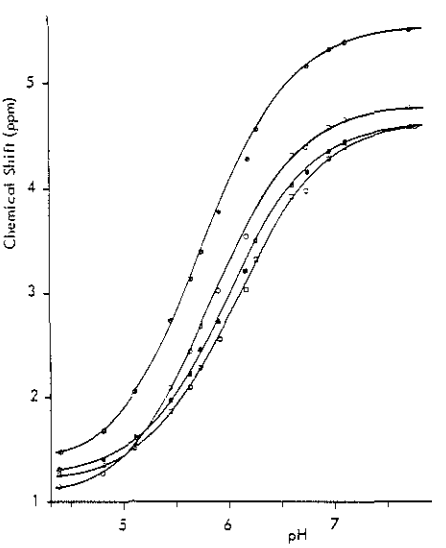

FIG. 6. Chemical shifts of Fru 1,6-P_2 resonances as a function of pH. For details see Fig. 4.

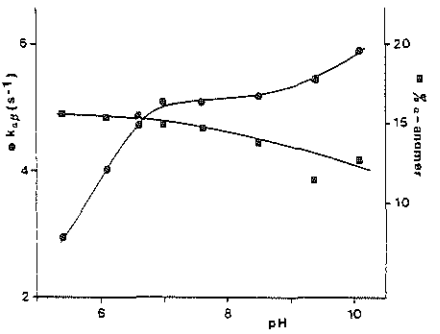

FIG. 7. Influence of pH on the rate constant of the anomerization and anomeric composition of Fru 1,6-P_2. Conditions as in Fig. 4.

linebroadening of 0.38 Hz for the β resonance, but only 0.04 Hz for the α resonance of the 1-phosphate at a Mg^{2+}/Fru 1,6-P_2 ratio of 6. This might indicate that $k_{\alpha\beta}$ is decreased upon Mg^{2+} binding. This is substantiated by the observation that at a Mg^{2+}/Fru 1,6-P_2 ratio of 6 the fraction α anomer is 17% and at a ratio of 20 this fraction is 19%. However the effect is small.

It is well known that the chemical shift of phosphate monoesters is very sensitive to the degree of ionization of these acids. Figure 6 represents an acid base titration curve of Fru 1,6-P_2. The pK values for the α and β conformations of the 1-phosphate are 5.8 and 5.9, respectively. These values are both 6.0 for the 6-phosphate.

The effect of pH on the rate constant $k_{\alpha\beta}$ and anomeric composition is shown in Fig. 7. It can be concluded that the degree of ionization influences this rate constant. Furthermore at high pH the anomeric equilibrium shifts slightly in the direction of the β anomer.

Conformation of the Phosphate Groups of Fru 1,6-P_2

^1H–^{31}P coupling constants are sensitive to the time-averaged preferred conformation of phosphate esters along the C–O bonds (16). We measured the effect of temperature, pH, and divalent cation binding on the coupling constants (J_{HP}) of the resonances of the 1-phosphate group. Figure 8 gives the relevant nomenclature on the orientation of the 1-phosphate. The fractional population of the transrotamer (P_t) can be estimated from (16):

$$P_t = \frac{24 - (J_{1HP} + J_{1'HP})}{18}.$$

Coupling constants and calculated P_t values are given in Table II. It can be seen that the 1-phosphate of the α anomer prefers gauche orientations, while the time-averaged preferred orientation of the β anomer is trans.

The time-averaged preferred orientations are dependent on a number of conditions. An increase in temperature from 6 to 34°C slightly increases the contribution of gauche orientations. At low pH both phosphates tend to increase the population of their trans rotamer, which is also observed in the presence of divalent cations. These latter effects are more pronounced on the α-phosphate than on the β-phosphate.

The effect of divalent cation binding and protonation was further studied by ^1H NMR. Figure 9a shows a 90-MHz ^1H NMR spectrum of a Fru 1,6-P_2 solution. Up till now none of the resonances has been assigned to particular protons of the sugar. After addition of $ZnCl_2$ some minor changes around 3.85 ppm can be observed (Fig. 9b). However the resonance at 4.00 ppm clearly shifts downfield to 4.07 ppm (arrow). Under these conditions only the resonance of the 1-phosphate of the α anomer shifts (see Fig. 5). Therefore the resonance at 4.00 ppm in the ^1H NMR spectrum is tentatively assigned to protons of the α anomer. Upon addition of $Mg(NO_3)_2$ also a downfield shift of this resonance is observed (not shown).

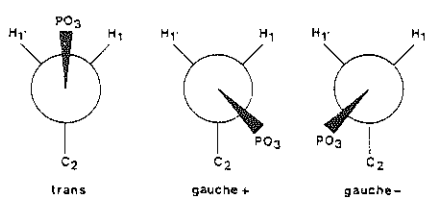

FIG. 8. Newman projections of the dominant orientations of the 1-phosphate group of Fru 1,6-P_2 viewed along the C_1–O_1 bond.

TABLE II

^1H–^{31}P COUPLING CONSTANTS AND TIME-AVERAGED PREFERRED ORIENTATIONS ALONG THE C_1–O_1 BOND OF FRU 1,6-P_2

T (°C)	pH	Addition	α Anomer		β Anomer	
			J in Hz	P_t in %	J in Hz	P_t in %
6	7.0	—	7.8	47	5.9	68 a
23	7.0	—	7.9	46	6.0	67 b
34	7.0	—	8.0	44	6.0	67 a
23	5.4	—	6.5	61	5.6	71 a
23	9.4	—	8.3	41	6.2	64 a
23	7.0	$ZnCl_2$ (0.17)[a]	7.5	50	6.0	67 b
23	7.0	$Mg(NO_3)_2$ (3.3)[a]	7.2	54	5.9	68 b
23	7.0	$Mg(NO_3)_2$ (5)[a]	7.0	56	5.8	79 b

Note. Samples contained 60 mM Fru 1,6-P_2, 100 mM KCl in the presence of 5–10 mM EDTA (a). In some cases a Dowex-treated Fru 1,6-P_2 sample was used in the absence of EDTA (b). H_1 and H_1, are virtually isochronous in their coupling to the phosphates and are given as one value. P_t is the fraction transrotamer.

[a] Value in parentheses represents the ratio of cation to Fru 1,6-P_2 concentration.

At pH 5.4 again some shifts around 3.85 ppm are visible but the resonance at 4.00 ppm virtually does not change its position (Fig. 9c). From this we conclude that divalent cations bind in a different way to α-Fru 1,6-P_2 than protons do.

DISCUSSION

Kinetics and Mechanism of Mutarotation

The anomeric composition of Fru 1,6-P_2 has been determined by several investigators (Table III). The composition of 15%

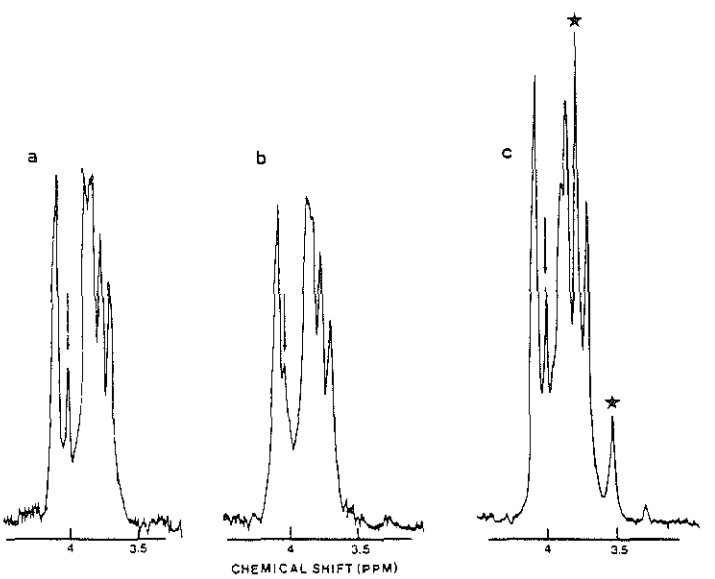

FIG. 9. ^1H NMR spectrum of Fru 1,6-P_2. (a) Spectrum of a Dowex-treated Fru 1,6-P_2 solution (40 mM) in the presence of 100 mM KCl at pH 7.0. (b) Idem after adding $ZnCl_2$ to a $ZnCl_2$/Fru 1,6-P_2 ratio of 0.15. (c) Spectrum at pH 5.4 in the presence of 10 mM EDTA. ★, Extra resonance intensity due to EDTA protons.

TABLE III

DISTRIBUTION OF THE FURANOSE FORMS OF FRU 1,6-P_2

$\alpha:\beta$	Method	Reference
23:70	^{13}C NMR	2
16:84	^{13}C NMR	3
20:80	^{13}C NMR	7
10:90	^{31}P NMR	11
15:85	^{31}P NMR	This study

α and 85% β anomer reported here agrees well with the most accurate values obtained by Midelfort *et al.* (3) using ^{13}C NMR. The mutarotation rate constants derived by us are also in good agreement with those obtained by these authors.

The D_2O effect of 2.1–2.6 reported here has been frequently observed in the mutarotation of nonphosphorylated sugars (17). A D_2O effect greater than 1 indicates that proton transfer is a rate-controlling step in the mutarotation. In comparison to nonphosphorylated sugars the mutarotation rates of phosphorylated sugars are very rapid (3, 17, 18). Baily *et al.* (18) have proposed that this is due to intramolecular phosphate catalysis. In their model study on the mutarotation of α-D-glucose they found that this reaction can be accelerated by inorganic phosphate; the dibasic species being a better catalyst than the monobasic species. A similar behavior is reported here for Fru 1,6-P_2 (see Fig. 7). We also found that the time-averaged preferred orientation of the 1-phosphate group of the α anomer is sensitive to the degree of ionization of this group. More gauche orientations coincide with an increased mutarotation. It cannot be excluded that the orientation of the 1-phosphate is also important in the phosphate catalysis. Considering this type of catalysis it can be expected that binding of divalent cations has some influence on the mutarotation rates and anomeric composition. However, our measurements with Mg^{2+} indicate that these effects are small (see also Ref. (7)).

From the titration curves with Mg^{2+} and Zn^{2+} (Figs. 4 and 5) we conclude that the 1-phosphate has the highest affinity for divalent cations. From model building it can be seen that divalent cations that are bound to the α anomer might coordinate with the 3-OH group. The distance of the 1-phosphate of the α anomer to this hydroxyl group and its conformation (see Table I) makes this less likely to be so for the β anomer. This could explain the relative high affinity of the α anomer for divalent cations. Supporting evidence is obtained by ^1H NMR: Coordination with the 3-OH group could result in a deshielding effect on protons located on C-3 and C-4. Indeed in the ^1H NMR spectrum a downfield shift was observed of a resonance that was tentatively attributed to α-anomeric protons.

From the upfield shift of the resonances observed in "crude" (i.e., not treated with Dowex) preparations of Fru 1,6-P_2, we conclude that these preparations are contaminated with divalent cations. One of these ions could be Zn^{2+}, which is then responsible for the kinetic hysteresis of FBPase (see accompanying paper (25)).

In Vivo Situation

In vivo the free Mg^{2+} concentration is about 1 mM (19) and the concentration of Fru 1,6-P_2 in rat liver is only 20 μM (20) so that the Mg^{2+}/Fru 1,6-P_2 ratio is about 50. At such a high ratio the anomeric composition shifts in the direction of the α anomer. The β/α ratio will be about 4.0.

Our measurements on the linewidth of the phosphate resonance and the α/β ratio in the presence of Mg^{2+} indicate that the kinetics of Fru 1,6-P_2 are affected to a small extent by this cation. This means that in the *in vivo* situation, when Fru 1,6-P_2 is completely saturated with Mg^{2+}, the mutarotaion rate constants could be slightly different from the values obtained by ^{13}C NMR (3) and ^{31}P NMR. From the considerations given above it is concluded that cellular changes in Mg^{2+} have no effect on the mutarotation rate and anomeric composition of Fru 1,6-P_2.

Relation with Enzyme Activity

FBPase shows rather complex kinetic properties, including activation by chelating agents accompanied by a shift of the pH optimum from more alkaline to neutral

pH (19). The inactivation has been attributed to binding of Zn^{2+} to the enzyme (8). Apart from these inhibitory sites it was necessary to postulate the existence of activating sites (8) to account for the activity in the presence of Zn^{2+} alone. Binding studies revealed three classes of binding sites(9, 10).

It might be speculated that, depending on the ionic conditions, the hydrolyses of Fru 1,6-P_2 occurs via a dissociative or an associative mechanism, each mechanism characterized by certain kinetic parameters. Metal ions can inhibit a dissociative mechanism or activate an associative mechanism or vice versa (21, 22), and so change the kinetics of rat liver FBPase.

As pointed out by Mildvan (22), there are differences in space requirements at the catalytic site between an associative and dissociative mechanism. From Table II it appears that the time-averaged orientation of the 1-phosphate of α-Fru 1,6-P_2 along the C_1–O_1 bond is strongly dependent upon the pH. The sharp pH optimum in FBPase activity in the presence of EDTA (23, 24) might indicate that a particular conformation of the 1-phosphate group is necessary for hydrolyses.

As Zn^{2+} inhibits at extremely low concentrations (K_i = 0.3 μM, Ref. (8)), we assume that, after turnover, Zn^{2+} remains bound to the enzyme near the catalytic site. Evidence for this proposal is given in the accompanying paper (25).

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Hysteretic Behavior of Rat Liver Fructose 1,6-Bisphosphatase Induced by Zinc Ions

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The measurement of the time dependency of the activity of rat liver fructose 1,6-bisphosphatase shows that the enzyme under certain conditions exhibits kinetic hysteretics. After addition of the substrate, the enzyme is initially in a state characterized by a "high" K_m of about 2 μM. During the reaction the enzyme is converted in a slow process to a low K_m form (K_m is about 0.5 μM). The transition is accompanied by a decrease in V. It is concluded that the hysteretic behavior is caused by binding of the Zn^{2+} substrate complex to the enzyme. The earlier reported effect of glucagon treatment on the activity of fructose 1,6-bisphosphate (O. D. Taunton, F. B. Stifel, H. L. Greene, and R. H. Herman (1974) *J. Biol. Chem.* **249**, 7228–7239) was reinvestigated, taking into account the hysteretic behavior. Under conditions where the pyruvate kinase activity is decreased by glucagon injection, no activity change of fructose 1,6-bisphosphatase is observed. It can be suggested that for studies concerning the effects of incubation or hormone treatment on fructose 1,6-bisphosphatase, the complex kinetics of the rat liver enzyme has to be taken into account.

Fructose 1,6-bisphosphatase (D-fructose-1,6-bisphosphate 1-phosphohydrolase, EC 3.1.3.11) catalyzes the hydrolysis of fructose 1,6-bisphosphate into fructose 6-phosphate and phosphate. The enzyme requires a divalent cation such as Mg^{2+}, Mn^{2+}, Zn^{2+}, or Co^{2+} for catalytic activity (1, 2). Zn^{2+} in the presence of Mg^{2+} or Mn^{2+} is a very potent inhibitor (2). Half-maximal inhibition is obtained with 0.3 μM of Zn^{2+} (2). The observed stimulatory effect of chelators as EDTA, histidine, or fatty acids in the neutral pH range are attributed to removal of tightly bound Zn^{2+} by these agents (2, 3). Some authors reported a nonlinear product formation, tending to decrease in time (4–6). Han *et al.* (6) reported that this phenomenon is dependent on the presence of Zn^{2+}. Recently we investigated the kinetics of FBPase isolated from control and glucagon-treated rat livers. We used assay media in which kinetic changes induced by hormones (7) or cAMP-dependent protein kinase (8) were described. It appeared that especially in these media a pronounced nonlinear product formation occurs (Van den Berg, unpublished). As this phenomenon could be important for a proper interpretation of kinetic changes by phosphorylation, we investigated the nature and the factors influencing this behavior more closely.

MATERIALS AND METHODS

Chemicals and enzymes. Fru 1,6-P_2,[2] $NADP^+$, phosphoglucose isomerase, and glucose 6-phosphate dehydrogenase were obtained from Boehringer (Mannheim).

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[2] Abbreviations used: Fru 1,6-P_2, fructose 1,6-bisphosphate; FBPase, fructose 1,6-bisphosphatase; SDS, sodium dodecyl sulfate.

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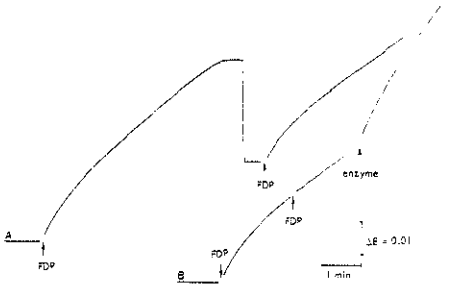

FIG. 1. Progress curves of the FBPase reaction. Partially purified FBPase was assayed in a spectrophotometric assay in the presence of 1 mM $MgCl_2$ and 15 μM Fru 1,6-P_2. A and B represent different experiments.

Assay. FBPase activity was measured in a medium containing 50 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 0.2 mM $NADP^+$, 7 U of phosphoglucose isomerase and 17 U of glucose 6-phosphate dehydrogenase in a volume of 3.0 ml. The reaction was, unless indicated otherwise, started by adding Fru 1,6-P_2. The increase in A_{340} was followed in an Aminco DW 2 spectrophotometer, thermostated at 30°C. In some experiments the dual wavelength mode was used at 370/340 nm. Specific activity was measured according to (8).

Purification procedure. FBPase was purified as described earlier (8). In most experiments we used partially purified enzyme. Such a preparation was purified as far as the $(NH_4)_2SO_4$ precipitation step. The 50% $(NH_4)_2SO_4$ precipitate was dissolved in 25 mM Tris–HCl, pH 7.5, and 5 mM 2-mercaptoethanol. Salts were removed by gel filtration (Sephadex G-25). The column was equilibrated with the aforementioned buffer. The pure enzyme preparation was unsuitable for direct kinetic studies as it contained Fru 1,6-P_2 introduced during the last specific elution step with Fru 1,6-P_2. Part of the substrate was tightly bound to the enzyme as it could not be removed by gel filtration. For this reason the enzyme preparation was incubated in the presence of 5 mM $MgCl_2$ and 0.1 mM EDTA in order to hydrolyze Fru 1,6-P_2. Finally fructose 6-phosphate and phosphate were easily removed by gel filtration. The final preparation had a specific activity of 6 U/mg and showed a neutral pH optimum. In a SDS–gel (9) a strong protein band with M_r of 42,000 and a faint protein band with M_r of 150,000 could be identified.

RESULTS AND DISCUSSION

The activity of FBPase is usually measured by a spectrophotometric assay, in which the hydrolytic splitting of Fru 1,6-P_2 is coupled to the production of NADPH. Coupled enzyme reactions have the advantage that the reaction is directly visualized. If the coupling enzymes are not present in excess, a lag phase will appear in the formation of NADPH. Unfortunately such a lag phase has been interpreted earlier as evidence for kinetic hysteresis of FBPase (compare Refs. (10 and 11)).

We tried to measure more realistic initial rates, especially at subsaturating substrate concentrations. For this large amounts of coupling enzymes were added and it was verified that the reaction rates were independent of the amount of the coupling enzymes used. It appears that, instead of a lag phase, a burst in product formation is observed (Fig. 1). After about 1 min the reaction rate is almost constant until all substrate is converted. Addition of a second amount of substrate at the end of the reaction gives a similar behavior, which excludes that an irreversible change in the enzyme has occurred. Addition of Fru 1,6-P_2 during the reaction does not result in a burst (Fig. 1, second trace). This excludes the possibility that Fru 1,6-P_2 contains some of the intermediate products or a related compound that is quickly converted by the coupling enzymes. This conclusion is further strengthened by the observation that the burst is also observed

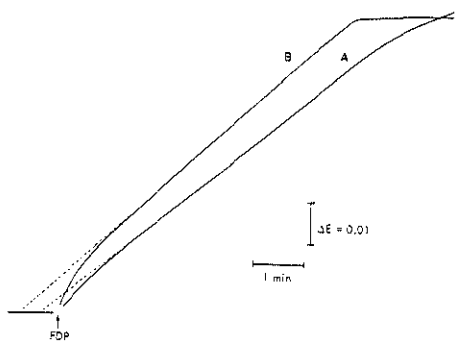

FIG. 2. Progress curves of the FBPase reaction using preparations of different purity. FBPase activity was measured in the presence of 1 mM $MgCl_2$ and 20 μM Fru 1,6-P_2. Curve A: 100,000*g* supernatant fraction; Curve B: pure enzyme in the presence of 5 μM $ZnCl_2$.

when the reaction is initiated by the addition of FBPase (not shown) or when more enzyme is added during the assay (Fig. 1, second trace). The observation was always reproducible either in a crude supernatant (Fig. 2, trace A) or with partially purified enzyme (Fig. 1). The observation was also made with a pure enzyme preparation. However, in this case a small amount of Zn^{2+} had to be added (Fig. 2, trace B). This can be understood in the light of later observations (see below). Commercial preparations of rabbit muscle FBPase (Boehringer, Mannheim) did not show this behavior (not shown). The nonlinear Fru 1,6-P_2 hydrolysis was earlier observed by others (4–6). Carlson *et al.* (4) reported that the change in reaction velocity is also observed when the enzyme is assayed by the release of inorganic phosphate. So we conclude that the observed nonlinear product formation must be attributed to an intrinsic property of rat liver FBPase. Until now no study has been reported to clarify the nature of this phenomenon. Figure 3A gives the family of progress curves using different amounts of enzyme. Figure 3B shows that both the initial rate and the rate measured in the linear part of the curve are proportional with the protein concentration. At high protein concentrations the rate in the second slow phase cannot be measured because the substrate is completely hydrolyzed before this phase is reached. The linear part of the curves converge at a point on the time axis at 50 s before the addition of substrate (Fig. 3A). So the enzyme reaches spontaneously a state with lower catalytic activity as soon as Fru 1,6-P_2 is added. Such a slow change in kinetic behavior in response to the addition of an effector is called "hysteresis" (12). The transition can be described:

Fructose 1,6-bisphosphate high activity $\underset{k}{\rightleftharpoons}$ Fructose 1,6-bisphosphatase low activity.

The velocity as a function of time is given by the expression

$$V_t = V_f + (V_i - V_f)e^{-kt}, \qquad [1]$$

where V_i is the initial rate and V_f the final linear rate (10, 13). The evaluation of k is explained in Fig. 4. In this particular case k equals 0.0365 s^{-1}. In this figure we also plotted the calculated progress curve using the integrated Eq. [1],

$$P_t = V_f t + \frac{V_i - V_f}{k}(1 - e^{-kt}), \qquad [2]$$

in which P_t is the product formed at time t. The parameters V_i, V_f, and k were derived from the experimental curve. Indeed, the calculated curve fits well with the experimental one. However, we have to realize that a good fit does not prove that the

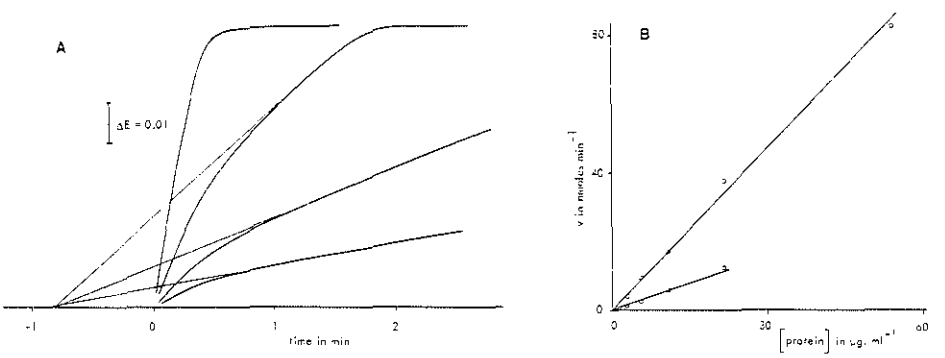

FIG. 3. Protein dependency of the FBPase reaction. Partially purified FBPase was assayed at 1 mM $MgCl_2$ and 20 µM Fru-1,6-P_2. (A) Progress curves with extrapolated linear parts. Protein concentrations were from left to right 36, 18, 7.2, 3.6 µg/ml. (B) Initial velocity (○) and final linear velocity (□) as a function of the protein concentration.

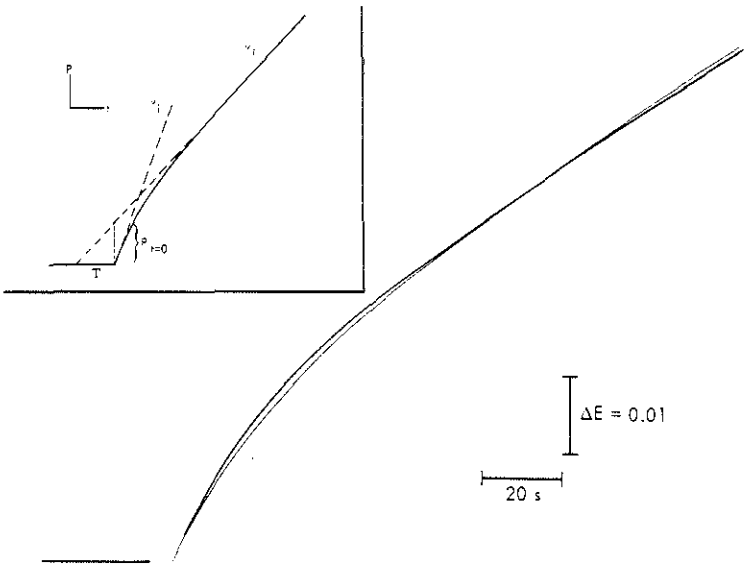

FIG. 4. Fitting of the progress curve according to a hysteretic model. The parameters v_i and v_f were measured as indicated in the insert. The rate constant k is given by * $k = (v_f - v_i)/P_{t=0}$. Using the parameters V_i, V_f, and k, a progress curve (thick line) was fitted using Eq. [2] (thin line). Protein concentration was 9 μg/ml.

foregoing model is correct. More stringent tests must be performed, such as characterization of the two forms.

Carlson *et al.* (4) observed that large linear initial rates are obtained when the activity is measured in the presence of 2-mercaptoethanol or fatty acids. We confirmed these results (not shown) and found that dithiothreitol has the same effect. We can interpret this result as that these compounds decrease k. We investigated whether the rate constant could also be influenced by other factors.

Figure 5 shows the Mg^{2+} dependency of the progress curves. It is clear that the rate of the second slow phase increases with increasing Mg^{2+} concentrations. The initial rate also seems to increase; however, as in this experiment the curve is strongly curved from the start at Mg^{2+} concentrations below 2.5 mM, we cannot measure accurate initial rates. The value of V_i is, however, important for the calculation of the rate constant k (Fig. 4). Therefore two extreme situations were distinguished: (a) V_i is independent of Mg^{2+} and equals the maximal velocity obtained in the presence of EDTA, or (b) V_i is equal to the rate obtained by drawing a tangent to the progress curve at zero time. The apparent rate constants calculated in both ways are presented in Table I. In order to find out if Mg^{2+} influences the initial velocity or not, the progress curves were fitted using either of the assumptions. It appears that the best fit is obtained if we suppose that at every Mg^{2+} concentration V_i equals the maximal velocity measured in the presence of EDTA (not shown). However, the difference with the calculated curve using assumption (b) is marginal, so that a clear discrimination between the two possibilities is not possible. For this it will be necessary to use a stopped flow device. Nevertheless, from Table I the important conclusion can be drawn that Mg^{2+} prevents the transition of the enzyme from a high-activity to a low-activity form.

In Fig. 5 it is also shown that in the presence of EDTA the progress curve is

* $k = (v_f - V_i)/v_f T$ or

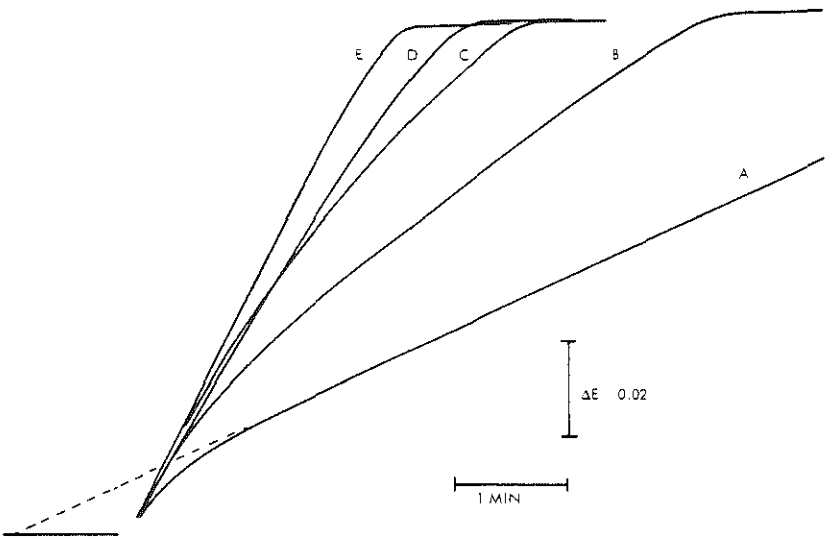

FIG. 5. Mg^{2+} dependency of the FBPase reaction. Progress curves of the FBPase reaction in the presence of 30 μM Fru 1,6-P_2 at: A, 0.6 mM; B, 1.2 mM; C, 2.5 mM; D, 10 mM $MgCl_2$; E, 10 mM $MgCl_2$ + 50 μM EDTA. Protein concentration was 9 μg/ml.

essentially linear. This effect of EDTA was observed under a variety of conditions; i.e., subsaturating, saturating, or inhibitory concentrations of Fru 1,6-P_2 or $MgCl_2$. Furthermore it appears that histidine, which is a well-known activator of the enzyme (14), has the same effect as EDTA, i.e., no hysteresis is observed. On the other hand in the absence of EDTA the hysteretic effect could always be observed, unless no precautions had been taken to clean the cuvets carefully. Trace amounts of fatty acids are sufficient to obtain full activity (4) and to mask the hysteretic behavior (not shown). As in the presence of 50 μM EDTA the greater part of the Mg^{2+} ions is not complexed, it is unlikely that removal of Mg^{2+} from some site of the enzyme causes the suppression of the hysteresis. More likely another tightly bound cation is complexed by EDTA. Recent experiments have shown that FBPase can bind divalent cations as Zn^{2+} or Co^{2+} with high affinity (2). It has been established that the liver enzyme has three binding sites for Zn^{2+} (15); two of which can be considered as inhibitory, while binding of Zn^{2+} to the third binding site leads to an activation. As commercial preparations of FBPase are contaminated with divalent cations (Van den Berg and Heerschap, accompanying paper (40)) we decided to investigate the possibility that Zn^{2+} causes

TABLE I

THE EFFECT OF THE DIVALENT CATION CONCENTRATION ON k

Addition	k (constant V_i) in s^{-1}	k (tangent) in s^{-1}
0.6 mM $MgCl_2$	0.054	0.030
1.2 mM $MgCl_2$	0.026	0.022
2.5 mM $MgCl_2$	0.016	0.016
5 mM $MgCl_2$		
+2 μM $ZnCl_2$	0.036	0.024
+3 μM $ZnCl_2$	0.053	0.029
+4 μM $ZnCl_2$	0.078	0.032
+5 μM $ZnCl_2$	0.093	0.036
+10 μM $ZnCl_2$	0.263	0.047

Note. k was calculated as described in Fig. 4 using the assumption that V_i equals the maximal velocity or that V_i is equal to velocity obtained by drawing a tangent to the progress curve.

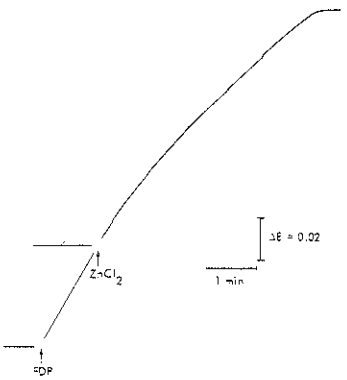

FIG. 6. Time course of the inhibition of active FBPase by Zn^{2+}. Purified rat liver FBPase was incubated in the presence of 1 mM $MgCl_2$ and 20 μM substrate. At the time indicated by the arrow, 5 μM $ZnCl_2$ was added.

the hysteresis. Figure 6 shows that addition of 5 μM $ZnCl_2$ to a cuvet containing FBPase under turnover conditions leads to a slow decrease in activity to 52% of the original activity. The apparent rate constant of this transition, calculated as described in Fig. 4, is 0.018 s^{-1}, which is of the same order of magnitude as the rate constant connected with the hysteresis (Fig. 4). The influence of Zn^{2+} was further studied by analyses of the progress curves. Table I shows that the apparent rate constants of the transition increase with increasing concentration of Zn^{2+}. As in the experiments with Mg^{2+}, we cannot conclude whether Zn^{2+} affects the initial velocity. Fitting of the progress curves indicates that contrary to the case with Mg^{2+}, the best fit is obtained assuming that the initial velocity equals the tangent to the curve. These observations give rise to the hypothesis that binding of Zn^{2+} to the enzyme in the presence of Fru 1,6-P_2 induces the transition of an enzyme with high catalytic activity to an enzyme with low catalytic activity. Contaminating metal ions in the Fru 1,6-P_2 solution are probably sufficient to introduce such a transition. Indeed when a Fru-1,6-P_2 solution was used that was pretreated with a cation exchange no hysteresis was observed (not shown). Recently it has been suggested that commercial samples of ATP are also contaminated with metal ions. The inhibition of FBPase by ATP must, most likely, be ascribed to this contamination (16).

It has been established that FBPase hydrolyzes α-Fru 1,6-P_2 (17). As Zn^{2+} binds preferentially to the 1-phosphate of Fru 1,6-P_2 in the α position (Van den Berg and Heerschap, accompanying paper (40)) the obvious conclusion is that this compound binds to the enzyme at or near the catalytic site; after hydrolysis of the substrate Zn^{2+} is not easily released, introducing an inhibited enzyme form. So the substrate is necessary to introduce Zn^{2+} to a site that is otherwise not available. This is confirmed by the observation that, if the enzyme is assayed in the presence of Zn^{2+} alone, a hysteretic effect can also be observed upon addition of Fru 1,6-P_2 (not shown). The protective effect of Mg^{2+} against inactivation (Fig. 5, Table I) can be explained as competition of Mg^{2+} with Zn^{2+} for binding to Fru 1,6-P_2. The observed inhibition of the enzyme at very high Mg^{2+} concentrations (18) might be explained by binding of Mg^{2+} at the catalytic site via the same mechanism as de-

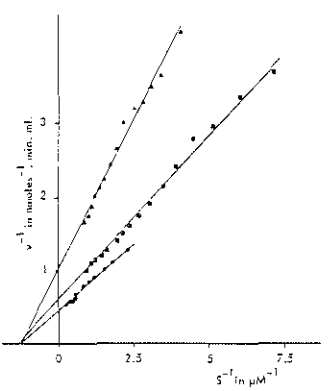

FIG. 7. Determination of kinetic constants of rat liver FBPase with a single enzyme reaction progress curve. Partially purified FBPase was assayed in the presence of 1 mM $MgCl_2$, 50 μM EDTA, and 5 μM (●) or 1.7 μM (■, ▲) Fru 1,6-P_2. Protein concentration (μg/ml) was: ▲, 1.3; ■, 2.6; ●, 2.6. Progress curves were used to obtain data pairs V and S according to $V = (S_i - S_j)/(t_i - t_j)$ and $S = (S_i - S_j)/\ln S_i/S_j$ (20).

scribed for Zn^{2+}. Libby *et al.* (19) suggested that inhibition of the enzyme by high Mn^{2+} concentrations could be correlated to binding of a substrate–Mn^{2+} complex to an allosteric site of the enzyme. This interpretation can be considered as a slight modification of the above-described hypothesis.

The current model implies an increased affinity of the enzyme for the Zn^{2+}–substrate complex. Therefore we determined the K_m for Fru 1,6-P_2. However the measurement of initial rates is complicated by the hysteresis. This problem was eliminated as we used an integrative method to calculate K_m and V from a single enzyme reaction progress curve (20). The validity of this method for the Fru 1,6-P_2 reaction is shown in Fig. 7. Here we determined the kinetic constants in the presence of EDTA at two different substrate concentations to exclude substrate inhibition and at different enzyme concentrations to exclude inactivation of the enzyme during the incubation. All progress curves deliver straight Lineweaver–Burk plots, intersecting on the abscissa giving a K_m of 1.4–1.6 μM which agrees with earlier reported values (21). A comparison of the K_m in the initial and final part of the progress curve was made. We assumed that the presence of EDTA is representative for the active form of the enzyme, while the low activity form is manifest in the part of the progress

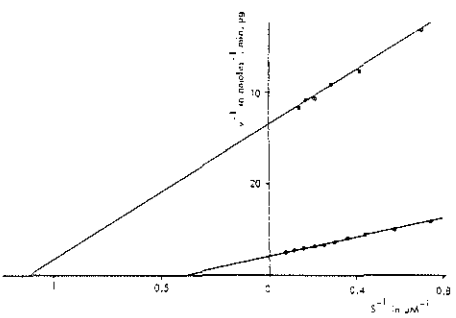

FIG. 8. Comparison of K_m of FBPase for its substrate in initial and final part of the progress curve. Partially purified enzyme was assayed in the presence of 1 mM $MgCl_2$, 10 μM Fru 1,6-P_2 in the presence (●) or absence (■) of EDTA. Progress curves were analyzed as described in Fig. 7.

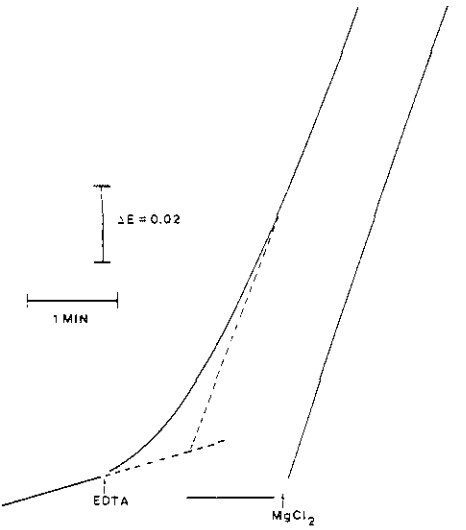

FIG. 9. Activation of rat liver FBPase by EDTA and $MgCl_2$. (A) Partially purified FBPase was incubated in the presence of 9 μM $ZnCl_2$, 1 mM $MgCl_2$, and 5 μM Fru 1,6-P_2. At the arrow 50 μM EDTA was added. (B) The enzyme was incubated in the absence of $MgCl_2$ with 6 μM $ZnCl_2$. At the arrow 10 mM $MgCl_2$ was added.

curve after the burst. We evaluate a K_m in the presence of EDTA of 2.6 μM while in the absence of EDTA a K_m of 0.9 μM is measured (Fig. 8). The V is 0.46 and 0.06 nmol min^{-1} μg^{-1}, respectively. In the presence of a low concentration of Zn^{2+} (μM range) the K_m is as low as 0.2–0.3 μM. So we conclude that in the presence of Zn^{2+} the K_m for Fru 1,6-P_2 drastically decreases. Such a change in kinetic properties can also be connected with the mechanism of hydrolysis of Fru 1,6-P_2. As described in the accompanying paper it is possible that in the presence of Zn^{2+} or high Mg^{2+} concentrations a dissociative mechanism is prevalent, while otherwise hydrolysis occurs by an associative mechanism or vice versa.

The binding of Zn^{2+} is easily reversible and an increase in activity can be observed (2). When 50 μM EDTA is added to the enzyme, which is inhibited by Zn^{2+}, a gradual increase in activity is observed (Fig. 9). The apparent rate constant of this tran-

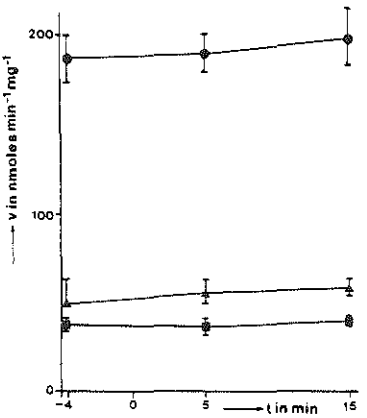

FIG. 10. The effect of glucagon on the activity of rat liver FBPase. Rats were injected with 0.3 mg glucagon at zero time exactly according to the scheme of Taunton *et al.* (7). Buffers, sampling, homogenizing procedure, and FBPase assay were exactly as described by Taunton *et al.* (7). FBPase activity was measured in the initial phase (●) or in the linear phase (■). Also the average amount of product formed per minute during the first 5 min (▲) is indicated. Results are expressed as the mean of three experiments ±SEM.

sition is 0.02 s^{-1}. The addition of Mg^{2+} to such an incubation leads to an immediate rise in activity (Fig. 9). From this experiment we conclude that Mg^{2+} does not activate the enzyme by replacing Zn^{2+}.

The question remains which binding site for Zn^{2+} is responsible for the hysteretic effect. In rabbit liver the addition of Fru 1,6-P_2 introduces additional binding sites for Zn^{2+} (22, 23). In rat liver it was claimed that Zn^{2+} binds also to this site in the absence of Fru 1,6-P_2, although the substrate increases the affinity for Zn^{2+} (15). These observations fit with the properties of the binding site for Zn^{2+} that is related to the hysteretic behavior reported here.

Using substrate analogs, Marcus (24) came to the conclusion that the positioning of the phosphate group located on C-1 is important in binding of the substrate. This fits well with the model presented here in which binding of Zn^{2+} to the phosphate group on carbon 1 introduces an enzyme form with quite different catalytic properties.

The question rises if the transition of a high- to a low-K_m form has physiological meaning. For instance in the process of gluconeogenesis it will be necessary that the Fru 1,6-P_2 concentration is well below 1 μM in order to prevent the activation of pyruvate kinase (25) and phosphofructokinase (26) by this metabolite. A transition of FBPase to a form with a K_m of 0.2–0.3 μM could be useful to maintain a low level of Fru 1,6-P_2. It has been reported that indeed the level of Fru 1,6-P_2 drops after the stimulation of gluconeogenesis by glucagon (27, 28). This fall in Fru 1,6-P_2 has been attributed to a rise in FBPase activity (7, 29). We repeated the experiments of Taunton *et al.* (7) in which rats are injected with glucagon. Liver samples are taken 5 and 15 min after the administration of glucagon. It appeared that in the assay medium for FBPase used by Taunton *et al.* (7) a strong hysteretic behavior is observed. Therefore we determined the activity in the initial and in the linear part of the progress curve and also the amount of product formed in the first 5 min was averaged, which is an indirect measure of the rate constant of the transition. It appears that no rise in activity occurs after the addition of hormone when the activity is measured by any of these methods (Fig. 10). The samples were also assayed for pyruvate kinase activity. Five minutes after the addition of hormone a 40 ± 2% (SEM, n = 3) decrease in activity was observed when the enzyme was measured at a subsaturating phosphoenolpyruvate concentration (2 mM) in the absence of Fru 1,6-P_2 (for method of measurement see (30)). This inactivation of pyruvate kinase after the addition of hormones is well established (6, 31–36) and results from cAMP-dependent phosphorylation of the enzyme (for a review see (37)). The reason for the discrepancy with the results of Taunton *et al.* (7) as far as FBPase is concerned is not clear. Hue reports (38) that he was not able to repeat the activation of mouse liver FBPase published by Chatterjee (29). Veneziale *et al.* (39) also found no effect of glucagon injection on the activity of FBPase while pyruvate kinase and phosphofructokinase activity were posi-

tively decreased. These findings weaken the evidence that glucagon introduces a stable activity change in rat liver FBPase and argue for the statement that when effects of incubation or hormone treatment are reported, the complex kinetics of FBPase has to be taken into account and that it has to be verified if changes in one of the multiple effectors of the enzyme might be responsible for the observed kinetic changes.

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CYCLIC AMP-DEPENDENT INACTIVATION OF HUMAN LIVER PYRUVATE KINASE

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SUMMARY

Human liver pyruvate kinase is rapidly (within 2 min) inactivated by incubation of a human liver supernatant with cyclic AMP, when measured at suboptimal substrate concentrations. Half-maximal inactivation is reached with 0.04 μM cyclic AMP. The apparent $K_{0.5}$ for phosphoenolpyruvate shifts from 0.5 mM to 1.1 mM by incubation with cyclic AMP. It is concluded that cyclic AMP-dependent protein kinase may catalyze the phosphorylation of human liver pyruvate kinase in vivo.

Recently it has been shown that purified L-type pyruvate kinase of rat[1] and pig[2] liver can be phosphorylated by a cyclic AMP-dependent protein kinase. The phosphorylated enzyme is characterized by a lowered affinity to its substrate phosphoenolpyruvate and its allosteric activator fructose-1,6-diphosphate[2,3]. This phenomenon can also be shown with rat hepatocytes incubated with glucagon[4,5] or rat liver homogenate incubated with cyclic AMP[6].

The similarity of the results obtained with cells with those obtained with purified L-type pyruvate kinase indicates that phosphorylation of L-type pyruvate kinase might be a mechanism by which in the liver glucagon stimulates gluconeogenesis. Phosphorylation of pyruvate kinase will lead to a lowered pyruvate kinase activity resulting in a lowered cycling at the level of pyruvate carboxylase, phosphoenolpyruvate carboxykinase and pyruvate kinase[7]. An impairment in the phosphorylation mechanism can lead to an increased cycling through the pyruvate-phosphoenolpyruvate cycle, which results in a reduced net lactate utilization. This might be one of the possible causes for lactate acidosis. No experimental data are known concerning the inactivation by cyclic AMP of human liver pyruvate kinase. Therefore, it is necessary to design experiments in which a possible phosphorylation mechanism in human liver samples can be tested. In this report we present the results obtained for human liver pyruvate kinase.

MATERIALS AND METHODS

Liver samples were obtained post mortem from human infants within

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three hours after death. There were no indications that the liver was involved in the cause of death. Material was frozen in liquid N_2 and stored at -70°C until use.

Homogenates (5% w/v) were prepared in 25 mM Tris-HCl pH 7.5. After addition of 1 mM 2-mercaptoethanol, the homogenate was centrifuged 20 min at 48 000 x g at 4°C.

Pyruvate kinase activity was measured at 30°C in an Aminco DW2 spectrophotometer by coupling with lactate dehydrogenase. In this way the pyruvate kinase activity of 0.2 mg supernatant protein can be accurately measured. Cuvettes contained in a final volume of 3 ml: 25 mM Tris-HCl pH 7.5, 200 mM KCl, 1.0 mM ADP, 0.13 mM NADH, 11 U lactate dehydrogenase and 0.2-0.5 mg protein. Mg^{2+} concentrations are indicated in the legends of the figures. After a 3 min incubation period the reaction was started by addition of the substrate phosphoenolpyruvate.

Incubations with cyclic AMP were started by mixing 0.1 ml supernatant with 0.1 ml medium containing 50 mM Tris-HCl pH 7.5, 125 mM KF, 10 mM theophylline, 10 mM $MgCl_2$, 0.4 mM ATP and 0.4 µM cyclic AMP. Incubations were performed during 2 min at 27°C and the activity of pyruvate kinase was measured. For the experiment described in Fig. 4 the medium contained 1.0 mM ATP and 10 µM cyclic AMP, while the incubation time was 5 min.

Protein was measured according to Lowry et al.[8] with bovine serum albumin as a standard.

RESULTS

Incubation of a human liver supernatant with cyclic AMP leads to a rapid inactivation of pyruvate kinase measured at suboptimal substrate concentrations (Fig. 1). In the absence of Mg-ATP no inactivation is observed. Inactivation is virtually complete within 1 min, while up to 5 min no further change in activity occurs. Controls incubated in the absence of cyclic AMP kept unchanged during the time of incubation.

Fig. 2 gives the dose-response curve of the inactivation. Half-maximal inactivation is reached with 0.04 µM cyclic AMP.

In order to investigate more closely the kinetic change in pyruvate kinase upon incubation, the phosphoenolpyruvate saturation plots are determined after incubation in the absence and presence of cyclic AMP. Supernatant incubated without cyclic AMP (Fig. 3A) as well as supernatant which is not incubated (Fig. 4) shows half-maximal activity ($K_{0.5}$) at 0.5 mM phosphoenolpyruvate. Upon incubation with cyclic AMP this value increases to 1.1 mM (Fig. 3B). The activity in the presence of an excess of the allosteric activator fructose-1,6-diphosphate is not altered upon incubation.

The discontinuity in the Hill plot (insert Fig. 3B) shows that after incubation of the enzyme with 0.2 µM cyclic AMP, two enzyme forms are present. By applying more extreme conditions for inactivation (Fig. 4), the discontinuity in the Hill plot disappears, while the $K_{0.5}$ increases still further.

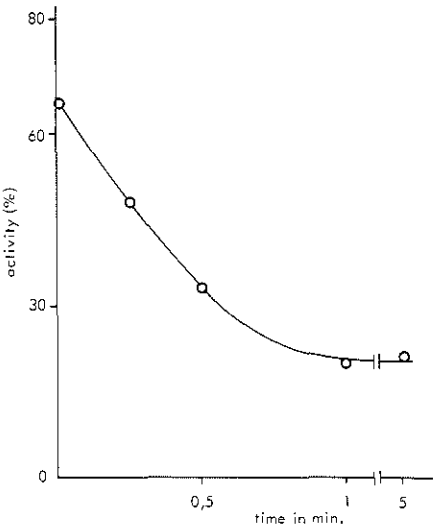

Fig. 1. Time course of the inactivation of human liver pyruvate kinase by incubation with cyclic AMP. Incubation of human liver supernatant was performed as described in Materials and Methods. Activity was measured at 0.5 mM phosphoenolpyruvate and 1.7 mM $MgCl_2$. Activity expressed as percentage of maximal activity, measured in the presence of 0.5 mM fructose-1,6-diphosphate.

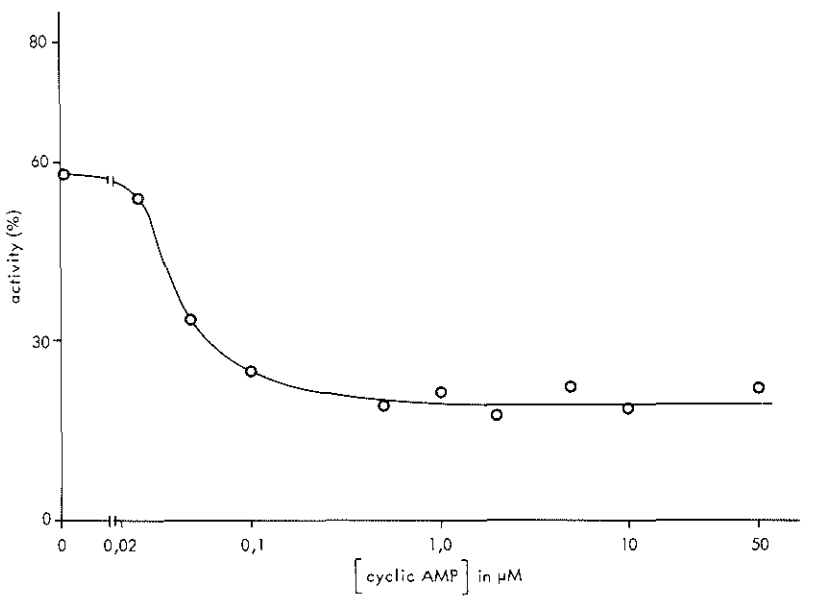

Fig. 2. Effect of cyclic AMP concentration upon the inactivation of human liver pyruvate kinase. Conditions and expression of results as in Fig. 1. Incubation time was 5 min.

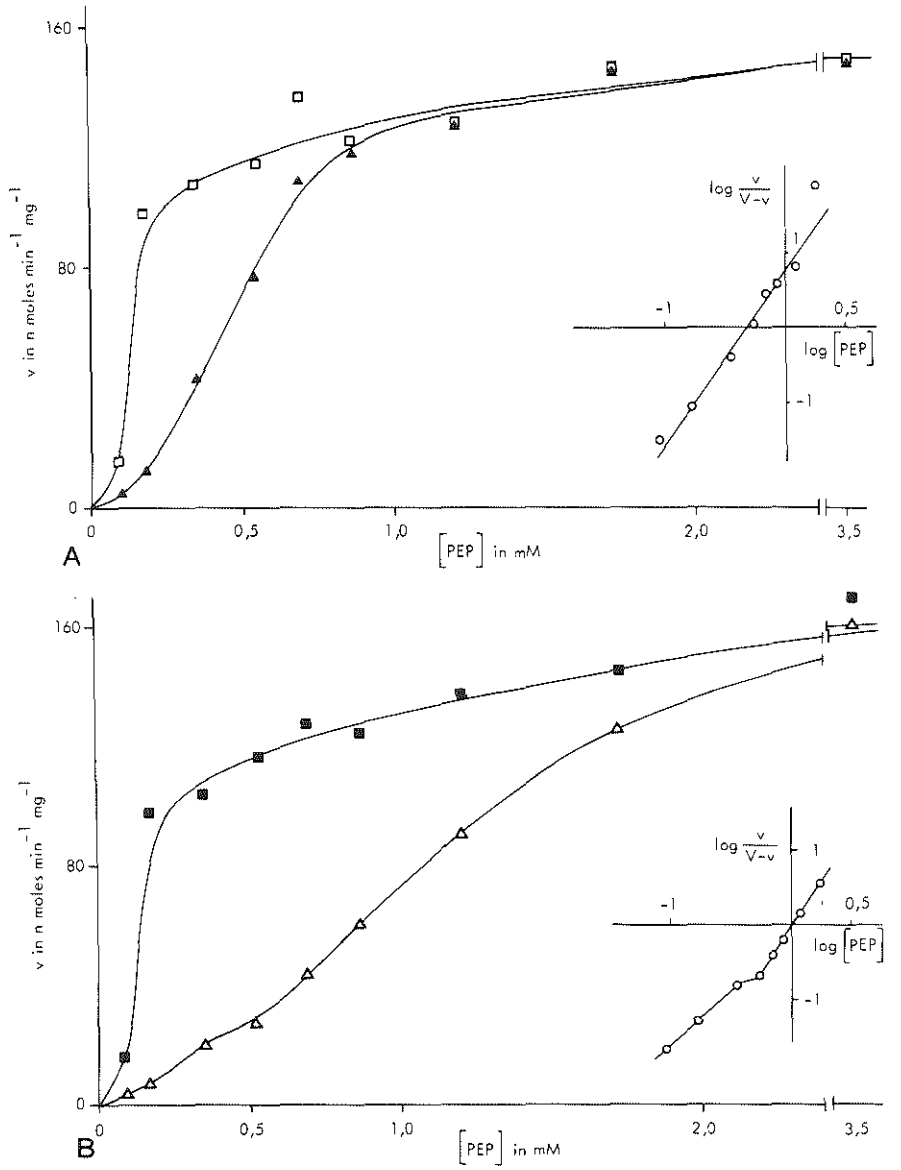

Fig. 3. Phosphoenolpyruvate saturation plot of human liver pyruvate kinase after incubation in the presence and absence of cyclic AMP. Incubation of human liver supernatant was performed as described in Materials and Methods. A: Incubation in the absence of cyclic AMP. B: Incubation in the presence of 0.2 µM cyclic AMP. Activity was measured at 1.7 mM $MgCl_2$ in the absence (triangles) and presence (squares) of fructose-1,6-diphosphate.

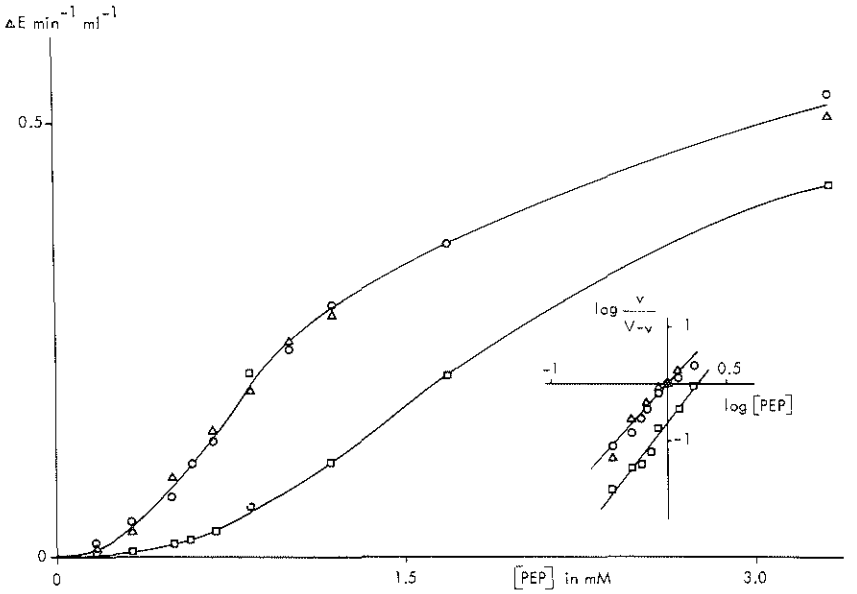

Fig. 4. Influence of incubation on phosphoenolpyruvate saturation of human liver pyruvate kinase. Incubation of human liver supernatant was performed as described in Materials and Methods except that $[Mg^{2+}]_{free}$ was kept constant at 1 mM (ref. 10). o—o, absence of cyclic AMP; □—□, 2 μM cyclic AMP; Δ—Δ, supernatant without incubation.

The described cyclic AMP-dependent inactivation of pyruvate kinase is also found in four other tested human liver samples.

DISCUSSION

The present results show that human liver pyruvate kinase can be inactivated in a human liver supernatant in a cyclic AMP-dependent way. This inactivation requires the presence of Mg-ATP, half-maximal rate of inactivation is obtained in the presence of 0.04 μM cyclic AMP. This apparent K_a is comparable to the values observed for cyclic AMP-dependent protein kinases, which makes it very likely that the cyclic AMP-dependent inactivation of pyruvate kinase is promoted by a human liver protein kinase. This implies that the inactivation is caused by phosphorylation of the enzyme. This conclusion is strengthened by the observed change in $K_{0.5}$ for phosphoenolpyruvate upon incubation while the maximal activity is unaltered.

Although no direct evidence is obtained for in vivo phosphorylation of human liver pyruvate kinase, the fact that pyruvate kinase in the supernatant is inactivated without further addition of protein kinase, and the high sensitivity to physiological amounts of cyclic AMP makes it very likely that this process is of physiological importance.

The importance of the phosphorylation of pyruvate kinase for the hormonal stimulation of gluconeogenesis is under discussion, however, it might be argued that a deficiency in this phosphorylation mechanism will lead to an increased phosphoenolpyruvate-pyruvate cycling. This results in a lowered lactate utilization by the liver i.e. a diminished lactate removal from circulating blood. This might occur in some patients with type B lactic acidosis[9], which makes it important to test the pyruvate kinase inactivation process in these patients.

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PROTEIN PHOSPHORYLATION IN HUMAN LIVER. A METHOD TO DETECT POSSIBLE REGULATORY ABERRATIONS

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Clin. Chim. Acta, submitted for publication

SUMMARY

Cyclic AMP-dependent and independent protein phosphorylation is studied in the cytosolic fraction of human post-mortem liver with a method which needs a small amount of liver.

On sodium dodecyl sulphate polyacrylamide electrophoresis 3 bands with molecular weight of 65,000 (pyruvate kinase), 58,000 and 35,000 are reproducibly visualized by autoradiography of which the phosphorylation is completely dependent on c AMP. Two proteins with molecular weight of 68,000 and 78,000 are phosphorylated independent of the presence of c AMP. The phosphorylation of the M_r 65,000 protein (pyruvate kinase) is partially inhibited by the glycolytic intermediate Fru 1,6-P_2 (10 μM), while the phosphorylation of the M_r 68,000 protein is completely inhibited.

A clinical case is presented with persistent high lactate and low fasting glucose levels in the blood. Administration of alanine, in contrast to fructose, did not lead to "de novo" glucose synthesis. The gluconeogenic enzymes pyruvate carboxylase, phosphoenol pyruvate carboxylase and fructose 1,6-bisphosphatase had activities in the normal range. It is suggested that an aberration in the regulation of the gluconeogenesis is responsible for the clinical features and that the protein phosphorylation system might be usefully applied to liver biopsies from patients with described abnormalities.

INTRODUCTION

Recently [1, 2] we investigated protein phosphorylation of the rat-liver soluble fraction by endogenous protein kinases. After dodecyl sulphate polyacrylamide electrophoresis and autoradiography phosphorylated bands were distinguished on basis of the difference in molecular weight. These studies indicated that the phosphoproteins could be subdivided in three groups: proteins that are phosphorylated by: a) c AMP-dependent kinase; b) c AMP-independent kinase and c) proteins in which both c AMP-dependent and -independent protein kinase are involved. It was further

shown that the glycolytic intermediate, Fru 1,6-P_2, influences the extent of phosphorylation of pyruvate kinase type L and inhibits the phosphorylation of a 68,000 M_r protein, of which the phosphorylation is c AMP-independent [2]. We suggested that phosphorylated hexoses can be considered as third messenger in the hormonal regulation of gluconeogenesis [3].
In the literature [cf. ref. 4] lactic acidosis is often mentioned but in most cases enzymatic investigations have been incomplete. More recently, Robinson et al. [5] reported that from 40 patients in which a possible defect of glucose-6-phosphatase or fructose 1,6-diphosphatase activity was ruled out, an enzymatic defect could be identified in only twelve cases. This leaves 28 patients with lactic acidosis in which no underlying deficiency could be detected. This study was hampered by the fact that the investigations were performed on fibroblast cultures. We had two patients with persistent lactacidemia and low fasting blood glucose, in whom all the gluconeogenic enzymes and pyruvate dehydrogenase activities in the liver biopsies were found to be normal. We speculate that the underlying unknown defect in these patients might be related to an aberration in the covalent regulation of the gluconeogenic enzymes. For such cases the detection system described in this paper might offer the possibility to investigate an eventual abnormality of protein phosphorylation in a small amount of material.

MATERIALS AND METHODS

Human liver samples were obtained within 3 h after death. There were no indications that the liver was involved in the course of death. Material was frozen in liquid N_2 and stored at -70°C until use. A piece of liver was homogenized (20% w/v) in a Potter-Elvehjem homogenizer in 250 mM sucrose, 25 mM Tris HCl, 2 mM 2-mercaptoethanol and centrifuged at 105,000 g for 60 min. Low molecular weight components were removed by gel filtration (Sephadex G25, medium). The column was equilibrated with 25 mM Tris HCl (pH 7.5), 5 mM $MgCl_2$ and 2 mM 2-mercaptoethanol. The samples were pre-incubated at 20°C during 30 min in order to

dephosphorylate the proteins fully before the phosphorylation experiments were started [3].

The phosphorylation experiments were performed as follows: 10 µl of the soluble fraction was preincubated at 6°C for 1 min with 10 µl of a mixture containing 25 mM Tris HCl (pH 7.5), 200 mM KCl, 5.0 mM $MgCl_2$, 10 mM theophylline, 40 mM phosphate and the additions as indicated in the legends to the figures. The final volume was 30 µl. The amount of protein per 30 µl is between 50-100 µg. The reaction was started by adding [γ-^{32}P]ATP (0.10 mM). The final specific activity of [γ-^{32}P]ATP was about 1 Ci/mole. The reaction was stopped with 15 µl of a solution containing 0.3 M Tris H_3PO_4 (pH 6.8), 3% sodium dodecyl sulphate, 50 mM 2-mercaptoethanol and 20% glycerol. Before electrophoresis the samples were heated at 90°C for 5 min.

SDS polyacrylamide slab gel electrophoresis was performed as in [6] in 7.5% gels. Gels were 0.75 mm thick. The gels were stained in a solution of 0.2% Coomassie brilliant blue R 250 in 50% methanol, 3.5 % acetic acid and destained in a mixture of 5% methanol and 7.5% acetic acid. For autoradiographs the gels were vacuum dried and the dried gels were exposed to Kodak XR-1 film.

Pyruvate carboxylase activity was measured according to ref. 7 and phosphoenol pyruvate carboxylase activity according to ref. 8; fructose 1,6-bisphosphatase activity and pyruvate dehydrogenase activity according to ref. 9 and 10, respectively.

RESULTS

The phosphorylation pattern of the soluble fraction of 4 different human livers is shown in fig. 1. Also is exhibited the phosphorylation pattern of rat liver soluble fraction, that was treated essentially in the same way as human liver. As reference proteins pyruvate kinase type L and phosphorylase are indicated. The former has been identified earlier with the aid of specific antibody (1) and the latter was identified by its Ca^{2+}-dependent phosphorylation and molecular weight (2).

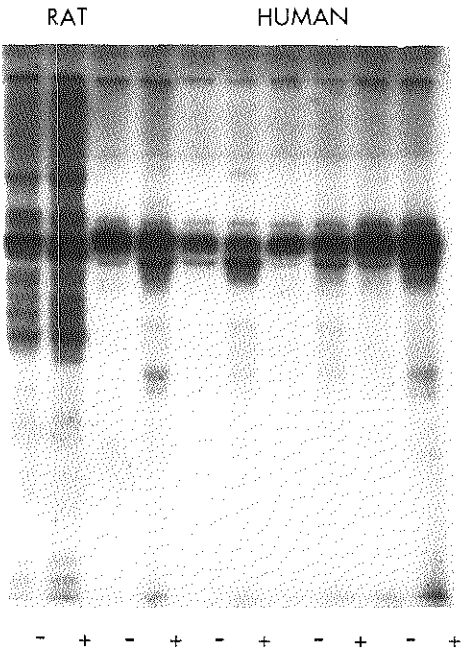

Fig. 1.

Phosphorylation pattern on SDS polyacrylamide gel electrophoresis of human and rat liver soluble fraction in the absence and presence of 10 μM cyclic AMP. Four different human samples were used.

A striking difference between the human and the rat liver is that, at least under these conditions, in the human liver samples much less proteins are phosphorylated. Considering the fact that the human samples were obtained 3 h after death and the rat sample directly after killing, protein degradation could have been responsible for these differences. Therefore, protein staining was applied on the SDS polyacrylamide gels (fig. 2).
Comparing the protein pattern of human and rat liver soluble fractions, one can conclude that both the rat and human soluble fraction do contain the high mol. weight proteins and no evidence for protein degradation with the human samples is obtained, which is also substantiated by the similarity of the protein pattern of the various human samples.

The phosphorylation of human pyruvate kinase in the human liver soluble fraction is highly dependent upon c AMP. The mobility of human pyruvate kinase (L-type) is slightly higher than

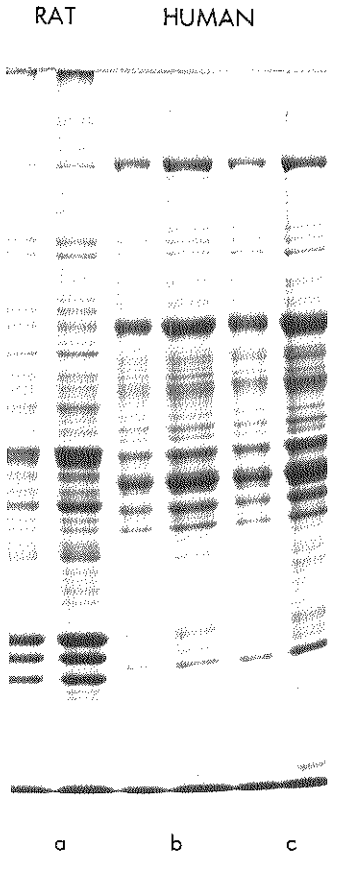

Fig. 2. The protein pattern on SDS polyacrylamide gel electrophoresis of human and rat liver soluble fraction. a) rat liver; b) and c) are human samples.

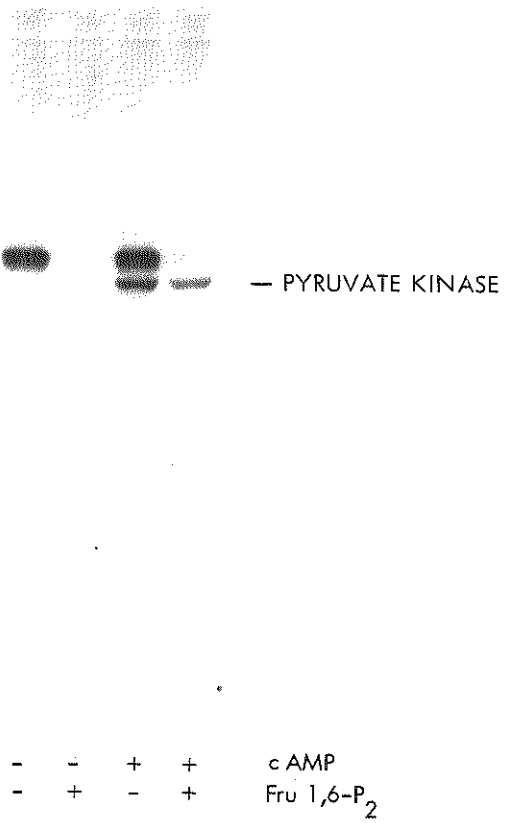

Fig. 3. The influence of Fru 1,6-P_2 (10 µM) on the phosphorylation of human liver soluble fraction proteins in the absence and presence of c AMP (10 µM). The various conditions are indicated in the figure.

the rat liver enzyme (resp. 65,000 and 63,000 M_r in these gels). Two other proteins with M_r of 58,000 and 35,000 show consistently in all samples a c AMP-dependent phosphorylation. The major phosphoprotein in human samples appears to be a 68,000 M_r protein of which the phosphorylation is c AMP-independent. In rat liver soluble fraction the phosphorylation of a 68,000 M_r

protein is also c AMP-independent. Careful inspection of the 68,000 M_r protein reveals that it is composed of two phosphoproteins, a major (68,000 M_r) and a minor (67,000 M_r) protein. In all samples also a phosphoprotein with a M_r of 78,000 can be observed of which the phosphorylation is not c AMP-dependent.

For the 68,000 M_r phosphoprotein from rat liver soluble fraction we found that its phosphorylation is inhibted by Fru 1,6-P_2, while the phosphorylation of rat liver pyruvate kinase (type L) is diminished [3]. Fig. 3 shows that these findings can be extended to human liver soluble fraction: the phosphorylation of the 68,000 M_r protein phosphorylation is completely inhibited by Fru 1,6-P_2 and the phosphorylation of pyruvate kinase (type L) is diminished.

A difference between human and rat liver phosphoproteins is the relative amount of ^{32}P incorporated into the 68,000 M_r and pyruvate kinase. For human liver about 1.9 times more ^{32}P is incorporated into 68,000 M_r protein than into pyruvate kinase. For rat liver this ratio is about 0.5.

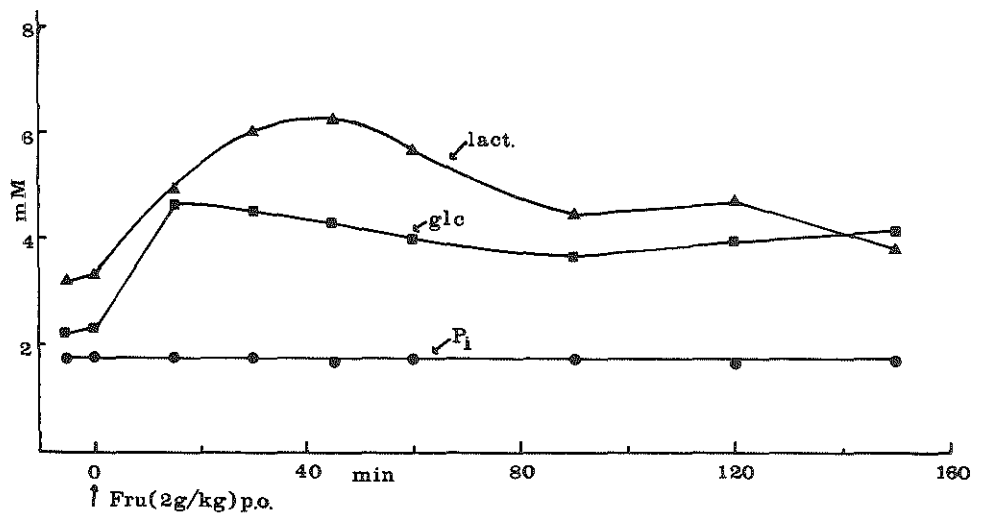

Fig. 4. The blood glucose (■-■), lactate (▲-▲) and P_i (●-●) responses on the loading with fructose (2 g/kg body weight) p.o.

Finally we like to point out for which cases this advanced method should be applied. A typical example is the following case.

This child had persistent elevated blood lactate levels (up to 3.7 mM) and low fasting blood glucose concentrations were repeatedly found. Glucagon administration resulted in a normal blood glucose increase, indicating a normal glycogenolysis. Loading with fructose orally showed a normal increase of blood glucose (from 2.2 to 4.7 mM) with a concomitant rise in lactate level from 3.2 till 6.2 mM, which is abnormally high (fig. 4). Intravenous administration of L-alanine did not lead to an increase, but to a small decrease in blood glucose and the lactate concentration rose till 5.0 mM (fig. 5). This finding gave strong

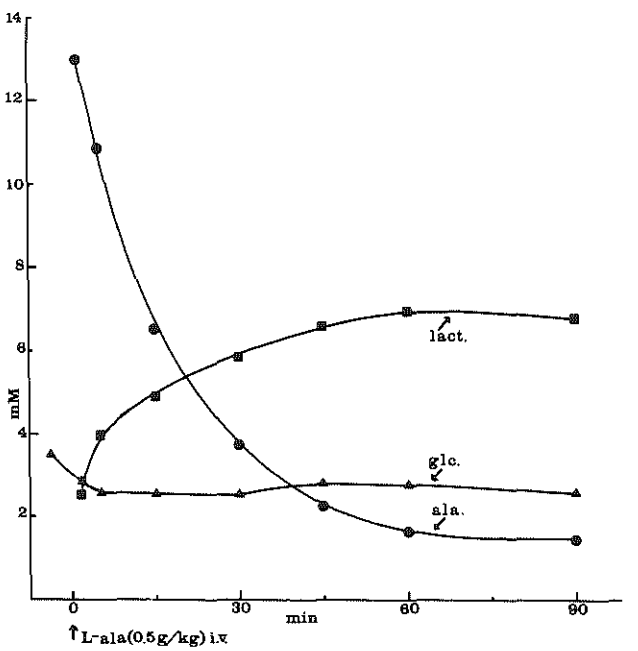

Fig. 5. The blood glucose (▲-▲), lactate (■-■) and alanine (●-●) responses on the loading with alanine (0.5 g/kg body weight) i.v.

evidence for an impaired gluconeogenesis on the level of phosphoenolpyruvate/pyruvate. A liver biopsy was performed. Table I summarized the enzymatic activities measured in this liver biopsy. No enzyme deficiency could be detected, which could

TABLE I

The activities of gluconeogenetic enzymes and pyruvate dehydrogenase in the liver of a child with persistent elevated lactate and low fasting glucose levels in the blood

Enzyme	Patient	Control
Pyruvate carboxylase with		
0.1 mM pyruvate	4.1	4.1 - 8.7 (n=6)
10 mM pyruvate	12.8	15.5 -31.6 (n=6)
Phosphoenolpyruvate carboxykinase	140	68-143 (n=11)
Fructose 1,6-bisphosphatase (- AMP)	32	21-51 (n=4)
Pyruvate dehydrogenase	1.65	1.03- 4.39 (n=3)
+ ATP (0.2 mM)	0.77	0.25- 1.65
+ Ca^{2+}(10 mM),Mg^{++} (10 mM)	2.88	2.53- 4.59

Activities are expressed as nmol/min/mg protein.

underly the elevated lactate concentration and low fasting blood glucose levels. Similar data were obtained from a second child also with high lactate and low glucose levels in the blood, in which all the enzymes involved in the utilization of lactate were normal. These two cases emphasize the need to look for an aberration in the regulation of gluconeogenic enzymes, but unfortunately this method was not at hand at the time the children were presented.

DISCUSSION

The most prominent differences between human and rat liver soluble fraction is the number of phosphoproteins which can be

detected under similar conditions. In rat liver, at least 21 phosphoproteins can be observed (2, and fig. 1), while in human liver samples only five evident and three weak phosphoproteins are detected. The possibility that in human liver most of the proteins are already phosphorylated is unlikely, because the samples have been preincubated with high Mg^{2+} in order to dephosphorylate the proteins [3]. Although from the protein staining pattern no indication for a general proteolysis is obtained, it remains possible that partial proteolysis has occurred, which could have caused a split of the phosphorylation sites, as recently shown for rat liver fructose 1,6-P_2ase [11]. The use of biopsy material will be necessary to investigate this possibility.

It is striking that the effect of Fru 1,6-P_2 on the phosphorylation of M_r 68,000 and pyruvate kinase (type L) is similar for rat and human liver. This similarity extends our earlier suggestion [3] that changes in the Fru 1,6-P_2 concentrations may have a very important regulatory role. As the Fru 1,6-P_2 concentration changes rapidly under different hormonal conditions [12, 13] it was proposed that Fru 1,6-P_2 [3] or a related phosphorylated hexose, such as fructose 2,6-diphosphate [14, 15] could be considered as a "third messenger" to translate or modulate extracellular hormonal changes into intracellular phosphorylated changes. The present results extend this view to human liver.

The patient we described showed persistent high lactate and low fasting glucose levels in the blood. The administration of L-alanine did not lead to an increase of the blood glucose, but to a marked increment of lactate, indicating an impaired gluconeogenesis. However, all the key enzymes involved in the process were normal, while the pyruvate dehydrogenase exhibited also a normal activity. This latter mitochondrial enzyme could be inactivated and reactivated under various conditions. This indicates that the phosphorylation system for this complex was intact. Aberrations in the gluconeogenesis on the levels of Fru 6-P / Fru 1,6-P_2 and Glc 6-P / Glc cycle could be ruled out on basis of fructose loading and administration of glucagon. The possibility remains that there was a failure in the regulation of the level of pyruvate/phosphoenolpyruvate cycle. In this cycle three enzymes are involved i.e. pyruvate carboxylase, phosphoenol-

pyruvate carboxykinase and pyruvate kinase. For the latter enzyme it is known that this enzyme in rat liver can be inactivated by phosphorylation [16-18] and this phenomenon is also valid for human liver [19]. Under gluconeogenic condition this enzyme should diminish in activity, otherwise a complete wasteful cycling would occur, which will result in elevated lactate and decreased fasting glucose levels in the blood. Our method offers now the possibility to detect the phosphorylation of pyruvate kinase and other relevant enzymes. It is suggested to apply the present system for patients with the described impaired administration test and in which normal enzyme activities are found.

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SAMENVATTING

De energie die mensen en dieren nodig hebben, wordt verkregen uit voedsel. Het voedsel wordt daartoe omgezet in een vorm die voor het lichaam geschikt is. Een groot gedeelte van ons dagelijks voedsel wordt bijvoorbeeld verteerd tot glucose. De glucose wordt vanuit het darmkanaal opgenomen in het bloed en naar de verschillende organen (bijv. de spieren) getransporteerd. Spieren kunnen de glucose afbreken, waarbij melkzuur ontstaat. Dit proces heet glycolyse. De energie die bij de glycolyse vrijkomt wordt gebruikt voor het verrichten van arbeid.

Melkzuur speelt een belangrijke rol in het vervolg van deze samenvatting. In veel gevallen zal het verder worden afgebroken tot koolzuur en water, waarbij energie vrijkomt die door het organisme nuttig gebruikt wordt. Onder bepaalde omstandigheden kan er echter uit melkzuur weer glucose gevormd worden. Dit proces wordt gluconeogenese genoemd. Het proces houdt in dat melkzuur naar de lever getransporteerd wordt. Daar aangekomen wordt het opgenomen in de levercel en via een aantal stappen omgezet in glucose. Dit proces kost energie.

Dergelijke omzettingen worden gestuurd/bevorderd door een bepaald soort eiwitten, die wij enzymen noemen. Bij de omzetting van melkzuur tot glucose zijn 10 enzymen betrokken en ontstaan evenzoveel tussenprodukten. Het opmerkelijke is dat in de lever (zoals hierboven gezegd is in verband met de spieren) ook glycolyse optreedt. Als nu glycolyse en gluconeogenese gelijktijdig optreden, zou er sprake zijn van een kringproces met als resultaat verlies van energie. Echter door een speciaal mechanisme wordt een dergelijk kringproces voorkomen. Hormonen spelen hierbij een belangrijke rol.

Wat is nu de zin van de gluconeogenese? Welnu, de hoeveelheid glucose in het bloed (glucosespiegel) mag niet al te sterk variëren. Als de glucosespiegel laag is, komt de glucosevoorziening en dus de energievoorziening van de hersenen in gevaar. De gluconeogenese zorgt ervoor dat de glucose in het bloed constant blijft in situaties waarin geen of onvoldoende aanvoer van glucose is vanuit het darmkanaal. Tijdens vasten werkt de gluconeogenese dan ook optimaal. De enzymsamenstelling van de lever is

dan zodanig veranderd dat een maximale gluconeogenese gegarandeerd is. Naast deze tamelijk langzame aanpassing kan de gluconeogenese ook binnen enkele seconden versneld worden. Dit is nodig om een plotseling verbruik van glucose (teneinde de trein te halen) op te vangen.

De vraag die in dit proefschrift gesteld wordt is: „Hoe schakelt de lever in korte tijd over van glycolyse naar gluconeogenese?" Hier is heel veel over bekend: Een lichte daling van de glucosepsiegel zet de pancreas aan tot de afgifte van het hormoon glucagon. Dit glucagon bereikt via de bloedbaan de lever, waar het zich aan de levercel hecht. Hierdoor wordt een enzym dat in de membraan van de levercel gelegen is, aangezet tot de vorming van de stof "cyclisch AMP". Cyclisch AMP stimuleert het enzym protein kinase. Protein kinase zorgt ervoor dat in bepaalde enzymen fosfaat wordt ingebouwd. De activiteit van deze enzymen verandert hierdoor, waardoor de gluconeogenese geactiveerd wordt.

Adrenaline kan ook de gluconeogenese stimuleren. Hierbij speelt niet cAMP maar calcium een rol. Preciezer gezegd gaat dit proefschrift over de vraag „in welke enzymen wordt fosfaat ingebouwd?" Dit probleem is benaderd door radioactief fosfaat te gebruiken. Enzymen die fosfaat inbouwen worden aldus radioactief gemerkt en zijn daardoor in een complex mengsel van eiwitten eenvoudig op te sporen.
De verwachting was dat een drietal enzymen fosfaat inbouwen onder invloed van cyclisch AMP, namelijk pyruvaat kinase, phosphofructo kinase en fructose 1,6-bisphosphatase. In appendix paper 1 wordt aangetoond dat pyruvaat kinase inderdaad fosfaat kan inbouwen, maar fructose 1,6-bisphosphatase niet. Fosfaat-inbouw in phosphofructokinase hebben wij niet direct kunnen aantonen of uitsluiten. De belangrijke conclusie uit appendix paper 2 is dat glucagon en adrenaline op verschillende manieren de fosfaat-inbouw bevorderen. Dit volgt uit de waarneming dat calcium de fosfaat-inbouw stimuleert in andere eiwitten dan cAMP doet.

In de gluconeogenese worden bepaalde tussenprodukten gevormd. Eén van deze produkten, fructose 1,6-bisphosphate blijkt de fosfaat-inbouw in pyruvaat kinase en ook in een niet nader geïdentificeerd eiwit te remmen. Dit staat beschreven in appendix paper 3. In appendix paper 4 staan bepaalde eigenschappen van fructose

1,6-bisphosphate beschreven, terwijl appendix paper 5 ingaat op het enzym dat deze stof kan omzetten, het eerder genoemde fructose 1,6-bisphosphatase.

Dit onderzoek werd begonnen vanuit het idee dat een niet goed functioneren van de gluconeogenese de oorzaak kan zijn van melkzuuracidose. In het bloed van patiënten die hieraan lijden wordt veel melkzuur aangetroffen. In een aantal gevallen kan dit geweten worden aan het niet goed functioneren van één van de gluconeogenese enzymen. In een aantal gevallen, die niet verklaard konden worden, zou het defect juist gelegen kunnen zijn in de beïnvloeding van de gluconeogenese door hormonen. Hoewel onze kennis over de regulatie van de gluconeogenese toegenomen is, weten wij nog steeds niet precies hoe de regulatie geschiedt. Onderzoek van patiënten zal dan ook geen definitief uitsluitsel over de relatie melzuuracidose - regulatie van de gluconeogenese kunnen geven.

Het onderzoek is uitgevoerd in de rat. In de laatste fase van het onderzoek hebben wij kunnen laten zien dat deze bevindingen ook gelden voor menselijk lever (appendix paper 6 en 7). Toepassing op patiëntenmateriaal is dus in principe mogelijk.

SUMMARY

The mechanism by which hormones affect the activity of glycolytic/gluconeogenic enzymes was investigated. The role of Ca^{2+} and cAMP as intracellular triggers of hormone action was established in an *in vitro* incubation system. In rat liver Ca^{2+}- and cAMP-dependent protein kinases are operational with different substrate specificity. Pyruvate kinase is one of the main substrate proteins for cAMP-dependent protein kinase. The phosphorylation of a protein with M_r 100,000 was dependent on the presence of Ca^{2+} and cAMP. This protein was tentatively identified as phosphorylase. Ca^{2+} also seems to inhibit the phosphorylation of some proteins. The cAMP-dependent phosphorylation of pyruvate kinase and the cAMP-independent phosphorylation of an unidentified M_r 68,000 protein can be modulated by Fru 1,6-P_2. It is postulated that Fru 1,6-P_2 acts as a third messenger.

Part of the regulation of the Fru 1,6-P_2 level resides in the kinetic properties of fructose 1,6-bisphosphatase. The enzyme shows hysteresis; i.e. it undergoes a reversible change between a low and a high affinity form, which is induced by Zn^{2+} ions. *In vivo* the anomerization of Fru 1,6-P_2 is most likely not in equilibrium. Under physiological conditions the β→α-anomerization rate constant is $1S^{-1}$ as determined with ^{31}P-NMR.

The findings in rat liver could be largely confirmed in human liver samples. The quantitative most predominant phosphoproteins are pyruvate kinase and the M_r 68,000 protein. The phosphorylation of pyruvate kinase is rapid at physiological cAMP concentrations. It is shown that human liver pyruvate kinase has a decreased affinity for its substrate phosphoenolpyruvate.

The *in vitro* phosphorylation of liver proteins can be used in principle as a screening procedure for aberrations in the phosphorylation mechanism in human liver biopsies. As an example a clinical case is presented with impaired gluconeogenesis from L-alanine, albeit gluconeogenic enzymes show normal activity.

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