EFFECTS OF GLUCOSE
METABOLISM IN NORMAL
AND CANCEROUS HUMAN LIVERS

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of the requirements for the degree
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by

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Declaration

I hereby declare that the work forming the basis of this thesis is my own and has not previously been submitted for any other degree at any university.

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CHAPTER I

INTRODUCTION

1. Pathways of glucose metabolism in normal liver

One of the many and varied metabolic functions of liver is its control of glucose metabolism via glycolysis, gluconeogenesis, glycogenesis and glycogenolysis. Although glycolysis occurs in almost all tissues, gluconeogenesis is restricted to certain organs such as liver and kidney cortex. As the liver is so much larger than the kidney cortex, it serves as the main source of glucose during starvation, or when carbohydrates are absent from the diet.

The first step involved in the metabolism of glucose is the phosphorylation of glucose to glucose-6-phosphate. This step is virtually irreversible ($K_{eq} > 63,000$) and is catalyzed by either glucose kinase or hexokinase. The reverse step or dephosphorylation reaction is catalyzed by a different enzyme, glucose-6-phosphatase.

The glucose-6-phosphate thus formed serves as a central point from which a number of pathways diverge or converge (Fig. 1.1). From Fig. 1.1 it can be seen that glucose-6-phosphate (G6P) can be converted to glucose-1-phosphate (G1P) with the aid of phosphoglucomutase, glucose-1,6-bisphosphate being a necessary co-factor in this reaction. The G1P formed from this step serves as a precursor for glycogen synthesis. G6P may also be oxidized via the pentose phosphate pathway to provide reducing potential for energy, free fatty acid and steroid synthesis. This pathway also provides pentoses for nucleotide synthesis. The first two reactions of this pathway are catalyzed by
FIG. 11. REACTIONS OF G6P METABOLISM.
glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, which are responsible for the conversion of G6P to 6-phosphogluconate and ribulose-5-phosphate respectively. Also, G6P may be converted to fructose-6-phosphate (F6P) in the presence of phosphoglucoisomerase to be metabolized via the Embden-Meyerhof pathway. On the other hand, G6P may be synthesized from non-carbohydrate sources via the gluconogenic pathway or from glycogen during glycogenolysis. The last steps of both these pathways involve the conversion of F6P to G6P and G6P to G6P, both of which are catalyzed by the same enzymes that are involved in the reverse reactions, namely phosphoglucoisomerase and phosphoglucomutase, respectively.

The ultimate proportion of G6P metabolized via these various pathways as well as the flux of these various pathways will depend on a number of factors, such as the dietary state of the animal, the maximal capacities of the enzymes, concentrations of intermediates and various factors that may affect the activity of these enzymes.

Unlike many substances that are metabolized via separate anabolic and catabolic pathways, many of the reactions involved in the synthesis and breakdown of glucose are shared. For instance, from Fig. 1.2, it is seen that the reactions catalyzed by aldolase, phosphoglucoisomerase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, phosphoglyceromutase, and enolase are common to both glycolysis and gluconeogenesis. On the other hand, as mentioned previously, the phosphorylation of glucose to glucose-6-phosphate and the reverse reaction are catalyzed by two different enzymes. Similarly, the conversion of fructose-6-phosphate to fructose-1,6-diphosphate and vice versa are also catalyzed by two different enzymes. Phosphofructokinase
FIG. 12 REACTIONS OF GLYCOLYSIS AND GLUCONEOGENESIS.
converts the fructose-6-phosphate to fructose-1,6-diphosphate. This 
reaction predominates during glycolysis and is thermodynamically uni-
directional \((K_{eq} = 10^3)\). The reverse gluconeogenic reaction, namely 
the conversion of fructose-1,6-diphosphate to fructose-6-phosphate, is 
catalyzed by fructose-1,6-diphosphatase (Demori, 1963). Another 
branch point of the glycolytic and gluconeogenic pathways occurs at the 
conversion of phospho-enol-pyruvate (PEP) to pyruvate and vice versa. 
When glycolysis prevails, PEP is mainly converted to pyruvate via the 
pyruvate kinase reaction. The equilibrium of this reaction has been 
shown to favour the conversion of PEP and ADP to pyruvate and ATP 
(YoQuata and Utter, 1959).

During gluconeogenesis, PEP is not synthesized by a process 
involved the reverse of this reaction but via oxaloacetate in the 
presence of PEP-carboxykinase and pyruvate carboxylase (Utter and Beal, 
1963 and Utter et al., 1964). However, Scrutton and Utter (1963) have 
pointed out that the maximal catalytic capacities of liver pyruvate 
carboxylase and PEP-carboxykinase are almost 10-fold less than the maxi-
mal capacity of pyruvate kinase. This would result in the PEP 
formed during gluconeogenesis being reconverted back to pyruvate; and 
hence the pathway would be short circuited, if the pyruvate kinase 
activity were not modulated by some other means in gluconeog 

Detailed kinetic studies have revealed that pyruvate kinase displays 
allosteric kinetics with respect to its substrate PEP. As these 
kinetistics are enhanced by allosteric inhibitors such as ATP and alanine 
and eliminated by activators such as fructose-1,6-diphosphate, it has 
been proposed that the activity of this enzyme could be closely modulated 
in vivo by feedback inhibition and feedback activation of these effec-
tors (Tanaka et al., 1967a and Illerme et al., 1970). (For details on
the kinetics of pyruvate kinase see page 19).

(a) Control of glycolysis and gluconeogenesis

The enzymes that catalyze unidirectional reactions have been implicated as playing a major role in the control of the fluxes of the pathways of glucose metabolism and particularly the glycolytic and gluconeogenic ones (Krebs, 1954 and Krebs et al., 1964).

Furthermore, as the glycolytic and gluconeogenic fluxes are affected by changes in the apparent concentration of enzymes that catalyze these unidirectional reactions, these enzymes have often been termed key regulatory enzymes. With the exception of hexokinase, all these key regulatory enzymes are subject to hormonal and dietary control. Under gluconeogenic conditions, such as starvation, diabetes and on corticosteroid administration, the levels of F6P-carboxykinase, pyruvate carboxylase, fructose-1,6-diphosphatase and glucose-6-phosphatase have been found to increase (Ashmore et al., 1954, Weber et al., 1961, Strago et al., 1963, Hennings et al., 1963 and Weber et al., 1965a). On the other hand, when animals are fed on diets rich in carbohydrates, or on insulin administration, the activities of the key glycolytic enzymes, such as pyruvate kinase, phosphofructokinase and glucokinase, increase (Krebs and Eggleston, 1962, Tanaka et al., 1967a and Weber and Singhal, 1965).

The finding that F6P-carboxykinase, pyruvate carboxylase, fructose-1,6-diphosphatase and glucose-6-phosphatase all increase under gluconeogenic conditions, and that insulin suppresses the activity of all these gluconeogenic enzymes and enhances the key glycolytic ones, led Weber et al. (1965b and c) to postulate that the synthesis of all the key glycolytic enzymes are controlled by one gene unit, whereas the gluconeogenic
ones are controlled by another operon and the bifunctional ones by a third. Furthermore, these authors suggested that insulin acts as a repressor of the gluconeogenic operon and an inducer of the glycolytic one. However, Sillen et al. (1969) have shown that both the glucose-6-phosphatase and pyruvate kinase levels were considerably elevated, when rats were fed on diets rich in glycerol and fructose. As glucose-6-phosphatase is a key gluconeogenic enzyme and pyruvate kinase a key glycolytic one, these findings do not agree with the functional gene unit concept of Weber et al. (1965b and c). These authors thus suggested that the regulation of the levels of the key glycolytic and gluconeogenic enzymes are controlled by not one, but many independent genes. Such a mode of regulation would have the added advantage of being more versatile.

As the activities of these key glycolytic and gluconeogenic enzymes are also affected reciprocally by various metabolites, many theories have been formulated on the regulation of these pathways by these metabolites. By correlating the results obtained from kinetic and other in vitro studies of the properties of the key glycolytic and gluconeogenic regulatory enzymes, it has been found that these are directly or indirectly affected by various nucleotides (Gershoni and Cevers, 1967). As the ATP/ADP ratio reflects the energy status of the cell, when this ratio is high (the ATP level will be low under such conditions), one will expect gluconeogenesis rather than glycolysis to prevail. As shown in Fig. 1.3, ATP is the substrate for pyruvate carboxylase hence this enzyme will be active. As the ATP/ADP ratio reflects other nucleotide ratios, the GTP/ADP ratio will also be elevated. GTP is the substrate for PEP-carboxykinase, the second gluconeogenic enzyme. Hence pyruvate will be converted to PEP via malosestate.
FIG. 13 Control of glycolysis and gluconeogenesis by F6P, ADP and AMP.

-ve and +ve indicate inhibitory and stimulatory effects on the enzymes respectively.
PEP will be prevented from being shunted back into pyruvate, as pyruvate kinase is inhibited by high ATP/ADP ratios. Furthermore, as the AMP level is low, fructose diphosphatase will be active as it will not be inhibited by AMP (Takata and Poggioli, 1965, Underwood and Næsholm, 1965 and Pontremoli et al., 1968). On the other hand, the phosphofructokinase will be depressed, firstly because ATP inhibits this enzyme, and secondly because the AMP level is low and cannot alleviate the inhibition by ATP (Layzer et al., 1969, Brock, 1969). During periods of active glycolysis, the ATP/ADP ratio will be low and the AMP level will be elevated. Under such conditions, the control mechanism mediated by these nucleotides will be reversed. The glycolytic enzymes, pyruvate kinase and phosphofructokinase will be activated whereas the key gluconeogenic enzymes, pyruvate carboxylase, PEP-carboxykinase and fructose-1,6-diphosphatase will be depressed.

Weber et al. (1966) have proposed a theory whereby the glycolytic and gluconeogenic fluxes are controlled by free fatty acid levels. During starvation and diabetes, the free fatty acid levels rise considerably. These enhance gluconeogenesis by inhibiting the key glycolytic enzymes glucose kinase, phosphofructokinase and pyruvate kinase. Furthermore, octanoate has been found by these authors to affect pyruvate kinase in such a way that the enzyme can be inhibited by its own substrate, PEP. This will ensure gluconeogenic utilization of PEP during gluconeogenesis. On the other hand, the free fatty acids do not affect the activity of the key gluconeogenic enzymes, glucose-6-phosphatase and fructose-1,6-diphosphatase. These free fatty acids also enhance gluconeogenesis by inhibiting glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase and thus preventing the diversion of glucose-6-phosphate into the pentose phosphate pathway.
The activities of these key glycolytic and gluconeogenic enzymes can also be modulated by various intermediates of the tricarboxylic acid cycle and fatty acid metabolism. Like the free fatty acids, these metabolites are all end products of glucose metabolism and can modulate the activity of the various enzymes through feedback control mechanisms. However, as the synthesis and breakdown of these intermediates are controlled by factors that control the fluxes not only of the pathways in which they occur but other pathways as well, the control of the fluxes of all these pathways are closely related (Fig. 1, b).

During gluconeogenesis, the long chain fatty acyl CoA and acetyl CoA levels are elevated. By inhibiting acetyl CoA carboxylase (Borle and Lysen, 1961) pyruvate dehydrogenase and citrate synthetase (Mielnik and Weiss, 1963 and Mieland, 1965), these elevated fatty acyl CoA and acetyl CoA derivatives will prevent acetyl CoA being utilized for fatty acid synthesis. They will cause a reduction in the amount of pyruvate being utilized via the tricarboxylic acid cycle and decrease intramitochondrial citrate synthesis. During starvation, the activity of citrate lyase, which cleaves citrate to oxaloacetate and acetyl CoA, is also considerably reduced (Spencer and Lowenstein, 1962 and Lowenstein, 1966). This, together with the decreased isocitrate dehydrogenase activity will result in accumulation of extramitochondrial citrate. The high extramitochondrial citrate levels will inhibit phosphofructokinase and activate pyruvate carboxylase (Wernsola and Severs, 1967). Inhibition of phosphofructokinase will result in decreased FDP levels. Thus fructose-1,6-diphosphatase will be activated and activation of pyruvate kinase by FDP will be prevented. Pyruvate kinase is homo-er, also inhibited by high citrate levels (Meber, 1969). This inhibition of
FIG. 1. Control of glycolysis and gluconeogenesis by various intermediates of glucose metabolism.

"-ve and +ve indicates inhibitory and stimulatory effects on the enzymes respectively."
pyruvate kinase, together with the activation of pyruvate carboxylase by high acetyl CoA levels will ensure the diversion of pyruvate into the gluconeogenic pathway. Again, under glycolytic conditions, these control mechanisms will not be effective and regulation will be reversed in favour of glycolysis.

2. Glucose metabolism of hepatomas

Under certain pathological conditions, derangement in glucose metabolism is often accompanied by changes in the flux of these regulatory enzymes. For instance, in diabetes, the key gluconeogenic enzymes of the liver are elevated, whereas in hepatomas, the activities of the key gluconeogenic ones are decreased and the key glycolytic ones are increased (Yobber et al., 1965a and Yobber, 1966). However, unlike the regulatory enzymes of normal liver cells, those of tumour cells lose their responsiveness to nutritional and hormonal regulations (Suda et al., 1966). Suda et al. (1966) have shown that hexokinase, pyruvate kinase and phosphofructokinase activities remain elevated in tumour-bearing diabetic rats whereas the glucose-6-phosphatase activity, which increases considerably in normal diabetic rats, remains unaltered in tumour-bearing rats.

Cancer cells were originally characterized as having high glycolytic activities with impaired respiration. These cells were thought to obtain most of their energy via glycolysis (Warburg et al., 1924 and Warburg, 1956).

The introduction by Morris (1963, 1966) of tumour lines of different growth rate has facilitated comparative studies to be carried out whereby changes at the molecular level can be correlated with the
growth rate of these tumours. Morphologically, the loss of degree of differentiation can be correlated with the growth rate, in that cells of slow-growing hepatomas resemble liver parenchymal cells whereas the fast-growing ones are poorly differentiated (Morris, 1963 and 1966, Knox et al., 1969 and 1970).

A close correlation has also been found between the growth rate of hepatomas and their glycolytic capacity. The slow-growing, highly-differentiated ones display low rates of glycolysis. This finding is contrary to the popular concept of high glycolytic capacities of cancer cells. On the other hand, the fast-growing, poorly-differentiated hepatomas have high glycolytic capacities similar to those envisaged by Marburg (Weber and Lea, 1966, Aisenberg and Morris, 1961, Burk et al., 1967, Lo et al., 1968 and Lin et al., 1962).

By correlating all the available data, Weber et al. (1964) and Weber (1966) have shown that a definite metabolic pattern prevails in cancer cells. The synthetic pathways of carbohydrate and lipid metabolism decrease with increased growth rate and loss of differentiation of the tumour cell whereas the catabolic ones increase. On the other hand, the catabolic pathways of protein and nucleic acid metabolism decrease progressively with increased growth rate whereas the synthetic ones increase.

The above concept has been verified with the glucose metabolising pathways of hepatomas. Glycolysis (catabolic pathway of glucose) increases with increasing growth rate. This is accompanied by increased lactate formation. Furthermore, increased glycolysis is also reflected in the increased hexokinase, phosphofructokinase and pyruvate kinase levels of hepatomas. Another degradative pathway that is enhanced, is
the pentose phosphate pathway, where the activity of glucose-6-phosphate dehydrogenase was found to be elevated. On the other hand, gluconeogenesis decreases with increasing growth rate of hepatomas. The decrease of gluconeogenesis is attributed to the decreased activity of key gluconeogenic enzymes, glucose-6-phosphatase, fructose diphosphatase, PD-carboxykinase and pyruvate carboxylase. These decrease with increased growth rate of the hepatoma. Glycogen deposition also decreases in tumour cells. This has partly been attributed to the decreased phosphoglucomutase activity. The Michaelis constant of this enzyme for G6P was found to increase in fast-growing, rat hepatomas (Weber et al., 1964). The specific phosphorylating enzymes, glucokinase and fructokinase also decrease with increased growth rate.

The finding that all the key glycolytic enzymes increase and the regulatory gluconeogenic ones decrease, whereas the bifunctional ones, remain unaltered lends further support to the functional unit concept proposed by Weber et al. (1965a, b and c).

Weinhouse (1966) has attributed the low glycolytic activity of slow-growing hepatomas to the low hexokinase activity in these cells, for it was found that in slow-growing, highly-differentiated hepatomas, both the hexokinase and glycolytic activities are low. In the fast-growing, poorly-differentiated hepatomas, the hexokinase and glycolytic rates are increased. Furthermore, the low glycolytic activity found in the slow-growing, highly-differentiated hepatomas could be increased by addition of purified hexokinase to homogenates of these cells.

Lo et al. (1968) have implicated pyruvate kinase as playing a major role in the control of the glycolysis. These authors pointed out that the Pasteur effect (regulation of glycolysis by respiration)
can partly be attributed to the transphosphorylating glycolytic enzymes, 3-phosphoglycerate kinase and pyruvate kinase competing with the respiratory system for ADP. In fast-growing, poorly-differentiated hepatomas, respiration is low and glycolysis is high. In the presence of FDP, these authors found that glycolysis and lactate production increased whereas respiration and respiratory phosphorylation remained low. From these findings, they concluded that most of the ATP produced is via glycolysis, and that the transphosphorylating enzymes compete successfully with the weak respiratory system for ADP. On the other hand, in slow-growing, highly-differentiated hepatomas, the reverse is found to be true, namely that in the presence of FDP, lactate production and glycolysis is low with most of the ATP being produced via respiratory phosphorylation. As the 3-phosphoglycerate kinase activity does not vary with growth rate and dedifferentiation of tumour cells, whereas both the pyruvate kinase and the glycolytic/respiratory activities do, it was concluded that of the two transphosphorylating enzymes, pyruvate kinase plays the major role in the control of glycolysis and the Pasteur effect. In slow-growing, highly-differentiated hepatomas, where the pyruvate kinase activity is low, the respiratory system will preferentially utilize the ADP and glycolysis will be decreased. On the other hand, as the pyruvate kinase level is high in poorly-differentiated hepatomas, the ADP will be preferentially utilized via glycolysis.

3. Glucose metabolism of regenerating liver

Although the glucose metabolism of regenerating liver resembles that of hepatomas in that glycolysis is enhanced, it differs from it, in that gluconeogenesis remains unaltered in regenerating liver and does not

Sude et al. (1966) have shown that the activities of the key glycolytic enzymes, glucokinase, phosphofructokinase and pyruvate kinase of regenerating liver increase and reach a maximum level within a week, after which, their activity decreases to a normal level. As these changes were found by these authors to persist after proliferation had stopped, it was concluded that these changes are not due to proliferation but due to the immaturity of the system.

4. **Glucose metabolism of foetal liver**

As in the case of regenerating liver, the glucose metabolism of hepatomas also resembles that of foetal liver in many respects. In both hepatoma and foetal liver, glycolysis is high. In foetal liver, glycolysis decreases with maturation of the foetus, whereas in hepatomas, glycolysis increases with increase growth rate and dedifferentiation. Just as the change in glycolytic activity in hepatomas has been partly attributed to change in the flux of the key glucose metabolizing enzymes (Weber, 1966), changes in glucose metabolism in foetal livers are associated with the appearance of certain enzymes and changes in the levels and forms of others with maturation of the foetus (Burch et al., 1963, Burch, 1965, Huxtable and Colacci, 1968, Fruhan and O'Toole, 1964; and Swiatek et al., 1970).

For instance, the increase of glycogen synthesis in foetal liver of guinea pig and rat at the end of gestation has been associated with
the increased levels of UDP-glycogen transglucosylase, UDPG-pyrophosphorylase, and decreased glucose-6-phosphate dehydrogenase activity. The decrease of glucose-6-phosphate dehydrogenase activity will prevent glucose-6-phosphate being preferentially utilized via the pentose phosphate pathway rather than the glycogenic one (Ballard and Oliver, 1963, Kornfeld and Brown, 1962, Jacquot and Kratchmar, 1965; and Burch et al., 1963). Furthermore, the absence of glucose-6-phosphatase activity in prenatal livers of guinea pigs and rats will prevent 6-P being shunted off to glucose in these species (Burch, 1965). Glucose-6-phosphatase activity has however been reported to be present in foetal livers of sheep, monkeys and humans (Auricchio and Rigillo, 1960, Ballard and Oliver, 1966 and Daskins, 1963).

The increased glycogen degradation that occurs immediately after birth has also been attributed to elevated glycogen phosphorylase activity in neonatal livers of guinea pigs and rats (Burch, 1965, Kornfeld and Brown, 1962, Jacquot and Kratchmar, 1965).

Decrease of glycolysis and increase of gluconeogenesis immediately after birth is reflected by the decreased hexokinase, pyruvate kinase and phosphofructokinase activities and in increase of the glucose-6-phosphatase, fructose diphosphatase and pyruvate carboxylase activities (Burch et al., 1963, Burch, 1965, Walker, 1965, Daskins, 1966, Seifert et al., 1970). Furthermore, glucokinase which is absent from fast-growing rat hepatomas (Weinhouse, 1966) is also absent from foetal livers and can only be detected a few days after birth. The appearance of this enzyme will enable the liver to carry out one of its major functions, namely the regulation of blood glucose levels. The increase of glucokinase activity is accompanied by a decrease of hexokinase.
activity (Walker, 1965). Furthermore, Sato et al. (1969) have shown that the glucokinase and hexokinase isoenzyme pattern of rat foetal liver is similar to that of the slow-growing, transplanted hepatomas. Another two enzymes that vary with maturation, are lactate dehydrogenase and 6-phosphogluconate dehydrogenase. In rat foetal liver, the lactate dehydrogenase/6-phosphogluconate dehydrogenase activity ratio is approximately 15, 3 days before birth. In new born rats, it is 1 and in adult liver, this ratio is 1.6. This ratio is also high in cancerous tissues (Burch et al., 1963). The high aldolase FDP/F1P ratio found both in hepatomas and foetus (21 in 5 day rat foetus) is consistent with the hypothesis that cancerous tissue reverts to an immature state.

5. Detailed information on specific enzymes of carbohydrate metabolism which show some alterations in hepatomas

(a) Glucokinase and Hexokinase

Both glucokinase and hexokinase catalyze the phosphorylation of glucose in the liver. Hexokinase is widely distributed in tissues and relatively non-specific. It has a high affinity for glucose ($K_m = 10^{-4}$ - $10^{-5}$ M) and is inhibited by high levels of glucose-6-phosphate (Sols, 1965 and Walker, 1965). Three hexokinase isoenzymes designated types I, II and III have been separated on DEAE-cellulose and by starch gel electrophoresis (Grossbard et al., 1966 and Gonzalez et al., 1967). Although all three hexokinase types have been shown to be present in all tissues, the proportion of these three isoenzymes differ in different tissue types (Grossbard et al., 1966, Katzman and Schimke, 1965 and Grossbard and Schimke, 1966). These three hexokinase types have been partially purified from rat tissue by Grossbard and Schimke (1966) and have been found to differ
in their kinetic properties, stability to heat and proteolytic inactivation.

Hexokinase is found in both soluble and particulate fractions of homogenates (Rose and Karas, 1967, Hyden and Borregaard, 1968, Kar-
patkin, 1967 and Kyer et al., 1966). Kosow and Rose (1968) have found that the kinetic properties of soluble and insoluble hexokinase II from escites cells differ, particularly with respect to the mode of inhibition by anhydroglucitol-6-phosphate.

Glucokinase is found only in gluconeogenic tissue such as liver. It has a high substrate specificity for glucose and mannose, a low affinity for glucose (Km = 0.01 - 0.02M) and is not inhibited by high glucose-6-phosphate levels (Sols, 1965 and Perry and Walker, 1966). As its activity is subject to dietary and hormonal regulation, it has been implicated in playing a major role in the regulation of blood glucose and glyco-
lysis. When the blood glucose and insulin levels are high, the activity of this enzyme is considerably increased. This and the fact that the enzyme has a high Michaelis constant will enable it to function best when the blood glucose levels are high. When gluconeogenesis prevails, not only will the concentration of this enzyme be reduced but its high Michaelis constant will not permit it to function efficiently.

In hepatomas, glucokinase cannot be induced by feeding animals carbohydrate-rich diets or by insulin administration. Furthermore, it is replaced by hexokinase, an enzyme that predominates in non-glucone-
genetic tissue, foetal, and regenerating livers. The hexokinase levels have been found to increase with increase growth rate and de differen-
tiation of hepatomas. On the other hand, the glucokinase activity has
been found to be low or absent from both fast- and slow-growing rat hepatomas (Neimhause, 1965 and Shatton et al., 1969). Not only do the glucokinase and hexokinase levels change with the growth rate of these hepatomas, but so does the proportion of the three hexokinase isoenzymes. In slow-growing, highly-differentiated rat hepatomas, the glucokinase and hexokinase isoenzyme pattern is found to be similar to that of normal liver, in that a glucokinase and three hexokinase isoenzymes are present (Shatton et al., 1969 and Sato et al., 1969). However, it has been reported that in these slow-growing hepatomas, the glucokinase isoenzyme does not separate into a fast and slow component and that hexokinase II is more active than normal liver hexokinase II (Sato et al., 1969). On the other hand, in fast-growing, poorly-differentiated hepatomas, the glucokinase isoenzyme is absent, the activity of hexokinase II is elevated, whereas that of hexokinase III is decreased and in some instances cannot be detected at all (Sato et al., 1969). The disappearance of hexokinase III has been attributed to gene suppression and the predominance of hexokinase II to gene derepression in these hepatomas.

(b) Aldolase

A similar change of one form of an enzyme for one that predominates in fast cell tissue, regenerating liver and non-gluconeogenic tissue has also been reported for aldolase. There are three distinct aldolases, namely type A, that predominate in muscle, type B, which is found in liver, and type C, that occurs in brain tissue. These three forms cleave FDP and FIP at different rates. Muscle type A cleaves FDP at a faster rate than FIP, giving a FIP/FDP activity ratio of over 50. Liver type B cleaves FDP and FIP at the same rate under a particular set of experimental
conditions, giving an activity ratio of unity. With type A aldolase, activity ratios between 5 and 10 have been reported (Blestein and Rutter, 1961, Schapira et al., 1963, Schapira et al., 1962 and Rutter, 1961).

Schapira et al. (1963) found high FDP/F1P activity ratios that resembled those found in muscle and foetal livers, in primary human-liver cancers and various rat hepatomas. This finding led to the suggestion that the gene responsible for synthesis of aldolase type A is repressed in normal liver and derepressed in hepatomas. Similar results have since been reported for a gradation of transplantable rat hepatomas. The fast-growing, poorly-differentiated hepatomas have been found to have high activity ratios of approximately 50 that resemble those of muscle type A aldolase. On the other hand, with the slow-growing, highly-differentiated hepatomas, FDP/F1P activity ratios of 1.3-6 were obtained that suggest the presence of both aldolase A and aldolase B. These proposals have been verified with immunological and electrophoretic techniques (Zeinhouse, 1966, Sugimura et al., 1966 and Katsumata et al., 1968).

That the aldolase present in hepatomas is identical with the muscle aldolase A was also confirmed by the findings of Kawabe et al. (1969), Grady et al. (1970) and Uchihara et al. (1970). These authors demonstrated that crystalline aldolases isolated from Rhodamine Sarcoma, Novikoff hepatoma and rat hepatomas induced with 3'-3'A-DAB, were all identical with muscle aldolase A but differed from liver aldolase B. These resembled aldolase A with respect to their Michaelis constant for both FDP and F1P, the FDP/F1P activity ratio, inhibition by FDP, electrophoretic mobility, isoelectric fractionation profiles, immunological response, amino acid composition, tryptic fingerprints and their response to carboxypeptidase
digestion. Similarly, Katsumata et al. (1968) have shown that aldolase, isolated from ascites hepatoma, has the same amino acid composition and tryptic fingerprint as that of muscle aldolase A.

(c) Fructose-1,6-diphosphatase

Although the fructose diphosphatase activity has been reported by Keber and Morris (1963) to be absent from fast-growing, poorly-differentiated hepatomas, Sato et al. (1967) found the activity of this enzyme to be considerable in Ehrlich ascites cells.

Partially purified fructose-1,6-diphosphatase from ascites tumour cells has been found to resemble muscle rather than liver fructose diphosphatase with respect to its kinetic properties (Sato and Taniki, 1968).

(d) Pyruvate kinase

In gluconeogenic tissue such as liver, the pyruvate kinase activity is subject to nutritional and hormonal control, whereas the pyruvate kinase activity of non-gluconeogenic tissue, i.e. muscle, is not affected by any of these factors (Krebs and Eggleston, 1965, and Tanaka et al., 1967a). Differences between liver and muscle pyruvate kinase have also been shown by kinetic studies. Furthermore, as can be seen from Table 1.1, pyruvate kinase isolated from a variety of sources such as rat liver and muscle, rabbit muscle, erythrocyte, leucocyte, adipose tissue, yeast and bacteria, reflect kinetic properties that resemble either the muscle or the liver pyruvate kinases or both.

(e) Rat muscle or type H pyruvate kinase

Rat muscle pyruvate kinase has been crystallized by Tanaka et al.
(1967a) and was found to have a molecular weight of 250,000. From Table 1.1, it can be seen that unlike liver type I pyruvate kinase, type II pyruvate kinase is precipitated at a higher ammonium sulphate saturation concentration (50-70%) but is eluted from DEAE-cellulose at a lower KCl level (0.02M KCl). It displays Michaelis-Menten kinetics with respect to either ADP or PEP, and has a low apparent Michaelis constant for PEP of 7.5 x 10^-5 M. The apparent Michaelis constant for ADP was found to be 2.7 x 10^-4 M.

When the muscle type II and liver type I pyruvate kinases were assayed under the same conditions, the muscle preparation was less susceptible to either ATP or PCH inhibition. (Tanaka et al., 1967a). A K_i (apparent) for ATP of 3.5 x 10^-3 M and a K_i (apparent) for PCH of 2.5 x 10^-5 M were obtained for muscle pyruvate kinase, whereas the liver type I pyruvate kinase was found to have an apparent K_i for ATP of 1.5 x 10^-2 M; and a K_i (apparent) for PCH of 7.4 x 10^-7 M.

(ii) Rat liver pyruvate kinase

Four pyruvate kinase isoenzymes have been found to be present in rat liver (Tanaka et al., 1967a). The two major ones have been designated type I or PK I and type II or PK II (Tanaka et al., 1967a and Susor and Butter, 1968). Normal liver contains 58.4% type I and 31.6% type II (Tanaka et al., 1967a, Suda et al., 1966 and Weber, 1968).

The II type pyruvate kinase is widely distributed in various tissues such as skeletal muscle, lung, spleen, testes, brain, intestinal mucosa and leucocytes (Table 1.2). Although liver type II pyruvate kinase is immunologically indistinguishable from the muscle enzyme, Susor and Butter (1968) have indicated that the electrophoretic mobility of the liver and
muscle types II pyruvate kinases differ slightly. Also, Jiménez de Asúa et al. (1971b) found that the liver and muscle types II pyruvate kinases respond differently to various amino acids. Whereas liver type II pyruvate kinase was inhibited by phenylalanine, alanine, tyrosine, tryptophan, valine, proline and threonine, muscle type II pyruvate kinase was inhibited only by phenylalanine. The allosteric inhibition of muscle type II pyruvate kinase by phenylalanine could be reversed by alanine, serine and cysteine (Rozengurt et al., 1970). This led Jiménez de Asúa et al. (1961b) to postulate that rat tissue contains two forms of type II pyruvate kinases that display different regulatory properties.

Whereas type II pyruvate kinase migrates towards the cathode, type I pyruvate kinase migrates towards the anode on electrophoresis and is only neutralized by type I pyruvate kinase antisera (Table 1.2). It is a regulatory enzyme for its level fluctuates with diet and is affected by hormones such as insulin and by the pathological state of the animal (Tanaka et al., 1967b; Suda et al., 1966 and Weber, 1966). Furthermore, type I pyruvate kinase displays allosteric kinetics with respect to PEP, indicating that the activity of the enzyme can be modulated by its substrate (Tanaka et al., 1967b, Susor and Rutter, 1968, Rozengurt et al., 1969 and Llorente et al., 1970). This cooperativity displayed by type I pyruvate kinase with respect to PEP can however be affected by various effectors, such as FDP. FDP was found to activate the enzyme, decrease the Michaelis constant for PEP from 6.3 x 10^{-3}M to 0.83 x 10^{-3}M and to change the kinetics from sigmoidal to hyperbolic. This activation by FDP is dependent on a number of factors, such as the pH, K^+, Mg^{2+}, Mg^{2+}, ATP and PEP levels (Tanaka et al., 1967b, Rozengurt et al., 1969, Susor and Rutter, 1968, Taylor and Bailey, 1967, Eggleston
The enzyme can also be activated by various hexose phosphates such as glucose-6-phosphate, glucose-1-phosphate, fructose-6-phosphate, fructose-1-phosphate and 6-phosphogluconate, though higher concentrations of these compounds are required (Taylor and Bailey, 1967, Weber et al., 1967 and Eggleston and Woods, 1970).

On the other hand, adenosine-5'-triphosphate (ATP) and alanine have been found to inhibit the enzyme allosterically, thus modulating the activity not only by inhibiting the enzyme but also enhancing the cooperativity of type I pyruvate kinase with respect to FEP. This allosteric inhibition of type I pyruvate kinase by alanine and ATP can be reversed by FDP (Tanaka et al., 1967b and Llorente et al., 1970). Fructose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, glucose-1-phosphate and inorganic phosphate can also partially alleviate the ATP inhibition (Kostar and Hallman, 1970). Other nucleotides, such as uracil triphosphate, guanosine triphosphate, uridine triphosphate, thymidine triphosphate and adenosine monophosphate also inhibit liver pyruvate kinase, but these are not as potent as ATP (Weber, 1969). Free fatty acids, acetyl CoA, oxaloacetate, citrate and divalent cations such as Ca⁺ and Mg⁺ also have an inhibitory effect on the liver pyruvate kinase (Weber et al., 1968, Weber, 1969 and Jiménez De Arda et al., 1970).

From Table 1.1, it is seen that type I pyruvate kinase has been crystallized by Tanaka et al., (1967a) and found to have a molecular weight of 208,000. The enzyme precipitates between 25-45% ammonium sulphate saturation and is eluted from DEAE-cellulose with 0.1M KCl.
### Table 1.2: Different Responses of Types L and H Pyruvate Kinase

<table>
<thead>
<tr>
<th></th>
<th>Type L</th>
<th>Type H</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Activity Increases</td>
<td>High CHO diets and insulin administration</td>
<td>Regenerating liver and transplantable rat hepatoma</td>
</tr>
<tr>
<td>(b) Electrophoresis</td>
<td>Migrates towards anode</td>
<td>Migrates towards the cathode</td>
</tr>
<tr>
<td>(c) Immunological responses</td>
<td>Not neutralized</td>
<td>Neutralized</td>
</tr>
<tr>
<td>i. Muscle pyruvate kinase antisera</td>
<td>Neutralized</td>
<td>Not affected</td>
</tr>
<tr>
<td>ii. Liver type L pyruvate kinase antisera</td>
<td>Neutralized</td>
<td>Not affected</td>
</tr>
<tr>
<td>(d) Kinetics with respect to PEP</td>
<td>Sigmoidal</td>
<td>Hyperbolic</td>
</tr>
<tr>
<td>(e) PEP</td>
<td>Activates, increases $V_{max}$ and lowers $K_m$</td>
<td>No effect</td>
</tr>
</tbody>
</table>
It displays Michaelis-Menten kinetics with respect to ATP; is inhibited by high levels of ADP and has an apparent Michaelis constant of $1 \times 10^{-4} M$ for ADP. Type L pyruvate kinase was found by these authors to display sigmoidal kinetics with respect to FEP and to have an apparent Michaelis constant of $8.3 \times 10^{-2} M$ for FEP.

Sussor and Rutter (1968) have found that type L pyruvate kinase (designated PyK B by these authors) may exist in two states which display different kinetics. The sensitive form of the enzyme displays sigmoidal kinetics with respect to FEP, has a high $K_{0.5}$ for FEP of $1.9 \times 10^{-2} M$ and is sensitive to FDP activation. The insensitive form of the enzyme displays Michaelis-Menten kinetics with respect to FEP, has a low $K_{0.5}$ of $1.5 \times 10^{-4} M$ for FEP and is not activated by FDP. The insensitive form of the enzyme was also found to be one-tenth as sensitive to ATP inhibition when compared to the sensitive form. The sensitive form of the enzyme could be desensitized by storage of the enzyme at $-20^\circ C$. As these authors found no difference in the elution profiles from Sephadex G-200 of muscle pyruvate kinase, and the sensitive and insensitive forms of liver pyruvate kinase, they concluded that this variation in the sensitivity is not due to varying degrees of association and dissociation of the enzyme. Llorente et al. (1970) have however shown that the desensitization of liver pyruvate kinase at $0 - 2^\circ C$ can be reversed by preincubating the enzyme at $37^\circ C$. Contrary to the above findings, Tanaka et al. (1967b) found that the enzyme loses its cooperativity and sensitivity to FDP activation when it is preincubated at $37^\circ C$ and not at $0^\circ C$. Furthermore, Bailey et al. (1968) found that liver pyruvate kinase could be desensitized with organic solvents such as methanol and glycerol.

Hess and Kutchbach (1971) have suggested that these different forms of liver pyruvate kinase may be attributed to differences in saturation
of the enzyme with FDP. These conclusions are based on the finding that in fresh homogenates of pig liver, an acid type pyruvate kinase having an isoelectric point of pH 5.1 and binding 2 moles FDP/mole pyruvate kinase predominates. During purification or incubation of the enzyme, this predominant form is converted to the minor, more alkaline type which has an isoelectric point of 6.1 and does not bind FDP. It was thus suggested that during purification or incubation, the FDP-saturated enzyme loses its FDP and reverts to the FDP-free form.

(iii) Rat hepatoma pyruvate kinase

Whereas in normal liver, the L isoenzyme forms two-thirds of the total pyruvate kinase activity, in hepatomas, the M isoenzyme predominates (Tanaka et al., 1967a, Suda et al., 1966 and Weber, 1966). The proportion of the M type increases with increased growth rate and de-differentiation of the tumour whereas that of the L type decreases (Farina et al., 1968 and Lo et al., 1968). In this respect pyruvate kinase resembles glucokinase, in that the regulatory L type pyruvate kinase, that is under dietary and hormonal control, is replaced by the M type isoenzyme, which predominates in fast and regenerating liver. However, unlike hepatoma aldolase, which is identical with the muscle aldolase type A, the type M pyruvate kinase of fast-growing rat hepatoma is thought to be different to the muscle type M isoenzyme. This conclusion is based on the findings of Taylor et al. (1969) who showed that 392kA rat hepatoma pyruvate kinase has different properties to the muscle type M isoenzyme. As can be seen from Table 1.3, the hepatoma enzyme resembles pyruvate kinase isolated from muscle with respect to its Michaelis constant for FDP, hyperbolic kinetics, solubility in ammonium sulphate and DEAE- Sephadex elution profile. On the other hand, it differs from muscle
<table>
<thead>
<tr>
<th></th>
<th>Hepatoma</th>
<th>Muscle</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (FDP)</td>
<td>$5 \times 10^{-5}$ M</td>
<td>$6.1 \times 10^{-5}$ M</td>
<td>$7 \times 10^{-4}$ M</td>
</tr>
<tr>
<td>Kinetics</td>
<td>Michaelis-Menten</td>
<td>Michaelis-Menten</td>
<td>Sigmoidal</td>
</tr>
<tr>
<td>Stability, $t_2$ at 37°C</td>
<td>1 mins</td>
<td>Stable</td>
<td>36 mins</td>
</tr>
<tr>
<td>FDP effect</td>
<td>Stabilizes</td>
<td>No known effect</td>
<td>Allosteric effector</td>
</tr>
<tr>
<td>($NH_4)_2SO_4$ fraction</td>
<td>55-70% saturation</td>
<td>55-70% saturation</td>
<td>0-55% saturation</td>
</tr>
<tr>
<td>DEAE-Sephadex fraction</td>
<td>0-100mM KCl</td>
<td>0-100mM KCl</td>
<td>100-200mM KCl</td>
</tr>
<tr>
<td>Electrophoretic</td>
<td>+ 12mM</td>
<td>+ 26mM</td>
<td>+ 50mM</td>
</tr>
<tr>
<td>migration in 10mM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris HCl buffer pH 7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-alanine $K_i$</td>
<td>$3.0 \times 10^{-1}$ M (protected by FDP)</td>
<td>$2 \times 10^{-2}$ M (FDP no effect) (protected by FDP)</td>
<td>$2.5 \times 10^{-1}$ M</td>
</tr>
<tr>
<td>FCMB $K_i$</td>
<td>$2 \times 10^{-7}$ M</td>
<td>$1.5 \times 10^{-5}$ M</td>
<td>$2 \times 10^{-7}$ M</td>
</tr>
<tr>
<td>ATP $K_i$</td>
<td>$1.5 \times 10^{-3}$ M</td>
<td>$1.7 \times 10^{-2}$ M</td>
<td>$5.6 \times 10^{-3}$ M</td>
</tr>
<tr>
<td>Copper Ions $K_i$</td>
<td>$1.5 \times 10^{-5}$ M</td>
<td>$10^{-1}$ M</td>
<td>$2 \times 10^{-5}$ M</td>
</tr>
<tr>
<td>Potassium $K_a$</td>
<td>11mM</td>
<td>12mM</td>
<td>10mM</td>
</tr>
<tr>
<td></td>
<td>Optimum 80mM, inhibits at high concentration</td>
<td>Optimum 70mM, inhibits at high concentration</td>
<td>Optimum 80mM, inhibits at high concentration</td>
</tr>
<tr>
<td>Calcium $K_i$</td>
<td>2.2mM</td>
<td>2.7mM</td>
<td>2.7mM</td>
</tr>
<tr>
<td>Sodium $K_i$</td>
<td>220mM</td>
<td>270mM</td>
<td>220mM</td>
</tr>
</tbody>
</table>
type K pyruvate kinase with respect to its stability, electrophoretic mobility and susceptibility to FGM, ATP and alanine inhibition.

Further proof that the hepatoma type K pyruvate kinase is not identical with muscle type K pyruvate kinase, was obtained by Criss (1969). By employing a pH isoelectric focusing technique, this author found four pyruvate kinase isoenzymes in normal liver and slow-growing, highly-differentiated hepatomas. However, in fast-growing, poorly-differentiated hepatomas, in addition to the aforementioned isoenzymes, an extra isoenzyme having an isoelectric point of 7.26 was found. As this isoenzyme was absent from regenerating and fasted livers, as well as from livers of diabetic and fasted rats, it was concluded that it was a new type of pyruvate kinase that was probably identical with the pyruvate kinase reported by Taylor et al. (1969).

(iv) Rabbit muscle pyruvate kinase

From Table 1, it can be seen that the physical and kinetic properties of rabbit and rat muscle pyruvate kinase are very similar, one major difference being that the specific activity of rabbit muscle pyruvate kinase (240 - 280 μg protein) is lower than that of rat muscle pyruvate kinase.

Rabbit muscle pyruvate kinase has also been crystallized and has been found to have a molecular weight of approximately 237,000 (Fista and Ochoa, 1956 and Warner, 1958). It has a tetrameric structure consisting of monomeric subunits having molecular weights of 57,000. These subunits appear to be very similar (Steinert and Deal, 1966).

The enzyme requires both Mg²⁺ and K⁺ for activity. Mg²⁺ + K⁺, but not Na⁺ can partially replace the activation by K⁺, whereas Na⁺ and
Go" can replace the activation by Mg" (Solomuk and Collin, 1955, Boyer, 1962 and Holmken and Storm, 1969). On the other hand, high levels of K° and Mg" inhibit the enzyme, the inhibition by high Mg" levels being affected by K° levels (Melchior, 1965). The actual mechanism of activation by these ions still remains to be elucidated. From magnetic resonance studies, Mildvan and Cohn (1965) have suggested that two moles of Na" are bound per mole of enzyme. Melchior (1965) has proposed that there are two binding sites for positive ions and that the enzyme is active when the one is occupied by K°. However, when both are occupied by Mg" or two K° ions, the enzyme is less active or inactive. Rabbit muscle pyruvate kinase is also inhibited by Ca" (Kachmar and Boyer, 1953). Furthermore, the inhibition by Ca" is competitive with Mg" and Na" (Mildvan and Cohn, 1965).

Further kinetic studies with rabbit muscle pyruvate kinase have revealed that, unlike liver type I pyruvate kinase, this enzyme displays Michaelis-Menten kinetics with respect to ADP and PEP. The Michaelis constants for ADP (3.0 - 9.8 x 10^-4) and PEP (7 - 10.4 x 10^-5) were found to be independent of the level of their second substrate (Reynard et al., 1961, Mildvan and Cohn, 1966 and Holmken and Storm, 1969). The quantitative variation in the Michaelis constant is due to the fact that these were found to differ in different buffer systems. Holmken and Storm (1969) have shown that in a glycyl-glycine buffer, pH 8.5, the Michaelis constant for ADP (3.3 x 10^-4) and PEP (1.4 x 10^-5) were lower than in Tris-Cl buffer, pH 8.5, where the Michaelis constant for ADP and PEP were found to be 9.8 x 10^-4 and 10.4 x 10^-5 respectively.

The evidence presented from the kinetic studies, where the Michaelis constants for ADP and PEP were found to be independent of the...
concentration of the second substrate, led to the suggestion of a random order of binding of the substrates to the enzyme (Hayward et al., 1961 and Mildvan and Cohn, 1965 and Holmosen and Storm, 1969). This was substantiated with the equilibrium binding studies of Hayward et al. (1961) who showed that ADP and PEP could bind independently to the enzyme. Furthermore, from these results, these authors concluded that the phosphoryl group is transferred directly from donor to acceptor.

Product inhibitor studies revealed ATP to be a competitive inhibitor of both ADP and PEP, and pyruvate a competitive inhibitor of PEP. From the random order of binding of the two substrates and product inhibitor studies, it was suggested that there is a binding site for ADP and ATP and a second site for PEP and pyruvate (Hayward et al., 1961 and Mildvan and Cohn, 1966). The competitive inhibition of ATP with PEP was attributed to the overlap of the transferable phosphoryl group of ATP and PEP (Hayward et al., 1961). On the other hand, ATP and pyruvate are thought to bind in a preferred order with ATP binding on first in the back reaction and coming off last in the forward reaction (Mildvan and Cohn, 1966).

The fact that adenosine nucleotides bind metal ions such as Mg⁺, Na⁺, K⁺ and Cu⁺ (Nelchior, 1954, Burton, 1959, O'Sullivan and Ferrin, 1964, Smith and Alberty, 1966 and Phillips, 1966) has led to a reappraisal of the kinetic data in terms of equilibrium concentrations of complex and simple ionic species present in solution (Nelchior, 1965). When K⁺ and PEP levels were kept constant, a close correlation was found between the MgADP levels and the rate of reaction. No relationship was found between the rate of reaction and the free Mg⁺ and ADP levels. This led Nelchior (1965) to formulate that MgADP rather than Mg⁺ or ADP participates in the reaction. Similarly, Hayward et al. (1961) have
also proposed that MgADP serves as the substrate. However, the suggestion that the metal nucleotide complex serves exclusively as substrate is still open to question, for results obtained from magnetic resonance studies of the Mn\(^{*}\) activated pyruvate kinase are in accord with random binding of metal, ADP, MgADP and FDP to the enzyme (Kildavan and Cohn, 1966).

(v) **Rat kidney pyruvate kinase**

Kidney, like liver, also plays a major role in gluconeogenesis. One might expect this enzyme to resemble liver type L pyruvate kinase. However, Jiménez De Asúa et al. (1971a) found that of the two types of pyruvate kinase present in rat kidney cortex, the type that predominates, viz. (PKI), resembles liver type L pyruvate kinase only in that it displays allosteric kinetics with respect to FDP. Unlike liver type L pyruvate kinase, its activity is not significantly affected by FDP and ATP, or its sigmoidal kinetics by pH. Although the enzyme is inhibited by alanine, this inhibition cannot be reversed by FDP. On the other hand, the minor isoenzyme (PKIII) does resemble the liver isoenzyme.

(vi) **Erythrocyte pyruvate kinase**

Although human erythrocyte pyruvate kinase has the same electrophoretic mobility as type L pyruvate kinase and resembles rat liver type L pyruvate kinase with respect to its solubility in ammonium sulphate, elution profile from DE-cellulose and immunological response, it differs from it in other respects (Koler et al., 1968). (Table 2.1).

The kinetic parameters reported for human erythrocyte pyruvate
kinases vary widely. For instance, Staal et al. (1971) found that the 
erthrocyte enzyme resembled rat liver type I pyruvate kinase in that 
it displayed Michaelis-Menten kinetics with respect to ADP and sigmoidal 
kinetics with respect to PEP. It had a high Michaelis constant \( (6.3 \times 10^{-12}) \) for PEP and was inhibited by high levels of ADP. The Michaelis 
constant for ADP was found to be \( 6.0 \times 10^{-4} \). Like rat liver type I 
pyruvate kinase, the allosteric properties displayed by the enzyme with 
respect to PEP could be affected by PEP and ATP. ATP activated the 
enzyme, changed the kinetics from sigmoidal to hyperbolic whereas ADP 
inhibited the enzyme allosterically. The enzyme could also be activated 
by glucose-6-phosphate, fructose-1,6-phosphate, 2,3-diphosphoglycerate 
and inorganic phosphate.

On the other hand, Campos et al. (1965) found that pyruvate 
kineae differed from rat liver pyruvate kinase in that it displayed 
Michaelis-Menten kinetics with respect to PEP. However, it resembled 
rat liver type L pyruvate kinase in that the Michaelis constant for ADP 
was dependent on the PEP level. Similarly, the Michaelis constant for 
PEP was found to be dependent on the ADP levels.

A third human erythrocyte pyruvate kinase preparation has been 
made by Ibsen et al. (1968), which resembled muscle and leucocyte pyru-
vate kinase. This enzyme was activated by \( K^+ \) and \( H^+ \) and required \( Mg^+ \) 
for activity. Like muscle pyruvate kinase, it displayed Michaelis-
Menten kinetics with respect to ADP and PEP and had a low Michaelis 
constant for PEP \( (4.6 \times 10^{-5}) \). Furthermore, the Michaelis constants 
for ADP and PEP were found to be independent of the concentration of 
their second substrate. These authors also found a linear relationship 
between the MgADP level and the pyruvate kinase activity, thus concluding
that \( K_{\text{gADP}} \) serves as a substrate. The Michaelis constant for \( K_{\text{gADP}} \) was found to be \( 3.3 \times 10^{-4} \text{ M} \).

Ibsen et al. (1968) have suggested that the differences in the kinetic parameters of their enzyme preparation could be due to an iso-enzyme variant or conformational isomer. Such electrophoretic variants have been reported by Tomes (1968). Furthermore, Steal et al. (1970) have reported other mutants with different kinetic and electrophoretic properties. However, these differences may partly be explained by the recent finding of Ibsen et al. (1971). These authors have shown human erythrocyte pyruvate kinase to be pleomorphic, existing in mono-, di-, tri-, tetra- and pentameric forms. These forms display different kinetics. The form that predominates in a preparation depends on the age of the sample and on the conditions of isolation. For instance, the tetrameric form is found to predominate in fresh, alumina gel purified preparations and also in frozen outdated cells. Such preparations are found to have a \( K_{0.5} \) for PEP of less than \( 3 \times 10^{-5} \text{ M} \) and to display Michaelis-Menten kinetics with respect to PEP that are not affected by FDP. The tetramer can be converted to trimer by ageing in alkaline pH. This trimer can be reconverted to its tetrameric form by ageing in solutions having a pH less than 7.0 or by preincubating with FDP. This trimeric form displays sigmoidal kinetics, has a higher \( K_{0.5} \) for PEP (2 x \( 10^{-4} \text{ M} \)) and is activated by FDP. A dimeric form was found to predominate in lysates of fresh blood cells. In such preparations the \( K_{0.5} \) for PEP and \( n_{\text{g}} \) value (interaction coefficient) are found to be dependent on the ATP level.

Taneja et al. (1967a and b) have shown that rat erythrocyte pyruvate kinase does not respond to dietary and hormonal changes and is not
activated by FDP.

(vii) Leucocyte pyruvate kinase

Human leucocyte pyruvate kinase resembles muscle pyruvate kinase with respect to its solubility in ammonium sulphate and DEAE elution profile (Table 1.1). It displays Michaelis-Menten kinetics with respect to ADP and PEP. It has a low Michaelis constant for PEP of $1.0 \times 10^{-4} M$ and a Michaelis constant for ADP of $1.76 \times 10^{-4} M$. Furthermore, the Michaelis constants for ADP and PEP are found to be independent of the level of the second substrate. (Campos et al., 1965, Koler et al., 1968 and Koler et al., 1969).

(viii) Adipose tissue pyruvate kinase

The procedure used for the partial purification of this enzyme resembles that of muscle with respect to its solubility in ammonium sulphate and elution profile from DEAE-cellulose (Table 1.1).

However, it resembles rat liver type I pyruvate kinase in that it was found to exist in two interconvertible forms (Pogson, 1966). These have been designated PyK A and PyK B. PyK A resembles the sensitive form of rat liver type I pyruvate kinase, since it has a high Michaelis constant for PEP ($6 \times 10^{-4} M$) and exhibits cooperative interaction with respect to PEP, $NH_4^+$, ATP and Mg++. It is also activated by FDP. On the other hand, PyK B resembles muscle and the insensitive form of rat liver type I pyruvate kinase for it has a low Michaelis constant for PEP ($6 \times 10^{-5} M$), displays Michaelis-Menten kinetics with respect to PEP and is not affected by FDP. It does however exhibit cooperative interaction with respect to $K^+$ and $NH_4^+$ (Table 1.1).
Like liver type 1 pyruvate kinase, these two forms are interconvertible. PyK 1 can be converted to PyK 2 with EDTA, ATP or citrate whereas PyK 1 can be converted to PyK 2 with FDP. On the other hand, adipose tissue pyruvate kinase differs from liver type 1 pyruvate kinase in that no difference is found in the susceptibility of PyK 1 and PyK 2 to ATP inhibition. A K_i value for ATP of 1.5 mM was obtained for both PyK 1 and PyK 2.

Recently however, Marco et al. (1971) have found no qualitative difference in the allosteric response of rat adipose tissue pyruvate kinase, in spite of the fact that they extracted the enzyme under the same conditions as did Pogson (1968). Furthermore, these authors reported allosteric inhibition of the enzyme by alanine that could be reversed with FDP. This inhibition by alanine could also be counteracted when assaying in imidazole buffer, pH 7.0 in the presence of 2mM MgATP, whereas in the presence of potassium phosphate buffer, pH 7, MgATP had no effect on this inhibition. Other hexose phosphates such as glucose-1-phosphate, fructose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate could not counteract this inhibition. The enzyme was also inhibited by phenylalanine.

(ix) Yeast pyruvate kinase

Pyruvate kinase has also been partially purified from baker's and brewer's yeast (Hunsley and Selbert, 1967 and Haeckel et al., 1968); (Table 1,1). The enzyme isolated from brewer's yeast has a specific activity of 200 U/mg protein and its molecular weight was estimated as being 200,000 by gel filtration (Haeckel et al., 1968) and 161,000 by sedimentation studies (Ashton and Piacanta, 1971). These later authors
show that it consists of 8 subunits each having a molecular weight of 30,000. The molecular weight of baker's yeast pyruvate kinase was estimated to be 135,000 by Hunsley and Sucler (1967) and between 160,000 - 165,000 by Kuczenski and Sucler (1970). Baker's yeast pyruvate kinase has been shown to be a tetramer with each polypeptide chain having a molecular weight of 42,000 - 45,000. The enzyme dissociates in the presence of malonic anhydride or 6M guanidine hydrochloride -0.1M 2-mercaptoethanol (Kuczenski and Sucler, 1970).

The kinetics of baker's and brewer's yeast pyruvate kinase resembles rat liver type L pyruvate kinase in that both display sigmoidal kinetics with respect to FDP. The sigmoidal kinetics are converted to hyperbolic in the presence of FDP, which activates the enzyme and lowers the Michaelis constant (Hess et al., 1966 and Hunsley and Sucler, 1969). Yeast pyruvate kinase is also activated allosterically by K+,

\[ \text{NH}_4^+ \] and \[ \text{K}^+ \]. The sigmoidal kinetics obtained with respect to these metal ions can be altered to hyperbolic in the presence of FDP. However, FDP only lowers the apparent Michaelis constant but does not affect the maximum velocity (Hunsley and Sucler, 1969 and Kacskal et al., 1968). Apart from fructose-1,6-diphosphate, only glucose-1-phosphate could activate this enzyme. Fructose-6-phosphate, fructose, glucose-1,

6-diphosphate, glucose-1-phosphate, glucose-6-phosphate, glucose and galactose-1,6-diphosphate had no effect on the enzyme. Ribulose-1,6-diphosphate had a slight inhibitory effect on the enzyme (Kacskal et al., 1968). On the other hand, ATP, citrate, NADP⁺ and Ca²⁺ inhibit the enzyme allosterically. Cytosine triphosphate, guanosine triphosphate, uridine triphosphate, inosine triphosphate, adenosine monophosphate also inhibit the enzyme (Kacskal et al., 1968). Although high ATP and Ca²⁺ levels inhibit the enzyme, low levels of these compounds activate the
(x) **Bacterial pyruvate kinase**

Pyruvate kinases isolated from bacteria resemble rat liver type I pyruvate in many respects. They also display specific characteristics that differ with different bacteria. For instance, two types of pyruvate kinase have been isolated from *Escherichia coli* K12 (Malcovati and Kornberg, 1967). The one type, PKI, resembles the sensitive liver type L isoenzyme in that it exhibits sigmoidal kinetics with respect to FDP that can be transformed to Michaelis-Menten kinetics in the presence of FDP. On the other hand, PKII resembles the insensitive form of rat liver type I pyruvate kinase in that it displays Michaelis-Menten kinetics with respect to FDP that are not affected by FDP. In contrast to pyruvate kinases isolated from other sources, that from *E. coli* B is not activated by Mg ions (Kasha and Samuel, 1964). This enzyme displays sigmoidal kinetics with respect to FDP. FDP only activates the enzyme by changing the maximum velocity. It does not affect the sigmoidicity of the kinetics. On the other hand, AMP, which was found to inhibit yeast and muscle pyruvate kinase, activates this enzyme. It decreases the Michaelis constant and changes the sigmoidal kinetics to Michaelis-Menten. Pyruvate kinase isolated from *Thiobacillus neapolitanus* also exhibits some distinctive properties (Cornish and Johnson, 1971). This enzyme displays sigmoidal kinetics that can be converted to hyperbolic in the presence of ribose-5-phosphate but not FDP. FDP has no effect on the enzyme. The enzyme in however activates by AMP and ribose-5-phosphate, fructose-6-phosphate and glucose-6-phosphate. Intermediates of the tricarboxylic acid cycle, such as succinate and fumarate also inhibit the enzyme.
Enzyme studies on human hepatomas

Most of the studies on glucose metabolizing enzymes of hepatomas and normal liver have been carried out in animals. Studies on glucose metabolizing enzymes in human hepatomas and livers have been sparse. This may partly be attributed to the limited availability of suitable human tissue and limitations encountered when working with such tissue. Whereas with animals, one can be selective as regards the age, sex, diet and pathology; and furthermore, their tissues can be used immediately after death, with humans, the type of material obtained from autopsies is varied and can only be obtained a few hours after death. Furthermore, the supply might be limited and the tissue may have to be deep frozen. In spite of these shortcomings, Shonk et al. (1964) have shown that the variation obtained in the levels of glucose metabolizing enzymes of normal human livers was similar to that found in rats, thus validating the use of such material. Furthermore, these authors have found that with the exception of phosphofructokinase, the levels of the more labile enzymes such as α-glycerolphosphate dehydrogenase, glycer-aldehyde-3-phosphosphate dehydrogenase, phosphoglycerate kinase and pyruvate kinase do not change in livers that have been kept at 24°C for 6.5 hours. Nor do the levels of these enzymes change when they are stored at -20°C for periods of up to 23-71 weeks. Although the phosphofructokinase activity was found to decrease considerably in livers kept at 24°C, the level of this enzyme does not decrease further after storage at -20°C.

The levels of glucose metabolizing enzymes present in human and rat livers were found by Boxer and Shonk (1966) to be very similar, the only exceptions being that the levels of α-glycerolphosphate dehydro-
glucose-6-phosphate dehydrogenase and fructose-1,6-diphosphatase were higher in rat livers. On the other hand, the enolase activity was found by them to be higher in human than in rat livers, whereas glucokinase was found to be absent from human livers. Because of this, these authors compared the glycolytic enzyme patterns of primary human cancers with those found in a gradation of slow- and fast-growing, rat hepatomas. Like the slow-growing, rat hepatomas, they found that the glucokinase and phosphofructokinase activities of these primary human hepatomas were similar to those of normal liver.

On the other hand, the pyruvate kinase levels found in these primary human hepatomas showed patterns that resembled both the slow- and fast-growing rat hepatomas. In some instances, the pyruvate kinase activities were considerably elevated, as in fast-growing rat hepatomas. On other occasions, the activity of this enzyme was similar in both cancerous and normal human livers (of slow-growing rat hepatomas).

Other enzyme activities, such as the lactate-glycerol phosphate dehydrogenase, phosphoglucomutase and fructose-1,6-diphosphatase showed patterns that resembled fast-growing rat hepatomas. Although the glucose-6-phosphate dehydrogenase level was found by these authors to be either normal or elevated in slow-growing and fast-growing rat hepatomas, it was low or normal in human hepatomas. From these data, these authors concluded that these primary hepatomas could not be divided into distinct classes, i.e., fast- or slow-growing, on the basis of their altered enzyme patterns.

7. Aim of the present study

No biochemical study has been carried out on primary human liver cancers except that of Boxar and Shook (1966). There is a high incidence
of primary liver cancer among the Bantu of Africa, particularly among those resident in Mozambique, where the incidence is 1:100 (Prata 1961). The pathology and the etiology of these may well be different to that of hepatomas occurring in other geographical regions.

It thus seemed worthwhile to investigate the activities of the glucose metabolizing enzymes in these primary liver cancers, in order to determine how the pattern obtained would compare with that obtained by Boxer and Shonek (1966) and with that of transplantable rat hepatomas. As the levels of these enzymes in normal liver are affected by the nutritional, physiological and pathological conditions, the levels of the enzymes measured in these hepatomas were compared with those in the respective host tissue. In addition, the enzymes were assayed in normal adult and fetal livers.

As discussed above, in the case of rate, the variation found in the levels of some enzymes may be due to different types of enzymes being produced (e.g. hexokinase and pyruvate kinase) in which case the conditions of assay might no longer be valid. In other instances, though there may not be any measurable differences in enzyme levels, the forms of the enzyme produced may vary as in the case of aldolase. For these reasons, the enzyme level estimations were supplemented with examination of the isoenzyme pattern.

Pyruvate kinase has been shown to play an important role in the control of glycolysis and gluconeogenesis and to have altered properties in rat hepatomas. Hence the major isoenzymes of this enzyme were partially purified from normal adult human livers and hepatomas. The kinetic properties of these were studied in order to determine whether in humans too, the regulatory properties of liver pyruvate kinase are
altered. The mechanism of action of both isoenzymes were studied in
detail by both kinetic and isotope exchange studies. Pyruvate kinase
was also isolated from foetal liver by using the exact procedure used
for hepatoma pyruvate kinase in order to assess how the properties of
this enzyme would compare with those of hepatoma pyruvate kinase.
# CHAPTER II

## LEVELS OF GLUCOSE METABOLIZING ENZYMES

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CHAPTER II

Levels of glucose metabolizing enzymes

I. Materials and Methods

1. Materials
   (a) Tissues:

   Normal adult human livers and hepatoma-containing livers were obtained from autopsies within 6 hours after death. The tissue of the hepatoma-containing livers was dissected. Those sections that appeared cancerous were separated from those that appeared normal when examined macroscopically. The latter were designated "host tissue".

   Two human foetal livers were obtained from aborted foetuses of approximately 5 months gestation period.

   All these tissues were kept frozen at -20°C.

   (b) Reagents:

1. Substrates

   The following reagents were obtained from Biochemica Boehringer: Glycerate-2-phosphate (2PGA) and glycerate-3-phosphate (3PGA) as the trisodium salts; phosphoenolpyruvate (PEP) as the monosodium salt; oxaloacetic acid; adenosine-5'-diphosphate (ADP) as the trisodium salt and adenosine-5'-triphosphate (ATP) as the disodium salt. The latter was neutralized with NaHCO₃ just before use. Dihydroxyacetone phosphate (DHAP) was obtained as the dimethyl ketal-dicyclohexylammonium salt. It was converted to free dihydroxyacetone phosphate by acid hydrolysis of
the katal group according to the instructions attached to the preparation. Its concentration was then estimated enzymatically with α-glycerolphosphate dehydrogenase.

From Sigma Chemical Co., the following reagents were obtained: fructose diphosphate (FDP) as the disodium salt, fructose-1-phosphate (F1P) as the disodium salt, fructose-6-phosphate (F6P) as the barium salt, which was converted to the sodium salt with sodium sulphate; glucose-6-phosphate (G6P) as the disodium salt; glyceraldehyde-3-phosphoric acid (GAP), which was neutralized with NaHCO₃ just before use; nicotinamide adenine dinucleotide, reduced form, Grade III, from yeast, as the disodium salt (NADH) and nicotinamide adenine dinucleotide phosphate from yeast as the monosodium salt (NADP⁺).

Pyruvic acid (potassium salt) was purchased from Calbiochem and glycerol-3-phosphate (trisodium salt) from Fluka and Buchs. Bovine plasma albumin was obtained from the Armour Pharmaceutical Company and dithiothreitol (DTT) from Calbiochem. Other standard reagents were obtained from BDH and Merck. These were of Analar grade.

ii. **Auxiliary enzymes**

These were all obtained from Biochemical Boehringer and included glucose-6-phosphate dehydrogenase (G6PDH), aldolase, α-glycerolphosphate dehydrogenase, α-glycerolphosphate dehydrogenase - triosephosphate isomerase mixture, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, fructose 1,6-diphosphatase, phosphoglucoisomerase and pig heart lactate dehydrogenase. The ammonium sulphate suspensions of these enzymes were diluted with homogenizing medium (for composition of homogenizing medium see methods) to which 0.1% bovine serum albumin had been added. The activi-
of the aforementioned enzymes were first assayed before diluting to the required value.

2. Methods

(a) Preparation of homogenates

The methods used for extracting and estimating the levels of glucose metabolizing enzymes were similar to those described by Shook and Boxer (1964) but some were modified.

Small pieces of tissue, 0.5g - 1.0g were removed from the frozen livers. The tissues were rapidly weighed and homogenized with an Ultra-Turrax homogenizer for a few seconds while being kept in an ice-bath. Homogenization was carried out in a solution containing 0.15M KCl, 0.05M KHC03, 0.005M Na2-EDTA and 1mM DTT. The homogenate was centrifuged in an MSE high-speed refrigerated centrifuge at 36,000g for 30 minutes at 4°C. The clear supernatant was then used to estimate the levels of different glucose metabolizing enzymes.

As the stability of the various enzymes in the homogenizing medium varied, the most labile ones were assayed first. Furthermore, in order to ensure uniform results, the enzymes were usually measured in the same order each time. As it was impossible to estimate the activity of all the enzymes on the same day, the more stable ones were kept at 4°C and assayed on the following day. Fresh dilutions of the stored supernatants were made.

All the supernatants were diluted with homogenizing medium. Most of the enzymes present in these supernatants were assayed by coupling to
systems that involved either the oxidation or reduction of di- or tri-
phosphoryridine nucleotides. The activity was therefore determined
by following the change in the optical density at 360 m\(\mu\). Exceptions
were enolase and phosphoglycerate mutase, which were determined by
following the PEP formation at 230 m\(\mu\). The activities of the enzymes
were estimated at 30\(^\circ\)C in a Unica S.P. 800 or Beckman D.B. recording
spectrophotometer. All the assays were done in duplicate.

A unit of enzyme activity is defined as the amount of enzyme
required to convert 1 \(\mu\) mole substrate per minute.

Specific activity is expressed as Units/g. wet wt.

(b) Specific enzyme determinations

1. Glyceraldehyde phosphate dehydrogenase (GAPDH)

The glyceraldehyde phosphate dehydrogenase activity was estimated
according to the following reactions:

\[
\begin{align*}
\text{GAPDH} & \rightarrow \text{GAP} + \text{PH}_2 \\
\text{ADP} & \rightarrow 1,3 \text{ di-PGA} \\
\text{PEP} & \rightarrow \text{3PGA} \\
\text{phosphoglycerate kinase} & \\
\end{align*}
\]

The assay system consisted of 50mM triethanolamine-NCl buffer, pH
7.4, 5mM EDTA, 10mM MgCl\(_2\), 0.15mM NADH\(_2\), 2.0mM 3PGA, 2.9mM ADP, 3.5mM
phosphoglycerate kinase and equivalent to approximately 1g
tissue.

After preincubating for 5 minutes, the reaction was initiated by
the addition of supernatant to both the blank and test cuvettes. The blank cuvette contained everything except PFA.

ii. Pyruvate kinase

The pyruvate kinase activity was estimated according to the following reactions:

\[
\begin{align*}
\text{ADP} & \xrightarrow{\text{pyruvate kinase}} \text{PSP} \\
\text{ATP} & \xrightarrow{\text{pyruvate}} \text{pyruvate} \\
\text{NADH}_2 & \xrightarrow{\text{lactate dehydrogenase}} \text{NAD}^+ \\
\text{lactate} & \xrightarrow{\text{NADP}} \text{lactate} \\
\end{align*}
\]

The assay mixture contained 50mM tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.4, 5mM EDTA, 10mM MgCl2, 1U lactate dehydrogenase, 0.15mM NADH2, 2.9 mM PSP, 2.9mM ADP and supernatant equivalent to approximately 1mg tissue.

The blank cuvette contained everything except ADP. The reaction was started by the addition of ADP. The reaction mixture was equilibrated at 30°C for 5 minutes before the addition of the ADP. During this time interval, any pyruvate present in the PSP could be reduced to lactate.

iii. Enolase

This enzyme catalyses the reaction:

\[
\text{PFA} \xrightarrow{\text{Enolase}} \text{PEP}
\]

It was estimated by measuring PEP formation at 230nm.

As the activity of this enzyme was found to increase with time, the supernatant (equivalent to about 2mg tissue) was preincubated at 30°C for
15 minutes with 50mM Tris-Cl buffer, pH 7.4 and 10mM MgCl₂. The reaction was then initiated by the addition of 2F6A (2.8mM).

The blank contained all the reagents except 2F6A.

iv. Aldolase

(a) FDP aldolase

The FDP aldolase activity was estimated according to the following reactions:

\[
\text{FDP} \rightarrow \text{GAP} \rightarrow \text{TEI} \rightarrow \text{DHAP} \rightarrow \text{NADH}_2
\]

The assay system contained 50mM triethanolamine-Cl buffer, pH 7.4, 50mM EDTA, 0.15mM NADH₂, α-glycerolphosphate dehydrogenase-α-glycerolphosphate isomerase mixture containing 0.6 and 4.8U respectively, 3mM FDP and supernatant equivalent to approximately 1-10mg tissue.

The activity of this enzyme was estimated against a blank that contained all the above reagents except FDP. The reaction was initiated by the addition of supernatant to both the test and blank cuvettes. The FDP was checked for triosephosphate impurities which could give falsely high results. This was done by using the above assay system without supernatant.

As 2 moles NADH₂ were oxidized per mole of substrate utilized the
15 minutes with 50 mM Tris-HCl buffer, pH 7.4 and 5mM MgCl₂. The reaction was then initiated by the addition of FDP (2.5mM).

The blank contained all the reagents except FDP.

iv. Aldolase

(a) FDP aldolase

The FDP aldolase activity was estimated according to the following reactions:

\[
\begin{align*}
\text{FDP} & \xrightarrow{\text{Aldolase}} \text{GAP} \\
\text{GAP} & \xrightarrow{\text{TPX}} \text{DIPF} \\
\text{DIPF} & \xrightarrow{\text{NADH₂}} \text{Glycerol-P} \\
\text{Glycerol-P} & \xrightarrow{\text{NAD⁺}} \text{Glycerol-FDH}
\end{align*}
\]

The assay system contained 50mM triethanolamine-HCl buffer, pH 7.4, 5mM EDTA, 0.15mM NADH₂, α-glycerophosphate dehydrogenase-triosephosphate isomerase mixture containing 0.8 and 1.8U respectively, 5mM FDP and supernatant equivalent to approximately 1-10mg tissue.

The activity of this enzyme was estimated against a blank that contained all the above reagents except FDP. The reaction was initiated by the addition of supernatant to both the test and blank cuvettes. The FDP was checked for triosephosphate impurities which could give falsely high results. This was done by using the above assay system without supernatant.

As 2 moles NADH₂ were utilized per mole of substrate utilized the
measured activity was divided by two.

(b) FIP aldolase

The reactions involved in the FIP aldolase assay system were as follows:

\[ \text{FIP} \rightarrow \text{Aldolase} \rightarrow \text{Glyceraldehyde \rightarrow \text{DHAP}} \rightarrow \text{HAD} \rightarrow \text{Glycerol-P} \rightarrow \text{HAD}^+ \]

The assay mixture consisted of 50mM triethanolamine-HCl buffer, pH 7.4, 5mM EDTA, 0.15mM NADH, 20 μg glyceraldehyde phosphate dehydrogenase, 1.5mM FIP and supernatant equivalent to approximately 2mg tissue.

The reaction was initiated by the addition of supernatant to both the blank and test cuvettes. The blank contained all the reagents except FIP.

v. Glucose-6-phosphate dehydrogenase (G6PDH)

The glucose-6-phosphate dehydrogenase activity was estimated directly as shown:

\[ \text{G6P} \rightarrow \text{HADP}^+ \rightarrow \text{G6PDH} \rightarrow 6 \text{P-gluconolactone} \rightarrow \text{HADPH}_2 \]

The assay system contained 50mM triethanolamine-HCl buffer, pH 7.4, 5mM EDTA, 10mM MgCl₂, 0.35mM NADP⁺, 5mM G6P and supernatant equivalent to
approximately 5-10 mg tissue.

The reaction was started by the addition of G6P. The blank consisted everything except G6P.

vi. Glucokinase and Hexokinase (GK and HK)

The activities of these enzymes were estimated according to the following reactions:

\[
\begin{align*}
\text{ATP} & \rightarrow \text{G}6\text{P} \\
\text{G}6\text{P} & \rightarrow \text{ADP} \\
\text{ADP} & \rightarrow \text{ATP} \\
\text{GK or HK} & \rightarrow \text{G}6\text{P} \\
\end{align*}
\]

The assay mixture consisted of 50mM triethanolamine-HCl buffer, pH 7.5, 5mM EDTA, 10mM MgCl₂, 0.25mM NAD⁺, 2.8mM ATP, 100 glucose-6-phosphate dehydrogenase, glucose (30mM glucose for the glucokinase assay and 1.5mM glucose for the hexokinase assay) and supernatant equivalent to 5-10 mg tissue.

The reaction was started by the addition of ATP to the test cuvette only. The glucokinase activity was determined by subtracting the activity obtained in the presence of low levels of glucose (1.5mM) from that obtained with the high glucose levels (300mM).

vii. Fructose diphosphatase (FDPase)

The FDPase activity was estimated according to the reactions shown schematically below:
The assay system contained 50 mM triethanolamine-HCl buffer, pH 7.4, 5 mM EDTA, 10 mM KCl, 0.3 mM NADP+, 1 U glucose-6-phosphate dehydrogenase, 1 U phosphoglucone isomerase, 0.3 mM F6P and supernatant equivalent to 2-10 mg tissue. The blank cuvette contained all the aforementioned reagents except FDP.

Because the fructose diphosphatase activity was found to increase with time for the first 10 minutes, (after which time the activity was found to be linear), the reaction was allowed to proceed for 10 minutes and only then was the activity recorded.

Any F6P present in the FDP preparation was estimated by setting up the assay system described above in the absence of supernatant.

viii. Phosphoglucone Isomerase

The phosphoglucone isomerase activity was estimated according to the following reactions:
The reaction mixture contained 50mM triethanolamine-HCl buffer, pH 7.4, 5mM EDTA, 10mM MgCl₂, 0.36mM NADP⁺, 400 units glucose-6-phosphate dehydrogenase, 0.25M F6P and supernatant equivalent to 1mg tissue.

As the F6P was found to be contaminated with G6P, all the reagents except the supernatant were added to the test cuvette. After all the G6P had been oxidised, supernatant was added to both the blank and test cuvettes. The linear rate then obtained represented the phosphoglucomutase activity. Only substrate (F6P) was omitted from the blank.

**Phosphoglycerate mutase**

The phosphoglycerate mutase activity was estimated spectrophotometrically at 230nm according to the following reactions:

\[ 3PGA \xrightarrow{\text{Phosphoglycerate mutase}} \text{2PGA} \xrightarrow{\text{Enolase}} \text{F6P} \]

The reaction mixture contained 50mM Tris-Cl buffer, pH 7.4, 2U enolase, 2.7mM 3PGA and supernatant equivalent to 2mg tissue.

Tris-Cl buffer, pH 7.4 was used instead of triethanolamine-HCl buffer, as the latter was found to have a high absorption at 230nm.

The blank contained all the reagents except 3PGA. The reaction was initiated by the addition of 3PGA.
x. α-Glycerolphosphate dehydrogenase

The α-glycerolphosphate dehydrogenase activity was estimated directly according to the reaction:

\[
\text{DHAP} \quad \rightarrow \quad \text{NADH}_2
\]
\[
\text{α-Glycerolphosphate dehydrogenase}
\]
\[
\text{Glycerolphosphate} \quad \rightarrow \quad \text{NAD}^+
\]

The following reagents were present in the reaction mixture: 50 mM triethanolamine-HCl buffer, pH 7.4, 5mM EDTA, 0.15mM NADH, 1.2mM DHAP and supernatant equivalent to 1-10mg tissue.

The blank contained all the reagents except DHAP. The reaction was initiated by the addition of supernatant to both the blank and test cuvettes.

The concentration of DHAP was estimated by assaying with 50mM triethanolamine-HCl buffer, pH 7.4, 5mM EDTA, 7U glycerol phosphate dehydrogenase and 0.15mM NADH2.

xi. Triosephosphate isomerase

The reactions involved in the triosephosphate isomerase reaction can be represented schematically as follows:

\[
\text{GAP} \quad \rightarrow \quad \text{Triosephosphate isomerase}
\]
\[
\text{DHAP} \quad \rightarrow \quad \text{NADH}_2
\]
\[
\text{Glycerol-1-phosphate dehydrogenase}
\]
\[
\text{Glycerol-P} \quad \rightarrow \quad \text{NAD}^+
\]
The triosephosphate isomerase activity was estimated by the addition of the following reagents to the test cuvettes: 30mM triethanolamine-HCl buffer, pH 7.4, 3mM EDTA, 0.15mM NADH, 7U glycerol-1-phosphate dehydrogenase, 0.6mM GAP and supernatant equivalent to 0.1mg tissue.

The blank contained everything except GAP. The reaction was initiated by the addition of supernatant to both the blank and test cuvettes, so that any triosephosphate isomerase contamination present in the glycerolphosphate dehydrogenase preparation would be detected before the addition of supernatant.

xii. Lactate dehydrogenase (LDH)

The lactate dehydrogenase activity was estimated directly according to the reaction:

\[
\text{Pyruvate} \xrightarrow{\text{Ldh}} \text{NADH}_2
\]
\[
\text{Lactate} \xrightarrow{\text{NAD}} \text{LDH}
\]

The activity of this enzyme was assayed in the presence of 30mM triethanolamine-HCl buffer, pH 7.4, 5mM EDTA, 2.5mM pyruvate, 0.15mM NADH, and supernatant equivalent to approximately 1mg tissue.

The reaction was started by the addition of pyruvate to the test cuvette only.

xiii. 3-Phosphoglycerate kinase

The reactions involved in the phosphoglycerate kinase assay system
can be represented schematically as follows:

\[
\begin{align*}
\text{ATP} & \quad \text{3PGA} \\
\text{Phosphoglycerate kinase} & \quad \text{ADP} \quad \text{1,3 di-RGA} \\
& \quad \text{NADH} \\
& \quad \text{GAPDH} \\
& \quad \text{P}^+ \quad \text{GAP} \quad \text{NAD}^+ \\
\end{align*}
\]

The reaction mixture contained 50mM triethanolamine-HCl buffer, pH 7.4, 5mM EDTA, 100mM MgCl₂, 0.15mM NADH₂, 4.3U glyceraldehyde-3-phosphate dehydrogenase, 2.5mM cysteine, 2.8mM 3PGA, 2.8mM ATP and supernatant equivalent to approximately 1mg tissue.

The blank cuvette contained everything except ATP. The reaction was begun by the addition of ATP to the test cuvette only.

xiv. Phosphoglucomutase

The phosphoglucomutase activity was estimated spectrophotometrically according to the following 2 reactions:

\[
\begin{align*}
\text{G6P} & \quad \text{phosphoglucomutase} \\
& \quad \text{G6P} \quad \text{NADP}^+ \\
& \quad \text{G6PDH} \\
& \quad \text{6 P-glucuronate} \quad \text{NADPH} \quad \\
\end{align*}
\]

The assay system contained 50mM triethanolamine-HCl buffer, pH
7.4, 6.2M EDTA, 10 mM KCl, 4 U glucose-6-phosphate dehydrogenase, 0.36 mM NADP⁺, 3 mM GIP and supernatant equivalent to approximately 1 mg tissue.

In order to obtain full activation of the enzyme by NAD⁺, the enzyme was preincubated in the cuvettes with all reagents except GIP. After 10 minutes, the reaction was initiated by the addition of GIP.

xv. Malate dehydrogenase

The malate dehydrogenase activity was estimated directly according to the following reaction:

\[
\text{oxaloacetate} \rightarrow \text{NADH}_2 \quad \text{malate dehydrogenase} \quad \text{malate} \rightarrow \text{NAD}^+
\]

The activity of this enzyme was determined by setting up the following assay system: 50 mM triethanolamine-HEPES buffer, pH 7.4, 10 mM NAD⁺, 0.15 mM NADH₂, 0.2 mM oxaloacetate and supernatant equivalent to approximately 1 mg tissue.

The reaction was started by adding oxaloacetate to the test cuvette only.

II. Results

The levels of different glucose metabolizing enzymes found in 36,000g supernatants of different samples of the same normal adult human liver are summarized in Table 2.1. It can be seen that some variation was obtained when the levels of these enzymes were estimated in different
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity in g/ml (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose kinase</td>
<td>0</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>138.3 ± 20.1</td>
</tr>
<tr>
<td>Phosphoglucone isomerase</td>
<td>40.6 ± 5.6</td>
</tr>
<tr>
<td>Fructose-1,6-diphosphatase</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>FDP-aldolase</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>FIP-aldolase</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Aldolase FDP/FLP</td>
<td>1.2 ± 0.1</td>
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<tr>
<td>Triosephosphate isomerase</td>
<td>600.0 ± 70.2</td>
</tr>
<tr>
<td>α-Glycerolphosphate dehydrogenase</td>
<td>22.7 ± 2.9</td>
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<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>123.5 ± 11.3</td>
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<tr>
<td>Phosphoglycerate kinase</td>
<td>70.2 ± 4.0</td>
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<tr>
<td>Phosphoglycerate mutase</td>
<td>15.3 ± 1.1</td>
</tr>
<tr>
<td>Enolase</td>
<td>15.4 ± 1.8</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>16.5 ± 2.7</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>180.6 ± 12.5</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>185.7 ± 6.4</td>
</tr>
</tbody>
</table>
samples of the same liver. The degree of variation differed for different enzymes.

Table 2.2 shows the results of a similar study where the activity of the glucose metabolizing enzymes was estimated in 36,000g supernatants of different samples of host and cancerous tissue of the same liver.

The results show some variation that again differed for different enzymes. The variations obtained in different samples of the same host and cancerous tissue were greater than those found in different samples of the same normal liver. In spite of this variation it can be seen that the phosphoglucomutase, fructose-1,6-diphosphatase, FBP-aldolase, triosephosphate isomerase, α-glycerolphosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase and malate dehydrogenase levels were statistically significantly decreased (P < 0.025) in supernatants of cancerous tissue when compared to those of their respective host tissue. On the other hand, the hexokinase, glucose-6-phosphate dehydrogenase and the pyruvate kinase levels, as well as the aldolase FBP/FIP activity ratio, were all statistically significantly elevated (P < 0.025) in supernatants of cancerous tissue. The phosphoglucomutase isomerase, phosphoglycerate kinase, phosphoglycerate mutase, enolase and lactate dehydrogenase levels showed no statistical difference between host and cancerous tissue in this particular hepatoma.

The levels of glucose metabolizing enzymes obtained in 36,000g supernatants of two different adult and foetal livers and in host and cancerous tissue of several hepatoma patients, are depicted graphically in Fig. 2.1. A solid line is used to join the activity of enzymes in a sample of cancerous tissue (C) with that of its corresponding host (H).
<table>
<thead>
<tr>
<th>Enzyme Name</th>
<th>Activity in U/g wet wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Host Mean ± S.E.</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>0</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>51.1 ± 3.0</td>
</tr>
<tr>
<td>Phosphoglucone isomerase</td>
<td>52.7 ± 13.7</td>
</tr>
<tr>
<td>Fructose-1,6-diphosphatase</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>FDP-aldolase</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>FIP-aldolase</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>Aldolase, FDP/FIP</td>
<td>2.5 ± 0.5</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>227.0 ± 11.0</td>
</tr>
<tr>
<td>α-Glycerophosphate dehydrogenase</td>
<td>6.3 ± 1.5</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>90.2 ± 6.2</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>74.2 ± 3.2</td>
</tr>
<tr>
<td>Phosphoglycerate mutase</td>
<td>13.1 ± 0.7</td>
</tr>
<tr>
<td>Enolase</td>
<td>13.6 ± 0.2</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>7.9 ± 0.5</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>119.5 ± 2.1</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>130.0 ± 19.8</td>
</tr>
</tbody>
</table>
Fig. 2.1. Levels of glucose metabolizing enzymes in 36,000g supernatants in extracts of host (H) and cancerous (C) tissues of primary human hepatomas and of normal (N) and fetal (F) livers.
From Fig. 2.1, it is seen that the phosphoglucomutase, fructose-1,6-diphosphatase, FDP-aldolase, α-glycerolphosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase levels were always lower in extracts of cancerous tissue as compared to those from their respective host tissues. Except for one case, all the triosephosphate isomerase levels were either the same or lower in hepatomas when compared to their respective host tissues. The aldolase FDP/FIP activity ratio was considerably elevated in hepatomas due to the lower activity with FIP. Pyruvate kinase levels were raised to some extent in eight out of nine hepatomas as compared to their respective host tissue types. The remaining glucose metabolizing enzymes viz. hexokinase, glucose-6-phosphate dehydrogenase, phosphoglucone isomerase, FDP-aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase and enolase showed no consistent level differences between cancerous and host tissue.

Although the levels of phosphoglucomutase, fructose-1,6-diphosphatase, FDP-aldolase and α-glycerolphosphate dehydrogenase in fetal livers were lower than those of normal adult livers, they were in general not as low as those found in cancerous tissue.

The statistical evaluation of the data obtained, when using the Student's "t" test and comparing the enzyme activities obtained in all the host and cancerous tissues of different hepatomas, verified that most of the differences observed were statistically significant (Table 2.3).

The decreased phosphoglucomutase, fructose-1,6-diphosphatase, FDP-aldolase, triosephosphate isomerase, α-glycerolphosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, lactate dehydrogenase and malate dehydrogenase activities found in supernatants of
TABLE 2.3: A COMPARISON OF THE ENZYME LEVELS IN HEPATOCELULAR AND CANCEROUS TISSUES OF SEVERAL HUMAN HEPATOMA PATIENTS

(Numbers in parentheses indicate number of patients)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity in U/g wet wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Host Mean ± S.S.</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>0</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>1.1 ± 0.1(9)</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>2.1 ± 0.8(9)</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>18.9 ± 9.7(8)</td>
</tr>
<tr>
<td>Phosphoglucone isomerase</td>
<td>73.9 ± 7.6(9)</td>
</tr>
<tr>
<td>Fructose-1,6-diphosphatase</td>
<td>3.3 ± 0.5(9)</td>
</tr>
<tr>
<td>FDP-aldehyde</td>
<td>2.5 ± 0.3(9)</td>
</tr>
<tr>
<td>FDP-aldehyde</td>
<td>1.8 ± 0.3(9)</td>
</tr>
<tr>
<td>Aldolase FDP/FIP</td>
<td>1.7 ± 0.2(9)</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>638.7 ± 11.6(7)</td>
</tr>
<tr>
<td>α-Glycerophosphate dehydrogenase</td>
<td>10.8 ± 2.8(8)</td>
</tr>
<tr>
<td>Glyceroldehyde-3-phosphate dehydrogenase</td>
<td>125.6 ± 11.8(9)</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>105.2 ± 11.8(8)</td>
</tr>
<tr>
<td>Phosphoglycerate mutase</td>
<td>14.1 ± 1.6(9)</td>
</tr>
<tr>
<td>Enolase</td>
<td>14.1 ± 1.2(9)</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>36.1 ± 3.5(9)</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>153.9 ± 19.5(9)</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>155.0 ± 10.4(8)</td>
</tr>
</tbody>
</table>
cancerous tissues were highly significant (P < 0.01). Although the elevated FDP/F6P activity ratio of cancerous tissue was also statistically significant, the elevated pyruvate kinase activities found in cancerous tissue were not statistically significantly different from host tissue when all the results were pooled. No statistically significant difference was found either between the hexokinase, glucose-6-phosphate dehydrogenase, phosphoglucone isomerase, FDP-aldolase and enolase levels of host and cancerous tissues.

Table 2.4 shows the statistical evaluation of the data when expressing the activities obtained in 36,000×g supernatants of cancerous tissue as a percentage of those obtained with their corresponding host tissue. The results thus obtained were very similar to those in Table 2.3 in that the lower fructose-1,6-diphosphatase, F6P-aldolase, triosephosphate isomerase, α-glycerolphosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase activities of cancerous tissue were highly statistically significantly different from those of host tissue. The elevated FDP/F6P aldolase activity ratio was also statistically significant. However, it was found that in addition to these enzymes, the elevated pyruvate kinase and lower phosphoglycerate kinase levels of cancerous tissue were statistically significantly different as well. The hexokinase, glucose-6-phosphate dehydrogenase, phosphoglucone isomerase, FDP-aldolase and enolase levels of cancerous tissue were not statistically significantly different from those of host tissue.

No glucokinase activity was found in any of the samples tested. Furthermore, in some instances negative results were obtained when the activity assayed in the presence of low levels of glucose (1.5mM) was
TABLE 2.1: LEVELS OF GLUCOSE METABOLIZING ENZYMES IN CUTANEOUS TISSUE EXPRESSED AS A PERCENTAGE OF THOSE IN KIDNEY TISSUE

(Numbers in parentheses indicate number of patients)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity (Mean ± S.E.)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexokinase</td>
<td>113.7 ± 17.4(9)</td>
<td>NS</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>272 ± 92.4(9)</td>
<td>NS</td>
</tr>
<tr>
<td>Phosphoglucone isomerase</td>
<td>57.7 ± 15.6(9)</td>
<td>NS</td>
</tr>
<tr>
<td>Fructose-1,6-diphosphatase</td>
<td>24.2 ± 9.2(9)</td>
<td>0.005</td>
</tr>
<tr>
<td>FDP-aldolase</td>
<td>106 ± 21.6(9)</td>
<td>NS</td>
</tr>
<tr>
<td>FDP-alcoholase</td>
<td>36.5 ± 20.7(8)</td>
<td>0.01</td>
</tr>
<tr>
<td>Aldolase FDP/FDP</td>
<td>656 ± 392.7(9)</td>
<td>0.01</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>292.2 ± 9.3(7)</td>
<td>0.005</td>
</tr>
<tr>
<td>D-Glycerolphosphate dehydrogenase</td>
<td>15.6 ± 2.2(8)</td>
<td>0.005</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate</td>
<td>73.3 ± 9.9(9)</td>
<td>0.01</td>
</tr>
<tr>
<td>dehydrogenase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>71.9 ± 6.9(8)</td>
<td>0.005</td>
</tr>
<tr>
<td>Phosphoglycerate mutase</td>
<td>64.9 ± 12.6(9)</td>
<td>0.01</td>
</tr>
<tr>
<td>Enolase</td>
<td>99.4 ± 14.6(8)</td>
<td>NS</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>186.8 ± 28.4(9)</td>
<td>0.005</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>65.2 ± 11.1(9)</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>56.5 ± 8.3(8)</td>
<td>&lt;0.005</td>
</tr>
</tbody>
</table>
subtracted from the activity obtained in the presence of high glucose levels (300mM). The reason for this is discussed later.

III. Discussion

1. Methods

Most of the glucose metabolizing enzymes were estimated by using the assay methods described by Shunk and Boxer (1964). A few of these were modified.

The glucokinase activity was taken as the difference in activity obtained with 300mM and 1.5mM glucose, and not as the activity obtained in the presence of 2.6mM glucose as was done by Shunk and Boxer (1964). In the glucose level used by these authors is far below the Michaelis constant of glucokinase for glucose, which is 40mM to 200mM (Gonzalez et al., 1967, Sols, 1965 and Parry and Walker, 1966), the activity estimated by them is mainly hexokinase. For this reason, hexose-6P phosphotransferase activity estimated in this manner has been termed hexokinase in the present study even though these authors have recorded it as glucokinase. However, the hexokinase activity is comprised of three isoenzymes, one of which, hexokinase III, is inhibited by high glucose levels (Grossbard and Schinke, 1956). Hence, when the glucokinase activity is low or absent and hexokinase III is high, the total hexose-6P phosphotransferase activity estimated at 300mM glucose will be lower, owing to the inhibition of hexokinase III by glucose. Subtraction of the hexokinase activity from the total activity might then yield negative results, and may account for the negative results sometimes obtained in the present study.
The phosphoglyceromutase activity was estimated by following the PEP formation at 230 μm in the presence of enolase. With this assay method only the auxiliary enzyme was used whereas two would be required if the method of Shoen and Boxer (1964) were employed. These authors assayed the back reaction by coupling the 2PGi formed from the phosphoglycerate mutase reaction to ATP in the presence of phosphoglycerate kinase (Fig. 2.2). The 1,3-diphosphoglycerate thus formed was reduced in the presence of NADH and glyceraldehyde-3-phosphate dehydrogenase, and the NAD formed recorded as the change in optical density at 340 μm.

![Chemical diagram]

**Fig. 2.2. Reactions involved in the metabolism of 2PGi**

The Michaelis constant for 2PGi for enolase and phosphoglyceromutase have been found to be $7 \times 10^{-5} M$ and less than $2 \times 10^{-3} M$, respectively for rabbit muscle (Grisolia, 1962 and Hold and Barkov, 1961). If these values are any reflection of the Michaelis constants for these enzymes in human liver, then it can be seen that the NAD 2PGi used by these authors would be saturating for enolase but not for phosphoglycerate mutase. Thus and the finding that the phosphi-
Glycinate release and enolase activities are similar in liver would indicate that 2AG will readily be converted to FEP. As the pyruvate kinase and lactate dehydrogenase activities are high in liver, FEP can then react with the ADP formed in their reaction mixture to form pyruvate and ATP. The pyruvate thus formed from this reaction could then be reduced in the presence of NADH and lactate dehydrogenase to form lactate. As NADH oxidation is being estimated, this would yield higher results. Although these authors estimated the activity of this enzyme over a three minute period, during which time the amount of ATP that may have accumulated for these side reactions to proceed was probably low, it was felt the assay method used in the present study is less subject to error, for side reactions could be minimized.

Enolase was another enzyme that was estimated directly by measuring FEP formation at 230nm instead of using the method described by Shonk and Boxer (1961). With this method no auxiliary enzymes were required and side reactions could be minimized. However, with both the above methods only low levels of protein equivalent to approximately 2ug of supernatant could be used because of the high absorbance at 230nm.

In the phosphoglucone isomerase assay, the fructose-6-phosphate concentration was reduced to 0.75mM for it contained 5-6% glucose-6-phosphate. As the phosphoglucone isomerase activity was estimated by coupling the glucose-6-phosphate formed to NADP in the presence of glucose-6-phosphate dehydrogenase, most of the NADP would have been reduced if the 2AG fructose-6-phosphate suggested by Shonk and Boxer (1961) were used.
It was found that the phosphofructokinase assay method described by Shank and Borer (1964), and which is generally used for the estimation of this enzyme, gave a high blank reaction. The assay method involves the cleavage of FDP formed from the phosphofructokinase reaction by aldolase and the conversion of the triose phosphates thus formed in the presence of triosephosphate isomerase and 6-glyceraldehyde dehydrogenase and NADH<sub>2</sub>. Oxidation of NADH<sub>2</sub> is then estimated as the change of optical density at 340nm.

The high blank could be due to impurities in the fructose-6-phosphate or auxiliary enzymes, or to interfering reactions. Moreover, FDP was found both in the present study and by Spalter et al. (1965) to be a potent inhibitor of aldolase which makes initial velocity measurements of phosphofructokinase difficult.

The activities of all these enzymes were estimated at saturating substrate levels except for triosephosphate isomerase and phosphoglycerate mutase which were estimated at 0.6mM GAP and 3mM 3PGA respectively, as was done by Shank and Borer (1964). The activities were not expressed relative to glyceraldehyde-3-phosphate dehydrogenase as recommended by Shank et al. (1964), for the activity of this enzyme was found to be statistically significantly reduced in cancerous tissue when compared to host tissue. A particular enzyme can only be used as a reference if its level is fairly constant from sample to sample and does not vary between host and cancer. As no other enzyme showed sufficiently constant levels to be used as an alternative reference, the results were expressed as units/g wet weight only.
II. Enzyme Levels

The activities of glucose metabolizing enzymes were estimated in two human adult livers and compared to those obtained by Shunk et al. (1951 and 1966), and Schmidt and Schmidt (1960 and 1970) in Table 2.4. Even though the livers used in the present study were obtained from humans that may have been of a different age and sex, or on a different diet and may have died at different pathological conditions, the results obtained still agreed closely with those of Shunk et al. (1951 and 1966), and Schmidt and Schmidt (1960 and 1970), thus supporting the concept that a definite enzyme pattern exists that is representative of human liver.

Although the levels of most of the enzymes in Table 2.4 are comparable, a few do vary. For instance, the hexokinase levels obtained in the present study were similar to those obtained by Shunk et al. (1951) but lower than those obtained by Schmidt and Schmidt (1970). Also, the phosphoglucose isomerase and phosphoglycerate mutase activities were lower than the levels quoted by Shunk et al. (1951). The lower phosphoglycerate mutase activity could probably be due to methodology, as discussed above.

Some of the variations in enzyme levels obtained in the present study, when different samples of the same tissue were used could be attributed to differences in cellularity or different distribution of enzymes. Such variations have been reported for different lobes of the same liver, as well as for different sampling sites (Schmidt and Schmidt, 1970 and Morrison, 1967). The higher variation obtained with samples of host tissue can perhaps be attributed to the host
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Adult 1 activity in U/g, net wt.</th>
<th>Adult 2 activity in U/g, net wt.</th>
<th>Schmidt and Schmidt 1960 and 1970 activity in U/g, net wt. (mean ± S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADPHS</td>
<td>65.5</td>
<td>600.0</td>
<td>614.0 ± 65.0</td>
</tr>
<tr>
<td>1-Glycerolphosphate dehydrogenase</td>
<td>13.5</td>
<td>22.7</td>
<td>15.3 ± 4.3</td>
</tr>
<tr>
<td>Glyceraldehyde phosphate dehydrogenase</td>
<td>65.2</td>
<td>123.7</td>
<td>61.5 ± 13.6</td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>53.2</td>
<td>70.3</td>
<td>55.0 ± 8.2</td>
</tr>
<tr>
<td>Phosphoglycerate mutase</td>
<td>6.2</td>
<td>15.3</td>
<td>27.0 ± 7.1</td>
</tr>
<tr>
<td>Enolase</td>
<td>21.3</td>
<td>16.7</td>
<td>21.0 ± 4.0</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>12.3</td>
<td>16.6</td>
<td>18.2 ± 3.0</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>79.6</td>
<td>180.5</td>
<td>133.0 ± 50.0</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>64.3</td>
<td>185.7</td>
<td>151.9 ± 44.0</td>
</tr>
</tbody>
</table>
tissue being under the influence of the cancer. Furthermore, if the stage of the cancer differs in different parts of the liver, then different sampling sites of cancerous tissue will also give different results.

iii. Detailed discussion of individual enzyme levels

From the results, it is seen that glucokinase, the glucose-ATP phosphotransferase with a high Michaelis constant for glucose, was absent not only from cancerous and host tissue of these primary human hepatomas, but from human adult and foetal livers as well. The failure to detect this enzyme in normal livers agrees with the results of Boxer and Shonk (1965) and Laurias and Cahill (1966).

From et al. (1967) however, reported the presence of this enzyme in livers of well-nourished humans, but found it to be low or absent from livers of poorly-nourished patients. As in the present study, this enzyme has also been reported to be absent from rat foetal livers and fast-growing rat hepatomas (Mäinhouse, 1965, Shatton et al., 1969 and Walker, 1965).

In the absence of glucokinase, the phosphorylation of glucose may be affected by hexokinase, which has a much lower Michaelis constant for glucose. The activity of this enzyme was higher in human foetal livers as compared to that of adult liver. However, no significant differences were found between the hexokinase levels of normal adult liver, host and cancerous tissue. Similarly, Boxer and Shonk (1965) found no differences in the activity of hexokinase in normal human livers and hepatomas. In transplantable rat hepatomas
on the other hand, the activity of this enzyme has been found to increase with increasing growth rate (Heinhouse, 1966, Shatton et al., 1969 and Shonk et al., 1965). As the hexokinase activity is compounded of a number of isoenzymes (Grossbard and Schimke, 1966), one cannot be certain that these are all being assayed for under optimal conditions. Furthermore, although no level differences were found in the activity of this enzyme in different tissue types tested, the proportion of these isoenzymes may have altered. That this is indeed the case is shown in Chapter III.

Glucose-6-phosphate dehydrogenase, which catalyzes the oxidation of glucose-6-phosphate to 6-phosphogluconolactone, was found to have a higher activity in extracts of foetal livers than in normal adult livers. Except for one level measured, the glucose-6-phosphate dehydrogenase activity was found to be higher in both host and cancerous tissues as compared to that of normal adult liver. The activity of this enzyme was also elevated in six out of the nine hepatomas tested relative to their respective host tissues. However, when the results obtained with host and cancerous tissue were pooled and subjected to statistical analysis, this deviation was not found to be significant. Boxer and Shonk (1966) found the activity of this enzyme to be considerably lower than that of normal liver in four out of ten human hepatomas and to be elevated in only three. The activity of this enzyme is generally elevated in rat hepatomas (Weber and Morris, 1963 and Shonk et al., 1965). As this enzyme is the first enzyme of the pentose phosphate pathway, raised levels of this enzyme could enhance the activity of the pentose phosphate pathway and lead to increased NADPH and pentose synthesis.
On the other hand, phosphoglucomutase, which catalyzes the conversion of glucose-6-phosphate to glucose-1-phosphate, was found to have a lower activity in extracts of foetal livers as compared to normal adult livers. The phosphoglucomutase levels were also found to be consistently lower in cancereous tissue as compared to those of their respective host tissues. These level differences between host and cancereous tissues were found to be highly significant ($P < 0.005$). Bomar and Shoup (1966) also found the activity of this enzyme to be lower in human hepatomas as compared to normal liver. The activity of this enzyme has also been found to be reduced in fast-growing rat hepatomas (Shoup et al., 1965 and Weber and Morre, 1953). Weber (1966) postulated that low levels of phosphoglucomutase could reflect a decrease of glycojen synthesis in tumour cells. However, glycojen deposition is also regulated by the activities of glycojen synthetase and glycojen phosphorylase, two enzymes which exist in two forms and whose activity is controlled by other regulatory enzymes and factors (Helzer, 1969 and Helzreich, 1969). Estimation of the levels of these enzymes, as well as the determination of their kinetic parameters could give a better insight into the control of glycojen synthesis.

No consistent differences between the different tissue types tested were found in the levels of the bifunctional enzyme phosphoglucoisomerase. Weber (1966) has also indicated that the activity of this enzyme does not alter in rat hepatomas.

The levels of the key glucosonicic enzyme, fructose-1,6-dihydro-
phosphatase were however found to be lower in extract of foetal than in normal adult livers. The levels of this enzyme were also found to be
consistently lower in cancerous tissue than those of their respective host tissues. When the results obtained with the different host and cancerous tissues were pooled, the differences found between host and cancerous tissue were highly significant (P < 0.005). These findings are in agreement with those of Boxer and Shonk (1966) who found the activity of this enzyme to be considerably reduced in the ten human hepatomas that they tested. Similarly the activity of this enzyme has been shown to decrease in rat hepatomas (Naber and Cantero, 1959, Shonk et al., 1965 and Boxer and Shonk, 1966).

As this is one of the key regulatory gluconeogenic enzymes, decrease in the activity of this enzyme could lead to decreased gluconeogenesis in these hepatomas as suggested by Naber (1966).

Although the FDP-aldolase levels were not significantly different in host and cancerous tissue, the activity of aldolase with FDP was consistently and statistically significantly reduced in cancerous tissue, giving high FDP/FIP aldolase activity ratios. These ratios agree with the findings of Balinsky and Bersohn (1967) and Schapira et al. (1963) in human hepatomas, as well as with the findings in transplantable rat hepatomas (Sugimura et al., 1966 and Katsushima, 1969). As pointed out in the introduction (page 17), two forms of aldolase exist, a muscle aldolase A, that hydrolyses FDP at a faster rate than FIP, giving an FDP/FIP activity ratio of over 50 and a liver aldolase B, which acts on both these substrates at the same rate giving activity ratios of 1 when these are being assayed under specific in vitro conditions. The finding of FDP/FIP aldolase activity ratios of 1 to 3 in host tissue, and between 2 and 11 in cancerous tissue would suggest that, in addition to liver aldolase B, muscle aldolase A is also present in host
tissue and may predominate in carcinomous tissue. These results could also indicate a gradual repression and derepression of genes in carcinogenesis rather than a deletion of genes. If genes were deleted only extreme FDP/F6P aldolase activity ratios of 1 and 50 would have been obtained. From the present results, it would appear that the gene responsible for the production of aldolase B is being suppressed, whilst that for muscle aldolase A is being derepressed as suggested by Tapia et al. (1963) and Matsushime et al. (1968). The FDP/F6P aldolase activity ratios were only slightly higher in extracts of human foetal liver as compared to those of human adult. These were however not as high as those reported by Schapira et al. (1963).

No consistent differences were found in the triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and phosphoglycerate mutase activities of host and carcinomous tissue. On the other hand, the α-glycerolphosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase levels were found to be consistently lower in carcinomous tissue as compared to their respective host tissue. However, when the results obtained with different host and carcinomous tissues were pooled and subjected to statistical analysis, the activities of all these enzymes were found to be significantly reduced in carcinomous tissue. A comparison of these enzyme levels in extracts of human adult and foetal livers, showed only the α-glycerolphosphate dehydrogenase and phosphoglycerate mutase levels to be lower in foetal livers. Of all these enzymes, only α-glycerolphosphate dehydrogenase and malate dehydrogenase levels were found to be lower in transplantable rat hepatomas.
(Shonk et al., 1965 and Weber, 1966). In humans, Baxter and Shonk (1965) found only the α-glycerolphosphate dehydrogenase levels to be lower in the ten primary cancers that they tested. One can speculate on the physiological significance of the low levels of these enzymes. Weber (1966) proposed that the low α-glycerolphosphate dehydrogenase activity could reflect decreased lipid synthesis.

In the case of glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, lactate dehydrogenase and malate dehydrogenase, one can argue that the levels are so high that it is doubtful whether the lower levels in cancerous tissue would affect the metabolic flux.

No consistent differences were found in the endonase levels of the different tissue types tested. Other workers have also found no differences in the levels of this enzyme in rat or human hepatomas (Baxter and Shonk, 1966, Weber, 1966 and Shonk et al., 1965).

No consistent differences were either found in the pyruvate kinase levels of fetal and adult livers. The pyruvate kinase activity was however found to be elevated in eight out of nine hepatomas, when host tissue was compared to its respective cancerous tissue. There was no statistically significant difference in levels when the activity was expressed as U/g wet wt, but the difference was significant when the activity was taken as the percentage change from host tissue. This can probably be attributed to the high variation in levels of host and of cancerous tissue of different hepatomas. Such variation can probably be expected, particularly if these enzymes, like those of normal liver, are still subject to dietary and hormonal regulation (Tanaka et al., 1967a, Sida et al., 1966 and Fitch and
Chaikoff, 1960). Boxer and Shunk (1966) found the activity of this enzyme to be enhanced in only three out of ten hepatomas. However, the levels, when elevated, were much higher than those found in the present study. Farina et al. (1966) have shown that the activity of this enzyme increases with growth rate in transplantable rat hepatomas. The increased activity of this enzyme, together with the increased phosphofructokinase and hexokinase levels have been implicated as reflecting increased glycolysis (Waber, 1966).

The differences found in the enzyme levels of these hepatomas show that their metabolic pattern is altered as compared to the host tissue. However, the enzyme pattern of these hepatomas differed in many respects from those of Boxer and Shunk (1966) and showed somewhat lesser variation. This could indicate that the primary human hepatomas tested in the present study were probably different to those tested by Boxer and Shunk (1966). This could partly be due to differences in aetiology of these cancers. Whereas all the human hepatomas tested in the present study were obtained from patients coming from one area (Nogentbique), those obtained by Boxer and Shunk came from several countries not including any African ones.

The enzyme level changes differed from those of transplantable rat hepatomas in several ways. The key regulatory enzymes of those human hepatomas did not all change dramatically as do those of fast-growing rat hepatomas. They were also unlike those of slow-growing transplantable rat hepatomas, which show virtually no change. These findings would suggest that the alteration in the enzyme pattern of these primary human hepatomas, like those reported by Boxer and Shunk (1966) cannot be equated to those of either fast- or slow-growing rat.
A comparison of the enzyme levels of normal adult human and fetal liver shows that the glucose-6-phosphate dehydrogenase and FDP/FAR aldolase activity ratios were elevated, whereas the 1,4-glycerolphosphate dehydrogenase and phosphoglucomutase activities were lower in 5 months old human fetal livers than in adult livers. As these enzyme level changes resemble those found in human hepatomas, one could speculate that the metabolic pattern of these human hepatomas partly resemble that of fetal livers. This could indicate that the metabolism of these hepatomas may revert to a more immature stage of development. However, the metabolic changes found in these hepatomas could also be attributed to their more anaerobic environment due to impaired blood supply.

Changes were found in the levels of key regulatory enzymes such as pyruvate kinase and fructose diphosphatase which could indicate enhanced glycolysis and decreased gluconeogenesis in these hepatomas. However, it was found that in addition to these regulatory enzymes, which catalyze unidirectional reactions, the levels of bifunctional enzymes, such as phosphoglucomutase, 1,4-glycerolphosphate dehydrogenase, triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase, phosphoglycerate kinase, malate dehydrogenase and lactate dehydrogenase were also all significantly lower in hepatomas. This would suggest that these too might affect the fluxes of the various pathways in which they occur. As the activities of these enzymes are being estimated under optimal in vitro conditions and as the direction of the reaction catalyzed by the bifunctional enzymes in
vivo will depend on a large number of factors such as the relative concentrations of substrates, cofactors, inhibitors and activators, it is difficult to assess how these would affect the glycolytic/glucosogenic fluxes or other pathways in which they occur. It is thus evident that these enzyme level changes only give an indication of metabolic changes occurring in these hepatomas.

As discussed in Chapter III, the isoenzyme pattern of some of these enzymes is also altered. Moreover, pyruvate kinase, which was studied in more detail (Chapter IV) was found to have altered properties in hepatoma as compared to normal liver. Hence the measurements of enzyme activities under one set of conditions in vitro gives only part of the answer to the possible in vivo activities.
CHAPTER III

ELECTROPHORETIC PATTERNS OF GLUCOSE METABOLIZING ENZYMES

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CHAPTER III.

Electrophoretic patterns of glucose metabolizing enzymes

I. Materials and Methods

1. Materials

The tissues and most of the reagents used were the same as those described under Materials and Methods in Chapter II. Additional materials were:— Adenosine-5'-monophosphate (AMP) which was obtained as the sodium salt from Sigma; 2-p-iodophenyl)-3(p nitrophenyl)-5-phenyl-tetrazolium salt (INT) from the National Biochem. Corp., Cleveland, Ohio; Phanexime methosulphate (PMS) from Sigma and agarose from the Saraves Lab. (Pty) Ltd. Connaught hydrolysed starch was obtained from the Connaught Medical research laboratory, Toronto, Canada.

2. Methods

The electrophoretic techniques employed for the isoenzyme separation of some of the glucose metabolizing enzymes present in samples of normal adult livers and hepatomas were similar to those described by Smith (1965). Some were modified as described below:

(a) Starch gel electrophoresis

2. Preparation of gels and horizontal electrophoresis

19g Connaught hydrolysed starch was suspended in 190ml gel buffer (see page 85) in a round bottomed glass flask. The suspension was heated, while constantly being swirled, over a low flame. It was allowed to boil until it became semi-transparent, was then degassed and poured into a mould that had been made with a medium sized (18.5 x 10.2 x 0.65 cm³) Shandon
frame.

The gel was covered and left to set overnight, or if urgently required it was cooled at 4°C for 1 to 2 hours. Slits were cut 6.5 cm from the end of the gel. Three strips of Whatman No.1 chromatography paper (9 x 5 cm) were used as application slips. These were dipped into the various enzyme preparations and placed into each slot. (For details of the procedure used for the extraction of the enzymes from the different tissues, see Methods, Chapter II). A minute amount of bromophenol blue was also dabbed on the side of the gel (in the same position as the slits) to serve as a marker. Finally, the samples were electrophoresed for a constant time interval, at constant anode, in a horizontal Shandon tank that had been filled with the required electrode buffer. Three folds of Whatman 3MM paper were used as wicks.

After the samples had been electrophoresed the application slips were removed and the starch sliced with a Shandon Slicer into four layers. The starch layers were separated by allowing the cut layer to adhere to a polythene film. These were then stained.

b. Staining and Fixation of gels

Since reduced tetrazolium salt forms an insoluble red precipitate, the position of the enzyme could be detected by coupling the reduced products of the enzymic reaction to PMS and INT as follows:-

```
Substrate Red
NADP⁺ HAD
→ PM Red
NAD
Substrate Oxid
NADH NAD⁺₂
→ PM Oxid
        INT
Insoluble red precipitate.
```
To minimize the quantity of reagents used, the stains were prepared in agarose, unless otherwise stated under the specific enzyme stain section. The agarose (0.85%) was solubilized by suspendng it in 75% of the required final volume of stain buffer and autoclaving it in a pressure cooker until the pressure reached 51b/cm². When the clear agarose solution had cooled down to 45°C, it was quickly mixed with the staining mixture and poured over the gel. The staining mixture consisted of the remaining 25% of the staining buffer to which PHS, INT, substrates and auxiliary enzymes had been added. The gel was then incubated, in the dark at room temperature, until the stained isoenzyme bands reached the required intensity. The gels were then fixed in 10% acetic acid and photographed with and without the agarose.

c. Specific enzyme stains

1. Glucokinase and Hexokinase

These isoenzymes could be detected with the following reaction system:

\[
\begin{align*}
\text{ATP} & \quad \text{glucose} \quad \text{MgCl}_2 \quad \text{G6PDH} \quad \text{PMS oxid} \quad \text{INT oxid} \\
\text{CK or HK} & \quad \text{G6P} \quad \text{NADPH} \quad \text{PHS red} \quad \text{INT precipitate}
\end{align*}
\]

Different layers of the same starch gel were developed in stains that were similar to those described by Katzcn and Schinke (1965). These consisted of 0.1M Tris-HCl buffer, pH 8.0, 0.75M NADP⁺, 7.5mM MgCl₂, 2mM KCl, 7U glucose-6-phosphate dehydrogenase, 1mg PHS, 0.05g INT, 3mM ATP, 0.85% agarose and glucose (0.5mL glucose for the hexokinase stain, and 0.1M glucose for the glucomokinase stain) to a total volume of 25ml. Two blanks
were set up, the one contained all the reagents except ATP and the second contained all except glucose.

ii. Pyruvate kinase (PK) and adenylate kinase (AK)

The pyruvate kinase and adenylate kinase isoenzymes were detected by making use of the following reactions:

![Diagram of the reaction]

The stain for detecting pyruvate kinase consisted of 0.2M Tris-HCl buffer, pH 8.0, 20mM MgCl₂, 3mM F6P, 1.5mM ADP, 0.1mM NADP⁺, 7U glucose-6-phosphate dehydrogenase, 7U hexokinase, 10mM glucose, 1mg F6P, 0.05g INT, 12mM ATP (to inhibit the adenylate kinase reaction) and 0.85% agarose to a total volume of 25mL. The blank stain contained all the reagents except F6P.

As ATP may also be synthesized via the adenylate kinase reaction, a different layer of the same starch gel was stained for this enzyme. The reaction mixture was similar to that described above except that both F6P and ATP were omitted. By comparing the isoenzyme patterns obtained with these two stains, the positions of pyruvate kinase isoenzymes could be determined.

iii. Lactate dehydrogenase

This stain was similar to the one described by Rabcz (1969) and con-
sisted of 0.05M potassium phosphate buffer, pH 7.5, 70% sodium lactate, 0.1mM NAD⁺, 0.1g INT and 1mg RPS in a total volume of 150ml.

The blank stain contained all the above reagents except sodium lactate.

iv. α-glycerolphosphate dehydrogenase

The stain was prepared in agarose and consisted of 0.1M potassium phosphate-HCl buffer, pH 7.0, 0.1M γ-phosphate (mixture of α- and β-), 0.1mM NAD⁺, 0.05g INT, 1mg RPS and 0.85% agarose in a final volume of 25ml.

The blank stain contained all the above reagents except glycerophosphate.

v. Triosephosphate isomerase (TPI).

The triosephosphate isomerase isoenzymes could be detected with the following reaction system:

A modified stain consisted of 0.2M Tris-HCl buffer; pH 8.0; 0.1mM NAD⁺, DHAP, 0.01M sodium arsenate, 70γ-glyceraldehyde-3-phosphate dehydrogenase, 1mg RPS, 0.05g INT and 0.85% agarose in a final volume of 25ml.
The DAP was prepared by dissolving 50 mg of dihydroxyacetone phosphate dimethyl ketal-dicyclohexylammonium salt in 2 ml H₂O. To this, 0.1 ml Dowex-1 resin in the H⁺ form was added. The solution was filtered, made up to 5 ml and incubated in a water-bath for 6 hours at 40°C.

vi. Halate dehydrogenase

The staining mixture consisted of 0.2M Tris-HCl buffer, pH 8.0, 0.1M NaN⁺, 1M malate, pH 7.0, Log PAS, 0.05g LIT and 0.85% agarose in a final volume of 25 ml.

II. Results

The electrode and gel buffers that gave the best results for the different enzymes tested, as well as the time, amperage and homogenate concentrations required, are summarized in Table 3.1.

Starch gel electrophoresis carried out under the above mentioned specified conditions, and developed in different stains gave the isoenzyme patterns shown in Figs. 3.1 - 3.7.

1. Glucokinase and Hexokinase

Figs. 3.1a and 3.1b illustrate the hexokinase isoenzyme patterns obtained with 35,000-g supernatants of normal adult liver, test and cancerous tissue when developed in the presence of either high (0.1M) or low (0.5mM) levels of glucose. From these figures, it is seen that only isoenzymes that migrate towards the anode were found. These have been designated types I, II and III in order of increasing mobility according to the nomenclature of Katzen and Schirme (1965).

Type I hexokinase appears to be the most active of the three iso-
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Gel buffer</th>
<th>Electrode buffer</th>
<th>Time (hrs.) electrophoresed</th>
<th>Current at 6°C</th>
<th>Homogenate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose kinase and Hexokinase</td>
<td>0.02M barbital pH 8.4, containing 2.5M EDTA and 0.1M DTT (1)</td>
<td>0.02M barbital pH 8.4, containing 2.5M EDTA and 0.1M DTT (1)</td>
<td>17</td>
<td>20 mamps</td>
<td>1:1</td>
</tr>
<tr>
<td>Pyruvate kinase and Adenylate kinase (2)</td>
<td>5mM DL-histidine adjusted to pH 7 with NaOH (2)</td>
<td>0.1M Citrate adjusted to pH 7.0 with NaOH (2)</td>
<td>2</td>
<td>20 mamps</td>
<td>1:3</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>0.076 M Tris-0.005M Citrate, pH 8.65</td>
<td>0.1M borate-NaOH, pH 8</td>
<td>3/4</td>
<td>20 mamps</td>
<td>1:10</td>
</tr>
<tr>
<td>Pyruvate phosphate dehydrogenase</td>
<td>0.076M Tris-0.005M Citrate, pH 8.65.</td>
<td>0.1M borate-NaOH, pH 8</td>
<td>3</td>
<td>20 mamps</td>
<td>1:3</td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>0.076M Tris-0.005M Citrate, pH 8.65.</td>
<td>0.1M borate-NaOH, pH 8</td>
<td>3</td>
<td>20 mamps</td>
<td>1:3</td>
</tr>
<tr>
<td>NADH</td>
<td>5mM DL-histidine adjusted to pH 7 with NaOH.</td>
<td>0.1M Citrate adjusted to pH 7 with NaOH.</td>
<td>17</td>
<td>3 mamps</td>
<td>1:1</td>
</tr>
</tbody>
</table>

(1) Katzen and Schinke (1965)

(2) Fielden and Harris (1966)
Fig. 3.1a. Starch gel electrophoresis of 36,000g. supernatants of adult human liver (N) and different host (AH, FH and TH) and cancerous tissue (AC, FC and TC) when developed in 0.1M glucose for glucokinase and hexokinase activity.

Fig. 3.1b. Starch gel electrophoresis of 36,000g. supernatants of adult human liver (N) and different host (AH, FH and TH) and cancerous tissue (AC, FC and TC) when developed in 0.5M glucose for hexokinase activity.
enzymes, and was found to be present in all the tissue types tested. Type II hexokinase was absent from supernatants of normal adult liver and if present in samples of host tissue, it stained very faintly when the electropherogram was developed in the different stains. On the other hand, type II hexokinase, was quite distinct in supernatants of cancerous tissue. Furthermore, from Figs. 3.1a and 3.1b, it can be seen that as the activity of hexokinase II becomes more pronounced so type III hexokinase decreases.

The electropherograms that were developed in 0.1M glucose and 0.5mM glucose were very similar, the only difference being that type III hexokinase (when present) stained slightly darker in the starch gel developed in 0.5mM glucose, presumably due to inhibition by high glucose levels. This similarity is due to the fact that the furthest anodic isoenzyme, type IV (glucokinase), which is reputed to have a high Michaelis constant for glucose (Katzman and Schimke, 1965), was found to be absent from supernatants of all the different tissue types tested (Fig. 3.1a).

Type V hexokinase, which migrates towards the cathode (Brown et al., 1967), was also found to be absent from the different supernatants tested.

No bands could be detected when the starch gels were developed in the blank stains.

ii. Pyruvate kinase and adenylate kinase

Fig. 3.2a illustrates the typical pyruvate kinase isoenzyme patterns obtained with 35,000g supernatants of normal adult liver, host and cancerous tissues.

When the starch gels were developed in the pyruvate kinase stain,
Fig. 3.2a. Pyruvate kinase electropherogram of 36,000g.
supernatants of normal adult human liver (N), and
various host (AH, FH and TH) and cancerous
tissue (AC, FC and TC).

Fig. 3.2b. Adenylate kinase electropherogram of 36,000g.
supernatants of normal adult liver (N) and
various host (AH, FH and TH) and cancerous
tissue (AC, FC and TC).
one cathodal and two anodal isoenzymes were obtained (Fig. 3.2a). Of the two anodal isoenzymes, only the one nearer the origin represents the pyruvate kinase isoenzyme. The further one corresponds to adenylyl kinase (Fig. 3.2b). This indicates that AMP does not inhibit adenylyl kinase completely.

The anodal and cathodal pyruvate kinase isoenzymes have been designated types L and M respectively, according to the nomenclature of Tanaka et al. (1967a).

The activity of the two pyruvate kinase isoenzymes were found to vary in supernatants of different tissue types. From Fig. 3.2a, it is seen that the L isoenzyme was predominant in supernatants of normal adult liver. Faint traces of type M pyruvate kinase were found in other electropherograms. On the other hand, type M pyruvate kinase was found to predominate in supernatants of cancerous tissue whereas type L pyruvate kinase was faint or absent. The pyruvate kinase electropherograms of 36,000g supernatants of host tissue were generally very faint (Fig. 3.2a). The proportion of types M and L pyruvate kinase in extracts of this tissue were found to vary with different electropherograms. In most instances both types L and M pyruvate kinases were present displaying approximately equal activity. On some occasions, type L appeared to predominate over type M, whereas at others, type M pyruvate kinase was more active.

Starch gels developed in the absence of FEP but in the presence of AMP (blank) were similar to those developed in the presence of FEP, the only difference being that the bands of activity were slightly fainter. This is probably due to endogenous FEP being present, for this blank reaction was eliminated on purification of this enzyme in a later study.
The electropherograms developed in the adenylate kinase stain were generally very streaky (Fig. 3.2b), but the furthest anodal isoenzyme that was found to be present when the gels were stained for pyruvate kinase, was still visible.

iii. Lactate dehydrogenase

Fig. 3.3 illustrates the results obtained when 36,000g supernatants of normal adult liver and different host and cancerous tissues were electrophoresed and the gels stained for lactate dehydrogenase activity.

From Fig. 3.3, it is seen that all five isoenzymes were present in all the samples tested. Also, there appears to be no significant difference in the activity of these different isoenzymes in supernatants of cancerous tissue when they were compared to those found in their respective host tissue. Slight, but not consistent differences were sometimes obtained in the activity of these different isoenzymes with different supernatants.

When these starch gels were developed in the blank lactate dehydrogenase stain, from which lactate was omitted, all five isoenzymes again appeared. However, these were much fainter.

iv. α-Glycerolphosphate dehydrogenase

The starch gel electrophoresis results obtained with 36,000g supernatants of normal adult human liver, host and cancerous tissue, when developed in the α-glycerolphosphate dehydrogenase stain are illustrated in Fig. 3.4a.

From Fig. 3.4a, it is seen that only the furthest anodic isoenzyme
Fig. 3.3. Lactate dehydrogenase isoenzyme patterns obtained with 36,000g. supernatants of normal adult human liver (N) and different host (TH, FH and AH) and cancerous (TC, FC and AC) tissues.
Fig. 3.4a. Starch gel electrophoresis of 36,000g supernatants of human adult liver (H), host (AH, FH and TH) and cancerous tissue (AC, FC and TC) when using the α-glycerolphosphate dehydrogenase stain.

Fig. 3.4b. Starch gel electrophoresis of 36,000g supernatants of human adult liver (H), host (TH) and cancerous (TC) tissue when using the α-glycerolphosphate dehydrogenase stain.
of the electropherogram represents the α-glycerolphosphate dehydrogenase activity. This enzyme was found to stain most intensely in supernatants of host tissue. On the other hand, its activity appeared to be considerably reduced or absent from supernatants of cancerous tissue, as this enzyme stained very faintly or was absent from gels developed in the α-glycerolphosphate stain. Furthermore, from Fig. 3.1a, it is seen that the intensity of the stain in supernatants of tumour tissue was always considerably less than that obtained with their respective host tissue. This is to be expected, as shown by the results obtained in Chapter II when levels were assayed.

In addition to the aforementioned enzyme, four faint anodic bands as well as a cathodic one appeared when the gels were developed in the α-glycerolphosphate stain. These were also found when the gels were developed in the blank α-glycerolphosphate stain. As this stain is similar to the lactate dehydrogenase blank stain and since Falkenberg et al. (1969) have shown that lactate dehydrogenase can react with some impurities in the starch gel and NAD to give NADH, these bands are probably lactate dehydrogenase isoenzymes.

From Fig. 3.1b, it is seen that dialysis of the different supernatant fractions did not eliminate the blank lactate dehydrogenase reaction. The stains of these lactate dehydrogenase isoenzymes were more intense in this electropherogram, as the gel was left in the stain overnight after the agarose had been removed from the α-glycerolphosphate dehydrogenase section.

V. Triosephosphate isomerase

Fig. 3.5 illustrates the triosephosphate isomerase isoenzyme.
Fig. 3.5. Starch gel electrophoresis of 36,000g. supernatants of normal adult human liver (N) host (TH) and cancerous (TC) tissue when developed in the triosephosphate isomerase stain.

Fig. 3.6. Starch gel electrophoresis of 36,000g. supernatants of normal adult human liver (N) host (AH, FH and TH) and cancerous tissue (AC, FC and TC) when developed in the malate dehydrogenase stain.
pattern obtained with 36,000g supernatants of normal adult liver, host and cancerous tissue. From Fig. 3.5, it is seen that three anodic triosephosphate isomerase isoenzymes were obtained. These were similar in all the tissue types tested and were unaffected by dialysis.

vi. Malate dehydrogenase

The malate dehydrogenase isoenzyme pattern obtained with 36,000g supernatants of normal adult liver and different host and cancerous tissues are shown in Fig. 3.6. From Fig. 3.6, it is seen that one anodic and one cathodic isoenzyme was obtained with all the tissue types tested.

III. Discussion

Isoenzymes of hexokinase, pyruvate kinase, α-glycerolphosphate dehydrogenase, lactate dehydrogenase, triosephosphate isomerase and malate dehydrogenase in 36,000g supernatants of cancerous and host tissue and normal adult human liver were separated by starch gel electrophoresis. Of all these enzymes, only the two key glycolytic regulatory enzymes viz. hexokinase and pyruvate kinase, displayed marked differences in their isoenzyme pattern with the different tissue types tested.

Comparative studies of the glucokinase and hexokinase isoenzymes revealed the presence of only hexokinase I and III in normal adult human liver. In host tissue, hexokinase I, III and faint traces of II were found, whereas in cancerous tissue hexokinase I, II and in some instances faint traces of hexokinase III were obtained. Glucokinase was absent from all the tissue types tested.
The absence of glucokinase from all the tissues tested confirms the kinetic assays where the activity of this enzyme could not be detected. Furthermore, the finding that hexokinase III (when present) stains more intensely in electrophoerograms developed in 0.5M glucose than in those developed in the presence of 100M glucose indicates that this isoenzyme is inhibited by high levels of glucose. This would lend further support to the explanation given for the negative glucokinase results (see Discussion, Chapter II).

The absence of glucokinase and hexokinase II, as well as the finding of both hexokinase I and III in normal adult human livers is consistent with the findings of Brown et al. (1967). These authors found glucokinase to be absent from livers of poorly-nourished patients. They reported the presence of hexokinase I and III in all samples of human liver but could only detect hexokinase II occasionally. In rat and dog livers on the other hand, all three hexokinase isoenzymes as well as glucokinase have been found to be present (Brown et al., 1967, Shattuck et al., 1969 and Sato et al., 1969).

The absence of glucokinase and hexokinase III and the predominance of hexokinase II in tumorous tissues of these human hepatomas is similar to the results that have been reported for fast-growing rat hepatomas by Sato et al. (1965). Both the regulatory enzyme, glucokinase, as well as hexokinase III predominate in liver. These are replaced by hexokinase II, which predominates in rat fastal liver and in muscle (non-glimcoseogenic tissue). This could reflect a loss in the regulatory requirements of control of glycalysis and glucoseogenesis in hepatomas. As muscle hexokinase II has a higher Michaelis constant for glucose than that of liver hexokinase III (Crossbard and Schinke, 1966), it will
probably be better adapted to cope with the high blood glucose levels in hepatomas now that glucokinase, which has a high Michaelis constant for glucose, is decreased or absent. Furthermore, as it is not inhibited by high glucose levels as hexokinase II is, it will probably also ensure a constant utilization of glucose by these highly glycolysing tumour cells.

The finding that hexokinase III is more active than hexokinase II in host tissue, and that hexokinase II predominates in cancer tissue, whereas hexokinase III is low or absent from this tissue could indicate changes affected by repression and derepression of genes rather than deletion of genes. If genes were deleted one would find either one or the other of these isoenzymes and not both together at the same time as was found in the present study. These results could also indicate that the host tissue is under the influence or affected by the presence of the cancer (see Discussion, Chapter II).

The finding of a gradation of changes in the pyruvate kinase isoenzyme pattern from the predominance of type I over type II pyruvate kinase in normal liver, to a similar proportion of these isoenzymes in host tissue could again indicate changes affected by repression and derepression of genes. These results also reveal the replacement of the regulatory type I isoenzyme that predominates in gluconeogenic tissue for one that is found in non-gluconeogenic tissue. A similar change in the proportion of types I and II pyruvate kinases has been reported for rat hepatomas by Suda et al. (1966).

The finding that mainly type II pyruvate kinase persists in tumour tissue corresponds to the results reported for fast-growing rat hepatomas (Yurina et al., 1968). The M isoenzyme of these human hepatomas
was found to migrate similarly to the minor or type II pyruvate kinase. Using cellulose acetate electrophoresis in 10mM Tris-HEPES buffer, pH 7.5, Taylor et al., 1969 showed that the pyruvate kinase of 3924A rat hepatomas has a different electrophoretic mobility to that of muscle type II. Also, Oriss (1969) has reported the presence of a new isoenzyme in fast-growing rat hepatomas.

The finding of a single anodic α-glycerolphosphate dehydrogenase enzyme of different intensities in the different tissue types tested (Fig. 3,Ja) confirms the kinetic assays where the activity of this enzyme was found to be considerably reduced in tumour tissue. These data also indicate that the activity differences found between the normal, host and tumour enzymes are not due to isoenzyme differences. This is also indicated with the finding of no difference in the lactate dehydrogenase, triosephosphate isomerase and malate dehydrogenase isoenzymes in the different tissue types tested. However, electrophoretic studies under different conditions may still yield differences. Furthermore, electrophoresis of other glucose metabolizing enzymes particularly the key regulatory ones such as fructose-1,6-diphosphatase and phosphofructokinase may also yield interesting differences with these different tissue types.
CHAPTER IV

PYRUVATE KINASE

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CHAPTER IV
PYRUVATE KINASE

I. Materials and Methods

A. Partial purification

1. Materials

The tissues and reagents used were as described in Chapters II and III, Materials and Methods section with a few additions.

The ion exchangers, DEAE- and SE-Sephadex A-50 were obtained from Pharmacia, Uppsala whereas CM-cellulose and Phosphocellulose were obtained from the Whatman Company. These were all purified by the method described by Paterson and Sober (1962).

Sephadex G-200 and Sephadex G-25 were obtained from Pharmacia, Uppsala.

Calcium phosphate gel was prepared according to the method of Keilin and Hartree (1938). Aquacide was obtained from Calbiochem.

2. Methods

a. Assay method

Throughout the purification procedure and in all the kinetic studies pyruvate kinase was assayed spectrophotometrically using a Unicam SP 200 recording spectrophotometer with water at 30°C circulating through the cell housing. The enzyme was assayed by coupling the pyruvate formed to lactate dehydrogenase and following the oxidation of NAD at 340 nm.

The standard reaction mixture contained in a final volume of 1 ml,
30mM triethanolamine- HCl buffer, pH 7.4, 10mM MgCl₂, 5mM Na₂EDTA (pH 7.0), 0.1M KC1, 3mM PEP, 3mM ADP, 40 u lactate dehydrogenase, 0.15mM NADH and a suitable amount of the pyruvate kinase preparation.

One enzyme unit is defined as the amount of enzyme catalyzing the oxidation of 1 μmol of NADH per minute, assuming the molar extinction coefficient for NADH to be 6.22 x 10³ at 340μm (Horakcr and Kornberg, 1948). The specific activity is the activity per milligram of protein. The protein content was determined by the method of Warburg and Christian (1931).

b. Partial purification procedure

All the following purification procedures were initially carried out on normal adult human liver. The final purification procedure adopted was later adapted to hepatoma. All manipulations were performed at 4°C unless stated otherwise.

i. Extraction of the enzyme

Approximately 5-10g of liver were usually employed. The enzyme was extracted in 5 volumes of homogenizing medium as described previously (Chapter II - Methods and Materials).

The clear supernatant obtained, after homogenization and centrifugation, was designated the crude fraction and was used for the purification.

ii. Acid fractionation

The crude fraction was divided into three portions. The pH of these was adjusted to 5.2, 4.6 and 4.2 respectively with cold 5M acetic
acid. After centrifuging at 36,000g for 10 minutes at \(4^\circ\)C, the pH of these supernatants were adjusted to 7 with solid \(\text{Na}_2\text{CO}_3\) and centrifuged again at 36,000g for 10 minutes at \(4^\circ\)C. The pyruvate kinase activity was estimated in the clear supernatants.

Typical results for this purification step are shown in Table 4.1. A purification factor of 1.6 was obtained together with 96.5% yield when the pH had been adjusted to 5.2. On the other hand, when the pH had been adjusted to 4.6 or 4.2 no purification and yields of 39.4% and 12.3% respectively were obtained.

iii. First ammonium sulphate fractionation

To the crude fraction that was being stirred gently at \(4^\circ\)C, solid ammonium sulphate was slowly added until 25% saturation was reached. 70g/100ml was taken to be equivalent to 100% saturation at \(4^\circ\)C (Benson et al., 1969). The solution was stirred for a further 10 mins and centrifuged at \(4^\circ\)C in an MSE high speed 18 refrigerated centrifuge at 36,000g for 10 mins. The precipitate, which generally contained very little pyruvate kinase activity was discarded. Solid ammonium sulphate was added to the clear supernatant to 80% saturation and the precipitate collected as above. The resulting precipitate, which contained the enzyme, was re-dissolved in 0.025M cacodylate-NaCl buffer, pH 6.2, containing 0.5M sucrose, 2.5mM EDTA and 0.1M DTT and dialysed for 1 hr against this solution.

Typical results of this purification are shown in Table 4.2. This step gave a 1.7-fold purification and 87% yield.

iv. Acetone fractionation

At low concentrations of electrolytes were essential in order to
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml.)</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity (activity/mg protein)</th>
<th>Purification factor</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>20</td>
<td>74</td>
<td>1514</td>
<td>0.047</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Crude homogenate</td>
<td>8</td>
<td>24.6</td>
<td>5048</td>
<td>0.048</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Crude homogenate adjusted to pH 5.2</td>
<td>8.2</td>
<td>23.8</td>
<td>352</td>
<td>0.058</td>
<td>1.4</td>
<td>96.5</td>
</tr>
<tr>
<td>readjusted to pH 7.0</td>
<td>8.0</td>
<td>24.0</td>
<td>352</td>
<td>0.068</td>
<td>1.4</td>
<td>97.8</td>
</tr>
<tr>
<td>Crude homogenate</td>
<td>0.2</td>
<td>9.8</td>
<td>325</td>
<td>0.030</td>
<td>-</td>
<td>39.8</td>
</tr>
<tr>
<td>Crude homogenate adjusted to pH 4.6</td>
<td>8.2</td>
<td>13.7</td>
<td>325</td>
<td>0.060</td>
<td>-</td>
<td>55.0</td>
</tr>
<tr>
<td>Crude homogenate</td>
<td>0.3</td>
<td>3.0</td>
<td>311</td>
<td>0.010</td>
<td>-</td>
<td>12.3</td>
</tr>
<tr>
<td>Crude homogenate adjusted to pH 4.2</td>
<td>8.3</td>
<td>3.1</td>
<td>311</td>
<td>0.010</td>
<td>-</td>
<td>12.8</td>
</tr>
<tr>
<td>Fraction</td>
<td>Vol. (ml.)</td>
<td>Total activity (umoles NADH oxidized/min.)</td>
<td>Total protein (mg./ml.)</td>
<td>Specific activity (activity/mg.protein)</td>
<td>Purification factor</td>
<td>Yield %</td>
</tr>
<tr>
<td>----------</td>
<td>-----------</td>
<td>------------------------------------------</td>
<td>--------------------------</td>
<td>---------------------------------------</td>
<td>-------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Crude</td>
<td>53</td>
<td>252</td>
<td>4425</td>
<td>0.057</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>25-50%  (NH₄)₂SO₄ Fraction</td>
<td>15.2</td>
<td>219</td>
<td>2230</td>
<td>0.095</td>
<td>1.7</td>
<td>87</td>
</tr>
</tbody>
</table>
obtain a good purification with acetone fractionation, the 25-50% ammonium sulphate fraction, which generally preceded this step, was dialyzed for 1 hr at 4°C as described above.

The method used was similar to that described by Tanaka et al. (1967a) viz.: acetone at -15°C was slowly added to a concentration of 23% (v/v) to the dialysate. During the addition, the solution was constantly being stirred while being kept on a salted ice-bath at 0°C. The solution was immediately centrifuged at 35,000g for 10 mins at -15°C and the precipitate discarded. The clear supernatant was cooled to -25°C in an acetone, dry-ice bath. Acetone (-15°C) was added to this supernatant to a concentration of 55% (v/v) and the precipitate collected as above. The acetone was removed from the precipitate by passing a stream of nitrogen over its surface. The precipitate was then dissolved in 0.02M tris-HCl buffer, pH 7.4 containing 0.5M sucrose, 2.5mM EDTA and 0.2mM DTT. As this solution was generally turbid, it was again centrifuged at 35,000g for 10 mins at 0°C and the precipitate discarded. The clear supernatant contained all the pyruvate kinase activity.

Typical results obtained with this procedure are summarized in Table 4.3. This step gave a 2.0 fold purification and 83.5% yield.

v. Second ammonium sulphate fractionation

By cutting finer ammonium sulphate fractions not only could the enzyme be purified further, but the two pyruvate kinase isoenzymes present in liver extracts could be separated. This procedure of separating types II and I pyruvate kinase has previously been described by Passeron et al. (1967).

The acetone fractionation step preceded this step.
### Table 4.1: Purification Results of the Acetone Fractionation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml)</th>
<th>Total activity (moles NADH oxidized/min)</th>
<th>Total protein (mg)</th>
<th>Specific activity (activity/mg protein)</th>
<th>Purification factor</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st (NH₄)₂SO₄ Fractionation 25-55% and dialysis</td>
<td>12</td>
<td>109.5</td>
<td>1040</td>
<td>0.11</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Acetone Fractionation 23-45%</td>
<td>8</td>
<td>91.2</td>
<td>292</td>
<td>0.31</td>
<td>2.8</td>
<td>83.5</td>
</tr>
</tbody>
</table>
To the partially purified enzyme, solid \((\text{NH}_4)_2\text{SO}_4\) was added to 25% saturation as described under the first ammonium sulphate fractionation. The ammonium sulphate concentration was then increased by approximately 6% increments until 60% saturation was attained. The precipitates obtained after each ammonium sulphate fractionation were redissolved in 0.025M Tris-HCl buffer, pH 7.4 containing 0.5M sucrose, 2.5mM EDTA and 0.1mM DTT, assayed for pyruvate kinase activity and electrophoresed in order to verify the isoenzyme separation.

As the \((\text{NH}_4)_2\text{SO}_4\) present in these solutions was found to affect the electrophoresis, these (0.1mL aliquots) were first desalted on a Sephadex G-25 column (2" x 0.2") that had previously been equilibrated with 0.025M Tris-HCl buffer, pH 7.4 containing 0.5M sucrose, 2.5mM EDTA and 0.1mM DTT. The electrophoretic technique and stain used, was similar to that described in Chapter III, Methods and Results sections, except that both the electrode and bridge buffer were adjusted to pH 6.0 instead of pH 7.0.

Typical results of the purification and separation of the two isoenzymes obtained by this procedure are summarized in Table 4.1 and are shown in Fig. 4.1. The best yield and purifications were obtained with the 25-32% and 31-37% ammonium sulphate fractions, which contained only type L pyruvate kinase. Both these steps gave an approximate 4-fold purification and a total yield of 62.5%. With the exception of the 69-72% ammonium sulphate fraction which gave a 1.2 fold purification and 12.1% yield, all the remaining ammonium sulphate fractions gave low yields and no purification. All these fractions contained only type K pyruvate kinase, the only exception being the 37-43% ammonium sulphate fraction, which contained faint traces of both isoenzymes (Fig. 4.1.).
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (ml.)</th>
<th>Total activity (molecules NADH oxid./min.)</th>
<th>Total protein (mg.)</th>
<th>Specific activity (activity/mg protein)</th>
<th>Purification factor</th>
<th>Yield %</th>
<th>Pyruvate kinase isoenzymes detected by Electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-45% n-propanol fraction</td>
<td>6</td>
<td>79.2</td>
<td>49%</td>
<td>0.16</td>
<td>1</td>
<td>100.0</td>
<td>Types L and M</td>
</tr>
<tr>
<td>2nd (NH$_4$)$_2$SO$_4$ Fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-25%</td>
<td>0.1</td>
<td>1.5</td>
<td>23.3</td>
<td>0.068</td>
<td>-</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>25-31%</td>
<td>0.45</td>
<td>23.4</td>
<td>31.9</td>
<td>0.67</td>
<td>4.2</td>
<td>29.6</td>
<td>Type T</td>
</tr>
<tr>
<td>31-37%</td>
<td>0.45</td>
<td>26.2</td>
<td>40.0</td>
<td>0.65</td>
<td>4.1</td>
<td>32.9</td>
<td>Type L</td>
</tr>
<tr>
<td>37-43%</td>
<td>0.45</td>
<td>0.46</td>
<td>36.5</td>
<td>0.019</td>
<td>-</td>
<td>0.6</td>
<td>Types L and M</td>
</tr>
<tr>
<td>43-49%</td>
<td>0.45</td>
<td>0.68</td>
<td>48.5</td>
<td>0.014</td>
<td>-</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>49-53%</td>
<td>0.45</td>
<td>9.6</td>
<td>52.8</td>
<td>0.16</td>
<td>2.2</td>
<td>12.1</td>
<td>Type M</td>
</tr>
<tr>
<td>53-60%</td>
<td>0.35</td>
<td>4.7</td>
<td>61.0</td>
<td>0.11</td>
<td>-</td>
<td>7.1</td>
<td>Type M</td>
</tr>
</tbody>
</table>
Fig. 4.1. Typical results of starch gel electrophoresis of the different (NH$_4$)$_2$SO$_4$ fractions. The pyruvate kinase isoenzymes were taken as the difference between gels developed in the pyruvate kinase stain (a) and the adenylate kinase (b) and blank stains (c).
vi. **Calcium phosphate gel adsorption**

The precipitate of the 25-50% ammonium sulphate fraction, that had been dissolved and dialysed against a 0.02M cacodylate-0.01M Tris, pH 6.05 mixture containing 0.5M sucrose, 2.5mM EDTA and 0.1mM DTT, was used in this step.

The calcium phosphate gel was equilibrated with the aforementioned cacodylate-Tris mixture.

Successive quantities of calcium phosphate were added to the enzyme, until it was completely adsorbed to the calcium phosphate. The enzyme was then eluted from the gel with 0.1M KCl that had been added to the equilibrating buffer.

A 1.6-fold purification and 13% yield was obtained with this method. The low yield is probably due to the inhibition of the enzyme by Ca$^{2+}$.

vii. **Ion exchange chromatography**

Proteins can be separated with ion exchange chromatography either by column chromatography or batchwise elution techniques. In either instance the ion exchangers were first equilibrated with the appropriate buffer (for details of the composition of this buffer see Table 4.5).

Aliquots of the first ammonium sulphate fractionation were used in this step. These were first dialyzed for 3 hrs at $4^\circ$C against the buffer used to equilibrate the ion exchangers.

2. **Column chromatography**

The ion exchangers were packed into columns (16 x 1cm$^3$) and washed
with equilibrating buffer. After the dialysate had been applied to the ion exchange column, the exchange column was washed, either until all the protein that did not adsorb to the exchanger was eluted, or if all the protein did adsorb to the exchanger, with 50 mM equilibrating buffer. The enzyme was then eluted from the column (DEAE-Sephadex) by increasing the ionic strength in a stepwise manner, by adding 0.05 M KCl, 0.1 M KCl, 0.15 M KCl and 0.2 M KCl to the equilibrating buffer. Enough buffer was used so that all the protein that was desorbed at a particular ionic strength could be eluted.

The protein could also be eluted by employing a linear gradient system, i.e., linear increase of the salt concentration and/or change of pH. A linear gradient from 0.02 M acetate buffer, pH 5 to 0.02 M malate, pH 7 was set up in order to elute the enzyme from a phosphocellulose column. 100 ml of each buffer system was used (Table I.5.).

With either system, 2 ml fractions were collected by passing the effluent from the column through a Uvicord spectrophotometer that was attached to an LKB fraction collector.

b. Batchwise elution technique

The ion exchanger was first sucked dry on a Buchner funnel. Sufficient enzyme was then added to it to form a thick suspension. This was stirred regularly during the half-hour interval that it was left to stand at 4°C. It was then sucked dry on a Buchner funnel and the filtrate assayed for pyruvate kinase activity. (This process was repeated with each new addition of buffer to the ion exchanger.) After the ion exchanger was washed twice with equilibrating buffer, the enzyme was eluted (from SE-Sephadex, CM-cellulose and phosphocellulose) with
<table>
<thead>
<tr>
<th>Type of Ion Exchange</th>
<th>Technique</th>
<th>Equilibrating medium</th>
<th>Mode of elution</th>
<th>Eluted at</th>
<th>Yield %</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-Sephadex A-50</td>
<td>Column</td>
<td>0.02M Tris-Cl, pH 7.4 containing 0.5M EDTA, 0.05M sucrose and 0.1M DTT</td>
<td>Increasing ionic strength</td>
<td>0.2M KCl</td>
<td>25.0</td>
<td>3.6 x</td>
</tr>
<tr>
<td></td>
<td>Batchwise</td>
<td>0.02M Caclodate - 0.01M Tris mixture, pH 6.05 containing 0.5M Sucrose, 2.5M EDTA and 0.1M DTT</td>
<td>Increasing ionic strength</td>
<td>0.05M KCl</td>
<td>4.0</td>
<td>1.0 x</td>
</tr>
<tr>
<td>SB-Sephadex A-50</td>
<td>Batchwise</td>
<td>0.02M Caclodate - 0.01M Tris, pH 6.06 containing 0.5M Sucrose, 2.5M EDTA and 0.1M DTT</td>
<td>Increasing ionic strength</td>
<td>0.1M KCl</td>
<td>26.0</td>
<td>1.3 x</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>Batchwise</td>
<td>0.02M Caclodate - 0.01M Tris, pH 6.05 containing 0.5M Sucrose, 2.5M EDTA and 0.1M DTT</td>
<td>Increasing ionic strength</td>
<td>0.05M KCl</td>
<td>40.0</td>
<td>1.6 x</td>
</tr>
<tr>
<td>Na+ form</td>
<td></td>
<td>0.02M Acetate buffer, pH 5.0 containing 2.5M EDTA, 0.25M Sucrose and 0.1M DTT</td>
<td>Linear gradient, 100mL, 0.02M Acetate, pH 5 containing 2.5M EDTA, 0.25M Sucrose and 0.1M DTT, 100mL, 0.05M Malate, pH 7.0 containing 2.5M EDTA, 0.25M Sucrose and 0.1M DTT</td>
<td>0.05M KCl</td>
<td>30.0</td>
<td>2.3 x</td>
</tr>
<tr>
<td>H+ form</td>
<td></td>
<td>0.02M Acetate buffer, pH 5.0 containing 2.5M EDTA, 0.25M Sucrose and 0.1M DTT</td>
<td>Linear gradient, 100mL, 0.02M Acetate, pH 5 containing 2.5M EDTA, 0.25M Sucrose and 0.1M DTT, 100mL, 0.05M Malate, pH 7.0 containing 2.5M EDTA, 0.25M Sucrose and 0.1M DTT</td>
<td>0.05M KCl</td>
<td>30.0</td>
<td>2.3 x</td>
</tr>
</tbody>
</table>

Note: The KCl was added to the equilibrating medium.
equilibrating buffer to which 0.05M, 0.1M, 0.15M and 0.2M KCl respectively had been added.

The different ion exchangers, equilibrating mixtures and mode of elution of the enzyme used, as well as the results obtained with these techniques are summarized in Table 4.5.

From Table 4.5 it can be seen that column chromatography of the enzyme on DEAE-Sephadex resulted in 37% of the pyruvate kinase activity not being adsorbed onto the DEAE-Sephadex. This fraction gave a 1.3 fold purification. The enzyme fractions eluted with 0.1M and 0.2M KCl gave a 3.2 and 3.6 fold purification, and yields of 63% and 25.3% respectively. With the batchwise elution technique of purifying the enzyme from DEAE-Sephadex, 92.8% of the pyruvate kinase activity was not adsorbed onto the DEAE-Sephadex. Elution of the enzyme with 0.1M and 0.2M KCl gave low yields of 8% and 4% and purification of 1.0 and 1.6 fold respectively.

Batchwise elution of the enzyme from SE-Sephadex gave a 1.3 fold purification and 26% yield. The enzyme was eluted from SE-Sephadex with 0.1M KCl.

When employing the batchwise elution technique with CM-cellulose, it was found that the enzyme could be eluted from both the Na+ and H+ forms of CM-cellulose with 0.05M KCl. The pyruvate kinase activity thus eluted from the Na+ form of CM-cellulose gave a 1.6 fold purification and 10% yield whereas a 2.3 fold purification and 36% yield was attained with the H+ form of CM-cellulose.

The enzyme could not be eluted from the phosphocellulose column.
with the linear gradient employed. However, with the batchwise elution technique, the enzyme could be eluted by increasing the ionic strength (0.2M KCl). This method gave a 26% yield and a 1.3-fold purification.

viii. Gel filtration

(a) Sephadex G-200 is a cross linked dextran that acts as a molecular sieve.

Initial attempts to purify the enzyme on a Sephadex G-200 column failed as the enzyme was found to be unstable on this column. Finally, the procedure of Taylor et al. (1969) was adopted, whereby the column was equilibrated with 0.5M (NH₄)₂SO₄. The 0.5M (NH₄)₂SO₄ was added to the 0.02M Tris-Cl buffer, pH 7.6 containing 0.5M sucrose, 1.5mM EDTA, and 0.1mM EIT that was originally used to equilibrate the Sephadex G-200.

The enzyme solution of the second ammonium sulphate fraction was used in this step. After this solution had been placed on the Sephadex G-200 column (95 x 1cm²), it was eluted from the column with the mixture used to equilibrate the column. 2ml aliquots were collected and assayed for pyruvate kinase activity. Those fractions that contained the highest specific activity were pooled.

Typical results obtained with this procedure are summarized in Table 1.6 and Fig. 1.2. The enzyme was eluted from the column after the first 20ml. When the most active fractions were pooled, a 2-fold purification and 76% yield was attained.

ix. Concentration of the enzyme

The eluates obtained from the different columns were concentrated down, by placing them in dialysis tubing and leaving them in acetonide
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml.)</th>
<th>Total activity (μmoles NADH oxid/min.)</th>
<th>Total protein (mg.)</th>
<th>Specific activity (activity/mg. protein)</th>
<th>Purification factor</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third (NH₄)₂SO₄ fractionation</td>
<td>6.9</td>
<td>17.6</td>
<td>59.5</td>
<td>0.22</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>25-37%</td>
<td>14.0</td>
<td>13.0</td>
<td>21.8</td>
<td>0.59</td>
<td>2.0</td>
<td>74</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 4.2. A typical elution pattern of normal adult human liver type L pyruvate kinase from Sephadex G-200 (95 x 1 cm²)
overnight at 4°C. No significant loss in activity was noted when this method was employed.

b. Isotopic Exchange

Isotopic exchange studies can be used as a means of obtaining further information on enzyme mechanisms. This technique was first proposed by Boyer (1959) and described in detail by Cleland (1967). Unlike initial rate studies, isotopic exchange studies are carried out under thermodynamic equilibrium conditions. All reactants are present at concentrations such that there is no net chemical reaction, but there will be a constant turnover of substrates and products. Addition of a labelled reactant to such a system will perturb this equilibrium and result in exchange of label with corresponding product. As the concentration of any reactant pair can be varied without affecting the equilibrium, the effect of increasing the concentration of individual reactant pairs on the initial velocity of exchange may be determined and from this pattern obtained, the mechanism.

1. Materials

The reagents used were similar to those described in Chapters II and III - Materials and Methods sections, with a few additions.

250 mc of 14C-pyruvate (12.1 mCi/mcule) was obtained from Amersham. This was dissolved in 1ml of 2% ethanol. It was diluted a further 5 times and 10 µl of this was used per assay.

14C ATP-γ-32P (17 Ci/mcule) was obtained from Amersham and dissolved in 1ml 2% ethanol. This was diluted 10 times and 10 µl of this
was used per assay.

DEAE-cellulose paper (DE-61) was purchased from Whatman. This was dipped into 5mM Na₂EDTA just before use and dried.

Scintillation grade 2,5-diphenyloxazole (POPOP) and 1,4 bis-(2-(1-
methyl-5-phenyloxazolyl)y-benzene (dimethyl POPOP) were obtained from the Packard instrument company.

MgATP was prepared by mixing equimolar amounts of MgCl₂ and ATP. The MgATP, ADP, FEP and pyruvate were all adjusted to pH 9 with NaHCO₃.

2. Methods

1. Determination of exchange rates at equilibrium

The equilibrium constant for the pyruvate kinase reaction:

\[ K_{eq} = \frac{[\text{Fgpyruvate}] [\text{ATP}]_{\text{at pH 9.0}}}{[\text{FEP}] [\text{ADP}]} \]

was taken to be 370 (McQuate and Utter, 1959). The reaction mixture contained in a total volume of 100 µl, 50mM glycine-NaOH buffer, pH 9.0, 12.5mM MgCl₂, 0.25mM EDTA, a suitable amount of enzyme and pyruvate, MgATP, ADP and FEP in various proportions, but always maintaining equilibrium conditions. The exchange rate between ATP-γ-³²P and FEP was estimated either in the presence of 2mM MgATP, 2mM FEP and pyruvate and ADP in a 37:1 ratio or with 75mM pyruvate, 0.5mM ADP and MgATP and FEP in a 2:1 ratio. For the determination of ¹¹C-pyruvate to FEP exchange rates, 7.4 pyruvate, 2mM FEP and ATP and ADP in a ratio of 100:1 were used. After preincubating the reaction mixture for 15 mins at 30°C, the
exchange reaction was started by adding 10 µl of either diluted \(^{14}C\)-pyruvate or ATP-\(^{32}P\). After quickly mixing, 15 µl aliquots were taken with a Hamilton syringe at various time intervals and spotted onto EDTA-treated, DEAS-cellulose chromatography paper (\(1/4\" x 1/4\"\)) to which 3 folds of Whatman's No.1 paper had been pinned on top. As the enzyme is Mg-dependant it was assumed and also confirmed by spectrophotometric studies that EDTA would stop the reaction by chelating the Mg\(^{2+}\).

11. **Assayiration and counting of pyruvate and PEP and of ATP and ADP**

The \(^{14}C\)-pyruvate and labelled PEP were separated by ascending chromatography in 0.1M Tris-HCl buffer, pH 7.4 for 3 hr at room temperature. On the other hand, the ATP-\(^{32}P\) and labelled PEP were separated by developing the chromatograms for 3 hrs at room temperature in a 0.1M ammonium formate buffer, pH 3.1 containing 50mM EDTA, by ascending chromatography.

After drying the chromatograms in the oven at 60°C, the positions of the phosphorus containing compounds were located by dipping marker strips containing these compounds into the ammonium molybdate stain described by Thomson (1969). This stain consists of 8 volumes of a solution containing 1g ammonium molybdate in 50ml H\(_2\)O, 3 volumes of concentrated HCl, 3 volumes of concentrated perchloric acid, 12N (32, 1.72) and 86 volumes of acetic acid. The strips were then exposed to ultraviolet light and the phosphate containing compounds appeared as blue spots. The nucleotides, ATP, could be detected by placing the marker strips directly under ultraviolet light without staining. Pyruvate was detected with a dinitrophenyl hydrazine reagent, consisting of a filtered solution of 0.5g, 2-h-dinitrophenyl hydrazine in 27 HCl. To increase the sensitivity of
this stain the marker strips were also dipped in an alkaline solution containing 2% NaOH in 90% ethanol (Smith and Smith, 1969).

After marking, the test strips containing the labelled PEP were cut out, fluted lengthwise, immersed in 15ml scintillator (7.5g PPO and 250mg dimethyl POPOP in 2l litres toluene) and counted in a Packard Tri-Carb liquid scintillation counter.

II. Results

1. Partial purification of pyruvate kinase from human liver

(a) Partial purification of liver type I pyruvate kinase from adult human liver

The purification steps that gave the best purification and yields were combined to give the purification procedure described below:

The enzyme was extracted and subjected to the first ammonium sulphate fractionation. It was then dialysed for 1 hr, subjected to acetone fractionation and a second ammonium sulphate fractionation. After the second ammonium sulphate fractionation, it was applied to a Sephadex G-200 column. The most active eluate fractions were combined and stored at 4°C until required for kinetic experiments. The enzyme was found to be stable for several months in this form.

The above mentioned steps have been described in detail under the methods section. As is shown in Table 4.7, this purification sequence gave an overall yield of 19% and a 3½ fold purification over the initial crude extract. This partially purified preparation was found to contain approximately 1.25% adenylyl kinase activity, 0.63% enolase
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity (nmole NADH oxidized/min.)</th>
<th>Total protein (mg)</th>
<th>Specific activity (activity/mg protein)</th>
<th>Purification factor</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>660</td>
<td>10,650</td>
<td>0.062</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>First ammonium sulphate fractionation</td>
<td>502</td>
<td>4,180</td>
<td>0.12</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>25 - 50% and dialysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetone fractionation</td>
<td>354</td>
<td>860</td>
<td>0.32</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>25 - 45%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second ammonium sulphate fractionation</td>
<td>183</td>
<td>120</td>
<td>1.52</td>
<td>21.5</td>
<td></td>
</tr>
<tr>
<td>25 - 37%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>120</td>
<td>60</td>
<td>2.13</td>
<td>34.3</td>
<td>19</td>
</tr>
</tbody>
</table>
and 1.56% aldolase activity.

(b) Partial purification of hepatoma type II pyruvate kinase

The procedure and sequence used to purify type II pyruvate kinase from cancerous tissue of hepatoma containing livers are summarized in Table 4.8.

From Table 4.8 it can be seen that the purification steps and sequence used were similar to those used to partially purify normal liver type L pyruvate kinase, the only difference being that most of the pyruvate kinase activity was found in the 50-70% precipitate of the first ammonium sulphate fractionation step and in the 50-65% fraction of the second ammonium sulphate fractionation step. Also with the acetone fractionation step, the enzyme could only be precipitated at a concentration of 49%.

By using the procedure and sequence shown in Table 4.8 a 35% yield and a 62.5 fold purification over the crude preparation was obtained. Partially purified preparations of this enzyme were found to contain 0.17% aldolase and 0.12% adenylate kinase activity.

(c) Partial purification of human foetal type II pyruvate kinase

From Table 4.9 it can be seen that 76.6% of the total pyruvate kinase activity of foetal liver was found in the 0-50% ammonium sulphate fraction, and 33.4% in the 50-70% ammonium sulphate fraction. This latter mentioned fraction contains only the M isoenzyme.

Although type N pyruvate kinase did not constitute the major proportion of the total pyruvate kinase activity of foetal liver, this isoenzyme was further purified in order to assess whether its properties
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity (Amoles NADH oxidized/min.)</th>
<th>Total protein (mg)</th>
<th>Specific activity (activity/mg protein)</th>
<th>Purification factor</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>510</td>
<td>915</td>
<td>0.88</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>First ammonium sulphate fractionation 50 - 70% saturation and dialysis</td>
<td>576</td>
<td>235</td>
<td>2.15</td>
<td>2.8</td>
<td>71</td>
</tr>
<tr>
<td>Acetone fractionation 0 - 45%</td>
<td>550</td>
<td>195</td>
<td>3.67</td>
<td>4.2</td>
<td>68</td>
</tr>
<tr>
<td>Second ammonium sulphate fractionation 50 - 65% saturation</td>
<td>305</td>
<td>49.4</td>
<td>6.18</td>
<td>7.0</td>
<td>30</td>
</tr>
<tr>
<td>Sephadex 0-200</td>
<td>280</td>
<td>5.1</td>
<td>54.90</td>
<td>62.5</td>
<td>35</td>
</tr>
</tbody>
</table>
Fig. 4.3 A typical elution pattern of hepatoma type M pyruvate kinase from Sephadex G-200 (95 x 1 cm²)
<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total activity (Units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (activity/mg protein)</th>
<th>Purification factor</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>265</td>
<td>3590</td>
<td>0.074</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>First ammonium sulphate fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 50% saturation</td>
<td>197</td>
<td>1650</td>
<td>0.119</td>
<td>1.6</td>
<td>74.5</td>
</tr>
<tr>
<td>50 - 70% saturation</td>
<td>60</td>
<td>540</td>
<td>0.113</td>
<td>1.5</td>
<td>22.6</td>
</tr>
<tr>
<td>Acetone fractionation of 50% - 70% ammonium sulphate fraction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 - 45% saturation</td>
<td>57.75</td>
<td>253</td>
<td>0.226</td>
<td>3.1</td>
<td>21.8</td>
</tr>
<tr>
<td>Second ammonium sulphate fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 - 65% saturation</td>
<td>30</td>
<td>83</td>
<td>0.360</td>
<td>4.9</td>
<td>11.3</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>13.5</td>
<td>4.2</td>
<td>3.22</td>
<td>43.5</td>
<td>5.1</td>
</tr>
</tbody>
</table>
would be similar to those of the hepatoma type K pyruvate kinase.

As can be seen from Table 4.9, the procedure and sequence used to partially purify this isoenzyme was the same as that used to partially purify hepatoma type K pyruvate kinase. This procedure gave 5.14 yield and a 43.5 fold purification over the crude preparation.

2. Stability of normal adult liver type L pyruvate kinase

As initial attempts to purify normal adult liver type L pyruvate kinase were tempered by the instability of the enzyme, attempts were first made to stabilize it.

It was found that if the precipitate of the first ammonium sulphate fraction was dissolved in 0.025M Tris-Cl buffer, pH 7.5 containing 2.5mM EDTA and 0.1mM DTT, the enzyme lost between 20-30% of its activity overnight when kept at 4°C. As shown in Table 4.10, this high percentage loss in activity could be almost completely eliminated by the addition of a high concentration of sucrose (0.25M) to the aforementioned buffer system. Also, from the Table, it can be seen that MgSO₄ (5mM), ATP (0.3mM), DTT (1mM) or low levels of sucrose (0.0175M) did not prevent the inactivation of the enzyme.

Attempts to stabilize the enzyme with 5mM E-aminocaproic acid failed. Furthermore, this compound was found to have an inhibitory effect on the enzyme (15% inhibition obtained).

3. Properties of human, normal adult liver type L, hepatoma and foetal types K pyruvate kinase

As discussed in the Introduction, rat liver pyruvate kinase is a
## Table 4.10: Stability of Normal Liver Pyruvate Kinase Activity After the First Ammonium Sulfate Fractionation at 4°C

<table>
<thead>
<tr>
<th>Condition</th>
<th>0% Loss</th>
<th>1 day</th>
<th>2 days</th>
<th>4 days</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme solution + 0.0175M sucrose</td>
<td>- 0%</td>
<td>23.5</td>
<td>43.75</td>
<td>- 0%</td>
<td>72.9%</td>
</tr>
<tr>
<td>Enzyme solution + 5% K$_2$SO$_4$</td>
<td>- 20.5</td>
<td>42.5</td>
<td>- 0%</td>
<td>- 0%</td>
<td>74.1%</td>
</tr>
<tr>
<td>Enzyme solution + 5% K$_2$SO$_4$ + 0.0175M sucrose</td>
<td>- 27%</td>
<td>48.5</td>
<td>- 0%</td>
<td>- 0%</td>
<td>72.9%</td>
</tr>
<tr>
<td>Enzyme solution + 5% K$_2$SO$_4$ + 0.305mM ATP</td>
<td>- 25%</td>
<td>43.5</td>
<td>- 0%</td>
<td>- 0%</td>
<td>73.5%</td>
</tr>
<tr>
<td>Enzyme solution + 5% K$_2$SO$_4$ + 0.305mM ATP + 0.0175M sucrose</td>
<td>- 26%</td>
<td>50%</td>
<td>- 0%</td>
<td>- 0%</td>
<td>69.2%</td>
</tr>
<tr>
<td>Enzyme solution + 0.25M sucrose</td>
<td>- 3%</td>
<td>-</td>
<td>34.25%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enzyme solution + 0.25M sucrose + 5% K$_2$SO$_4$ + 0.305mM ATP</td>
<td>- -</td>
<td>-</td>
<td>37.5%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Enzyme solution + 0.25M sucrose + 1% BHT</td>
<td>- -</td>
<td>-</td>
<td>35.2%</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
regulatory enzyme as evidenced by its allosteric kinetics with respect
to FBP, feedforward activation by FDP and feedback inhibition by ATP
and alanine (Klimente et al., 1970, Tanaka et al., 1967a, Susor and
Ritter, 1968, Rossenburt et al., 1969). Thus the effects that these
substrates have on human normal liver type L, hepatoma and fetal type
H pyruvate kinases were determined in order to assess the regulatory
nature of these isoenzymes.

A. Factors affecting the binding of FBP

(a) Normal liver type L pyruvate kinase

Normal liver type L pyruvate kinase exhibits homotropic cooperativity
with respect to FBP for plots of initial velocity versus the FBP
concentration gave S-shaped curves (Fig. 1.1). These could be affected
by various modulators.

1. The effects of ATP and FDP

Fig. 1.1 shows the results obtained when normal liver type L
pyruvate kinase activity was estimated as a function of the FBP concen-
tration at 1mM ADP either in the absence of effector (control) or in the
presence of FDP, ATP or ATP + FDP.

The S-shaped curve obtained when the initial velocity was plotted
against the FBP level was transformed to a hyperbola in the presence of
0.1mM FDP. FDP activated the enzyme and caused the enzyme to reach
maximum velocity at a lower FBP concentration. On the other hand, 1mM
ATP inhibited the enzyme, decreased the maximum velocity and enhanced the
sigmoidal nature of the curve. Although 0.1mM FDP eliminated the ATP.
Fig. 4.4. Effect of FDP and ATP on the initial velocity of normal human adult liver type L pyruvate kinase at various PEP concentrations. — Control, \( \Delta - \Delta \) 0.1mM FDP, \( \times - \times \) 1mM ATP, \( \circ - \circ \) 1mM ATP + 0.1mM FDP. Reaction mixtures contained 50mM triethanolamine-HCl buffer, pH 7.4, 10mM MgCl\(_2\), 50mM EDTA, 0.1M KCl, 1mM ADP, 0.15mM NADH and 4U lactate dehydrogenase in addition to PEP, effector and enzyme.

Inset: Hill plot of data.
inhibition at the low levels of PEP, it had no effect at the high PEP levels.

Sigmoidal kinetics can be assessed by using the classical Hill equation rearranged to the form

$$\log \left( \frac{v}{V_{\text{max}} - v} \right) = n \log (S) - \log K$$

where $v$ represents the reaction velocity, $V_{\text{max}}$ the maximum reaction velocity, $n$, the number of substrate binding sites and $K$, a constant (Hill, 1910; Chance, 1965 and Akahira, 1966). On plotting $\log \left( \frac{v}{V_{\text{max}} - v} \right)$ versus the log of the substrate concentration, several parameters can be obtained. The $K$ or the PEP concentration at half maximal velocity, is obtained at $\log \left( \frac{v}{V_{\text{max}} - v} \right) = 0$. The slope of the Hill plot, $n$, gives the interaction coefficient and represents the minimal number of binding sites.

From such a Hill plot (Inset Fig. 4.4), a $K$ for PEP of 0.75mM and a $n$ value of 2.1 was obtained in the absence of effector (control). In the presence of 0.1mM PEP the $n$ value decreased to 0.9 and the apparent affinity of the enzyme for PEP increased as the $K$ for PEP decreased to 0.11mM. On the other hand, ATP increased the $K$ for PEP to 1.11mM and the $n$ value to 3.1. In the presence of 1mM ATP and 0.1mM FDP, a $n$ value of 1.1 was obtained whereas the $K$ value for PEP was similar to that obtained in the presence of 0.1mM FDP alone, namely 0.11mM.

ii. Effects of DL-alanine and FDP

Fig. 4.5a shows the results of a similar study when using DL-alanine. From plots of initial velocity versus the PEP concentration at 1mM ATP (Fig. 4.5a), it can be seen that the sigmoidicity of the curve
Fig. 4.5a. Effect of DL-alanine and FDP on the initial velocity of normal human adult liver type L pyruvate kinase at various PEP concentrations.

- o Control, △ 2 μM FDP, □ 4 mM DL-alanine, x 4 mM DL-alanine + 2 μM FDP. Conditions were as in Fig. 4.4.

Fig. 4.5b. Hill plot of data.
obtained in the absence of effector (control) could also be enhanced in the presence of 1mM DL-alanine. Like ATP, DL-alanine also decreased the maximum velocity. This inhibition could be completely reversed with 2 mM FDP and the kinetics altered from sigmoidal to hyperbolic.

From the Hill plot of the data (Fig. 4.5b), a \( k_{1/2} \) value for PEP of 0.75 mM was obtained in the absence of effector (control). This \( k_{1/2} \) value for PEP increased to 1.1 mM in the presence of 1mM DL-alanine. As the Hill plots obtained were not linear, the \( n_H \) values of the control changed from 2.0 at the high PEP levels to 0.6 at the low PEP concentrations.

A change in the \( n_H \) value from 1.3 to 1.2 was also obtained in the presence of 1mM DL-alanine. But overall, at the same PEP concentrations, it can be seen that the \( n_H \) value of the control was lower than that obtained in the presence of 1mM DL-alanine. In the presence of 3 \( \mu \)M FDP or 2 \( \mu \)M FDP+ 1mM DL-alanine similar \( k_{1/2} \) values for PEP of 0.2 mM and \( n_H \) values of 0.6 were obtained, indicating that FDP reverses the DL-alanine inhibition of the enzyme.

iii. Effect of temperature on the allosteric properties

The allosteric properties of rat liver type L pyruvate kinase have been shown to be eliminated at -20°C (Szasz and Rutter, 1968), at 0-2°C (Llorente et al., 1970) and at 37°C (Tanaka et al., 1967b).

Temperature does not appear to affect the allosteric properties of the human type L pyruvate kinase, since similar results were obtained when the enzyme was kept at 0°C or at room temperature. In both cases, a \( k_{1/2} \) for PEP of 0.5 mM and a \( n_H \) value of 1.9 were found when the enzyme was assayed in the presence of 50mM triethanolamine-HCl buffer, pH 7.4, 5mM KCl, 10mM MgCl₂, 0.1M KMn, 1mM ATP and at different PEP levels.
(b) **Effects of ATP, DL-alanine and FDP on hepatoma type H pyruvate kinase**

The results obtained when hepatoma type H pyruvate kinase activity was estimated as a function of the FEP concentration at 1 mM ADP in the absence of effector (control) or in the presence of either FDP, DL-alanine, FDP + DL-alanine or FDP + ATP are shown in Fig. 4.6.

From Fig. 4.6 it can be seen that the activity of the enzyme was the same both in the absence of effector and in the presence of 0.1mM FDP. Furthermore, the inhibition obtained in the presence of 1 mM ATP was not affected by the presence of 0.1mM FDP. However, the inhibition obtained in the presence of DL-alanine could be almost completely reversed with 0.1mM FDP, indicating that the enzyme is sensitive to FDP under certain conditions only.

(c) **Effects of ATP, DL-alanine and FDP on foetal type H pyruvate kinase**

From Fig. 4.7, it can be seen that foetal type H pyruvate kinase exhibited properties very similar to those of the hepatoma enzyme when assayed under the same conditions. The plots of initial velocity versus the FEP concentration at 1 mM ADP gave hyperbolic kinetics. These were not affected by the presence of 0.1mM FDP. 1 mM ATP inhibited the enzyme. However, unlike hepatoma type H pyruvate kinase, this inhibition could be partly reversed by FDP at FEP levels below 0.25mM. On the other hand, the inhibition of the enzyme by 1 mM DL-alanine was almost completely reversed with 0.1mM FDP at all levels of FEP, again indicating that this enzyme is also affected by FDP under certain conditions only.
Fig. 4.6. Effect of ATP, DL-alanine and FDP on the initial velocity of hepatoma type M pyruvate kinase at various PEP concentrations. o — o Control, △ — △ 0.1mM FDP, x — x 4mM ATP; o — o 4mM ATP + 0.1mM FDP, △ — △ 4mM DL-alanine, △ — △ 4mM DL-alanine + 0.1mM FDP. Conditions were as in Fig. 4.4.
Fig. 4.7. Effect of ATP, DL-alanine and FDP on the initial velocity of human foetal liver type M pyruvate kinase at various PEP concentrations. ○—○ Control, □—□ 0.1 mM FDP, ×—× 4 mM ATP, ○—○ 4 mM ATP + 0.1 mM FDP, Δ—Δ 4 mM DL-alanine, Δ—Δ 4 mM DL-alanine + 0.1 mM FDP. Conditions were as in Fig. 4.4.
B. Sensitivity of pyruvate kinase isoenzymes to various effectors

The susceptibility of the three isoenzymes to various effectors was determined by estimating the pyruvate kinase activity at various effector concentrations in the presence of 50mM triethanolamine-HCl buffer, pH 7.4, 10mM MgCl₂, 5mM EDTA, 0.1M KCl, 0.15mM NADH, 1U LDH, 1mM ADP, 0.5mM FDP and enzyme.

(a) Activators

i. Triose phosphates

The results obtained when normal adult liver type L pyruvate kinase was estimated as a function of different triose phosphate levels are shown in Fig. 4.8.

From Fig. 4.8 it can be seen that glyceraldehyde-3-phosphate, glyceraldehyde-2-phosphate and dihydroxyacetone phosphate, all activated the normal adult human liver type L pyruvate kinase approximately two fold. The level of dihydroxyacetone phosphate required to activate the enzyme two fold was 10 times lower than the concentration of the other triose phosphates tested. Glyceraldehyde-phosphate and glyceraldehyde-3-phosphate had no affect on the enzyme.

Hepatoma and fetal types of pyruvate kinase were not affected by the presence of any of the aforementioned triose phosphates.

ii. Hexoses and hexose phosphates

The results obtained when normal adult liver type L pyruvate kinase was assayed under the conditions described above and in the presence of different hexoses or hexose phosphates are shown in Fig. 4.9 and summarized in Table 4.11.
Fig. 4.8. Effect of various triose phosphates on normal human adult liver type L pyruvate kinase activity. The triose phosphates were $x-x$ dihydroxy acetone phosphate; $o-o$ glyceraldehyde-3-phosphate; $o-o$ glycerate-2-phosphate; $\Delta-\Delta$ glycerol-1-phosphate and $\Box-\Box$ glycerate-3-phosphate. Reaction mixtures contained 50mM triethanolamine-HCl buffer, pH 7.4, 5mM EDTA, 10mM MgCl$_2$, 0.1M NaCl, 4U lactate dehydrogenase, 0.15mM NADH, 1.0mM ADP and 0.5mM PEP in 1.0ml in addition to enzyme and triose phosphates.
Fig. 4.9. Effect of hexose phosphates on normal human adult liver type I pyruvate kinase activity: □ Glucose-1-phosphate, △ 2-deoxy glucose-6-phosphate, × fructose-1-phosphate, ○ fructose-6-phosphate, • glucose-6-phosphate and △ fructose-1,6-diphosphate. Assay conditions were as in Fig. 4.8.
### Table 1.11: Effects of Sugar and Sugar Phosphates on Normal Human Adult Liver Pyruvate Kinase Activity

<table>
<thead>
<tr>
<th>Hexose or Hexose Phosphate</th>
<th>$K_a$ (mM)</th>
<th>Amount of Enzyme Activation at $R_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>1.0</td>
</tr>
<tr>
<td>F-1,6-P&lt;sub&gt;2&lt;/sub&gt;</td>
<td>$0.35 \times 10^{-3}$</td>
<td>2.7</td>
</tr>
<tr>
<td>G-6-P</td>
<td>0.66</td>
<td>2.7</td>
</tr>
<tr>
<td>F-6-P</td>
<td>0.76</td>
<td>2.7</td>
</tr>
<tr>
<td>F-1-P</td>
<td>1.05</td>
<td>2.7</td>
</tr>
<tr>
<td>2-deoxy G-6-P</td>
<td>2.88</td>
<td>2.0</td>
</tr>
<tr>
<td>G-1-P</td>
<td>7.94</td>
<td>1.7</td>
</tr>
<tr>
<td>Gal-6-P</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>No effect</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>No effect</td>
<td></td>
</tr>
</tbody>
</table>
From Fig. 1.9 it can be seen that fructose-1-phosphate, glucose-
6-phosphate, fructose-6-phosphate, and FDP (inset) all activated the
enzyme to the same maximal level. However, the concentration of FDP
required to activate the enzyme maximally was less than that of any of
the other hexose phosphates tested. 2-deoxyglucose-6-phosphate and
glucose-1-phosphate were not able to activate the enzyme to the same
maximal level as the aforementioned hexose phosphates even though higher
concentrations of these compounds were used.

From Table 1.11 it is seen that the $K_2$ value for FDP ($0.35 \times 10^{-3}$
mM) was approximately 2,000 fold lower than that of glucose-6-phosphate
(0.66mM), fructose-6-phosphate (0.76mM) and fructose-1-phosphate (1.05mM).
The $K_2$ values obtained for 2-deoxyglucose-6-phosphate (2.88mM) and glucose-
1-phosphate (7.94mM) were even higher. FDP, glucose-6-phosphate, fructose-
6-phosphate and fructose-1-phosphate all activated the enzyme 2.7 times
above the control (no effector) at their $K_2$ values, whereas 2-deoxyglucose-
6-phosphate and glucose-1-phosphate only activated the enzyme 2 and 1.7
fold respectively. Galactose-6-phosphate, glucose and fructose had no
effect on the enzyme.

None of the aforementioned compounds were able to activate the
hepatoma or foetal types II pyruvate kinases. Furthermore, when the con-
centration of these hexoses or hexose phosphates were increased to 100mM
they had a slight inhibitory effect on these isoenzymes.

iii. FDP and the effect of ATP and alanine on this activation

The effect that ATP and DL-alanine had on the FDP activation of
normal liver type I pyruvate kinase was determined by varying the FDP
level, keeping the FDP and ATP levels constant at 0.5mM and half repectively.
lively and determining the activity of the enzyme in the absence of inhibitor and in the presence of ATP, DL-alanine, or both (Fig. 1.10).

From plots of initial velocity versus the FDP concentration (Fig. 1.13) and from the EadH plots of the data (Fig. 1.10, inset), it can be seen that in the absence of effector, \( k_p \) and \( k_q \) values for FDP of 0.8 and 0.5 \( \mu M \) respectively were obtained. 16mM DL-alanine or 2mM ATP increased the \( k_p \) for FDP to 0.63 \( \mu M \) and the sigmoidicity of the kinetics with respect to FDP (\( k_p = 1.7 \)) whereas, when 16mM DL-alanine and 1mM ATP were used, a \( k_p \) for FDP of 2.2 \( \mu M \) was obtained. In the presence of both 16mM DL-alanine + 2mM ATP, the \( k_p \) increased to 3.2 \( \mu M \) and the \( n_p \) value to 1.5. This indicates that the inhibitory effect of ATP and DL-alanine is additive and that these inhibitors act at different sites.

(b) Inhibitors and the effect of FDP on this inhibition

The inhibition of normal adult human liver type I, hepatoma and fetal types II pyruvate kinases by ATP, ADP, citrate and DL-alanine and the effect FDP had on the inhibition are shown in Figs. 1.11 - 1k and summarized in Table 1.12.

i. ATP

From Fig. 1.11a it can be seen that when the activity of normal liver type I pyruvate kinase was plotted as a function of the ATP concentration, no cooperative interaction was obtained as a \( n_p \) value of 1.6 was obtained from the EadH plot of the data (inset, Fig. 1.11a). A \( k_q \) for ATP of 0.62 \( \mu M \) was obtained. FDP activated the enzyme, increased the \( k_q \) for ATP and allowed the cooperative interaction of the inhibitor to be expressed. The \( k_q \) and \( n_q \) values for ATP increased to 1.5 \( \mu M \) and 2 respectively in the presence of 0.2 \( \mu M \) FDP and to 1.5 \( \mu M \) and 2.5 respectively in the
Fig. 4.10. Effect of FDP, ATP and DL-alanine on normal human adult liver type L pyruvate kinase activity at various FDP concentrations. △ — △ Control, x — x 2mM ATP, o — o 16mM DL-alanine, □ — □ 8mM DL-alanine + 1mM ATP, ◦ — ◦ 16mM DL-alanine + 2mM ATP. Conditions were as in Fig. 4.8.

Inset: Hill plot of data.
Fig. 11. Effect of ATP and FDP on the pyruvate kinase activity. ○—○ Control, △—△ 0.2 μM FDP and □—□ 0.1mM FDP. Conditions were as in Fig. 4,6.

Insets. Hill plots of data

(a) Normal adult human liver type L pyruvate kinase.

(b) Hepatoma type M pyruvate kinase.

(c) Human foetal type M pyruvate kinase.
presence of 0.1M FDP. Although 0.2mM and 0.1mM FDP both activated this isoenzyme they only reverse the ATP inhibition at low ATP levels.

Fig. 4.11b shows the results obtained when the hepatoma type M pyruvate kinase was estimated under similar conditions. A Ks for ATP of 5.2mM was obtained from the Hill plot of the data (inset, Fig. 4.11b). As the Hill plot of the data was not linear, the nH value changed from 1.8 at the low ATP levels to 3.2 at the high concentrations of ATP. In either instance, the inhibitor exhibited positive cooperativity. As FDP had no effect on the enzyme, the Ks and nH values remained unaltered.

Similar results to the hepatoma type M pyruvate kinase were obtained with the foetal type M pyruvate kinase, the only difference being that 0.1mM FDP activated the enzyme at the high ATP levels (Fig. 4.11c). A Ks for ATP of 5.6mM and an nH value that changed from 1.3 at the low ATP levels to 2.5 at the high ones were obtained from the Hill plot of the data (inset, Fig. 4.11c).

ii. AMP

When the normal liver type L pyruvate kinase activity was estimated as a function of the AMP concentration, a Ks for AMP of 72mM was obtained from the Hill plot of the data (Fig. 4.12, inset). As the Hill plot was not linear, the nH value changed from 1.3 at the low AMP levels to 2.2 at the high AMP levels, indicating that the cooperative interaction of the inhibitor is only expressed at the high AMP levels. 0.1mM FDP activated the enzyme, decreased the Ks for AMP to 60mM and permitted the cooperative interaction of the inhibitor to be expressed even at the low AMP levels (nH = 1.9).
Fig. 4.12. Effect of AMP and FDP on the pyruvate kinase activity. 
- - - Control, A — A 2.0 mM FDP 
and c — c 0.1 mM FDP. Conditions were as in Fig. 4.6.

Insets. Hill plots of data

(a) Normal adult human liver type H 
pyruvate kinase.

(b) Hepatoma type H pyruvate kinase.

(c) Human foetal type H pyruvate kinase.
The results obtained when either the hepatoma or foetal types M pyruvate kinase activities were determined as functions of the ADP levels are shown in Fig. 4.12b and c. With hepatoma type M pyruvate kinase, a $K_a$ for ADP of 55mM and a $n_H$ value of 1.2 was obtained from the Hill plot of the data (inset, Fig. 4.12b). With the foetal type M pyruvate kinase, a $K_a$ for ADP of 52mM and a $n_H$ value of 1.5 was obtained (inset, Fig. 4.12c). FDP had no effect on either the hepatoma or foetal types M pyruvate kinases.

iii. Citrate

When normal liver type L pyruvate kinase activity was plotted as a function of the citrate concentration (Fig. 4.13a), $n_H$ and $K_a$ values for citrate of 0.9 and 3.2mM respectively were obtained from the Hill plot of the data. FDP activated the enzyme and increased the $K_a$ for citrate. The $K_a$ for citrate increased to 10.5mM in the presence of 2 mM FDP and to 11.0mM in the presence of 0.1mM FDP. FDP caused the cooperative interaction of the inhibitor to become manifest ($n_H = 2.1$). As $n_H$ values of 2.1 were obtained in the presence of either 2 mM or 0.1mM FDP, it would appear that maximum cooperativity had been attained.

Figs. 4.13b and 4.13c show the results obtained when the hepatoma and foetal types M pyruvate kinases were estimated as functions of the citrate concentration. From the Hill plot of the data, $K_a$ values for citrate of 11.5mM were obtained for the hepatoma isoenzyme (inset, Fig. 4.13b) and of 12.0mM were obtained for the foetal isoenzyme (inset, Fig. 4.13c). As the Hill plots were not linear (insets, Figs. 4.13b and c), the $n_H$ values change with both the hepatoma and foetal enzymes from 1.4 to 2.4 (hepatoma) or from 1.4 to 2.6 (foetal) at the low and high citrate levels respectively. As 0.1mM FDP activated both these isoenzymes at
Fig. 4.13. Effect of Citrate and FDP on the pyruvate kinase activity. e—e Control, A—A 2 μM FDP and o—o 0.1 mM FDP. Conditions were as in Fig. 4.8.

Inset, Hill plots of data

(a) Normal adult human liver type I pyruvate kinase.
(b) Hepatoma type H pyruvate kinase.
(c) Human fetal type H pyruvate kinase.
the high citrate levels only, it made the Hill plots linear and caused the $n_H$ value to revert back to 1.4 (i.e., causes the cooperative interaction obtained at the high citrate levels to be suppressed).

iv. DL-alanine

The results obtained when the normal liver type L pyruvate kinase activity was plotted as a function of the DL-alanine concentration are shown in Fig. b.11a. A $n_H$ value and a $K_s$ for DL-alanine of 1.8 and 0.63mM respectively were obtained. As can be seen in Fig. b.11a, 2µM TDP activated the enzyme and reversed the inhibition.

Figs. b.11b and b.11c show the results obtained when the hepatoma and foetal types II pyruvate kinases were estimated as functions of the DL-alanine levels. With the hepatoma type II pyruvate kinase, a $K_s$ value for DL-alanine of 0.52mM and $n_H$ values that changed from 1.7 at the low DL-alanine levels to 0.8 at the high were obtained from the Hill plot of the data (inset, Fig. b.11b). With the foetal type III pyruvate kinase, a $K_s$ value of 0.46mM and $n_H$ values that changed from 1.7 at the low DL-alanine levels to 0.8 at the high ones were obtained from Hill plots of the data (inset, Fig. b.11c). Thus both the hepatoma and foetal type II isoenzymes display positive cooperative interaction at low DL-alanine levels.

The summary of the results in Table b.12 shows that normal adult liver type L pyruvate kinase was more susceptible to AMP inhibition ($K_s$ = 0.63mM) than either the foetal ($K_s$ = 0.46mM) and hepatoma ($K_s$ = 5.6mM) types II pyruvate kinases. The L isoenzyme was also more susceptible to
Fig. 4.14. Effect of DL-alanine and FDP on the pyruvate kinase activity. •—• Control and ◦—◦ 2 μM FDP. Experimental conditions were as in Fig. 4.9.

Inset: Shown plots of data.

(a) Normal adult human liver type I pyruvate kinase.

(b) Hepatoma type II pyruvate kinase.

(c) Human foetal liver type II pyruvate kinase.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>FDP</th>
<th>$K_{i}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal liver type L</td>
</tr>
<tr>
<td>ATP</td>
<td>None</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>0.2 mM</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>4.4</td>
</tr>
<tr>
<td>GTP</td>
<td>None</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>60</td>
</tr>
<tr>
<td>Citrate</td>
<td>None</td>
<td>3.2</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td>12.8</td>
</tr>
<tr>
<td>Alumine</td>
<td>None</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td>High (reversal of inhibition)</td>
</tr>
</tbody>
</table>
to ultrate inhibition \( k_2 = 1.2 \text{mM} \) than either the foetal \( k_2 = 11.5 \text{mM} \) or hepatoma \( k_2 = 12.3 \text{mM} \) isoenzymes. On the other hand, it was less susceptible to ATP \( k_2 = 72 \text{mM} \), but showed a similar sensitivity to DL-
alanine \( k_2 = 0.6 \text{mM} \) inhibition as the N isoenzyme. \( k_2 \) values for
ATP of 52mM and 52mM, whilst \( k_2 \) values for DL-alanine of 0.52mM and 0.10
mM were obtained for hepatoma and foetal types II pyruvate kinases respec-
tively, indicating that the susceptibility of these two isoenzymes to
these inhibitors was similar.

FDP activated normal liver type L pyruvate kinase, decreased the
susceptibility of this isoenzyme to ATP \( k_2 = 4.6 \text{mM} \) and citrate \( k_2 =
11.6 \text{mM} \) inhibition. On the other hand, FDP increased its sensitivity to
ATP \( k_2 = 60.2 \text{mM} \). As a result of this, the \( k_2 \) values obtained resembled
those of hepatoma and foetal types II pyruvate kinases. Although FDP
activated both the hepatoma and foetal types II pyruvate kinases under
certain conditions (see above) it did not alter their \( k_2 \) values for ATP,
ATP and citrate.

0. MgCl\(_2\) requirements

Pyruvate kinase has an absolute requirement for magnesium. How-
ever, in order to assess whether magnesium binds as free magnesium or
complexed with ATP as MgADP, the activity of normal liver type L pyruvate
kinase was estimated as a function of the total MgCl\(_2\) level in the presence
of various constant ATP concentrations. The results thus obtained are
shown in Fig. 14.15 where it can be seen that an increase of activity with
increasing MgCl\(_2\) levels was obtained. Maximum activity was obtained
between 5 and 10mM MgCl\(_2\). At concentrations above 10mM MgCl\(_2\), the
activity decreased steadily in all cases.
Fig. 4.15. Effect of variation in the total magnesium concentration (MgCl₂) on the initial velocity of normal human adult liver type 6 pyruvate kinase at various fixed ADP levels. The reaction mixture contained 50mM triethanolamine-HCl buffer, pH 7.4, 0.1M KCl, 13.5mM EDTA, 0.15mM NADH and 4U LDH, in 1.0ml in addition to MgCl₂, ADP and enzyme, as well as:

(a) 5mM PEP
(b) 1mM PEP
As a 'shift' in the MgCl₂ levels required for optimum activity at various ADP levels could be overlooked over such a wide range of MgCl₂ levels, the activity of liver type L pyruvate kinase was estimated over a narrower range of MgCl₂, i.e., 1-10mM. The results obtained are shown in Fig. 1.16, from which it can be seen that in the presence of 0.15mM ADP, maximum activity was obtained at 1mM MgCl₂, whereas in the presence of 3mM ADP, the MgCl₂ level required for maximum activity was shifted slightly to 5.5mM.

1) The effect of ATP and FDP on the MgCl₂ requirements of liver type L pyruvate kinase

Fig. 1.17 shows the results obtained when the pyruvate kinase activity was estimated as a function of the MgCl₂ concentration in the presence of 1mM FDP, 1mM ADP as well as in the presence of constant levels of either FDP, ATP or ATP + FDP.

From Fig. 1.17, it can be seen that in the absence of effectors, maximum activity was obtained between 5 and 10mM MgCl₂. 0.1mM FDP activated the enzyme and permitted full activity of the enzyme to be obtained at 3mM MgCl₂. 1mM ATP inhibited the enzyme and increased the MgCl₂ optimum to 6mM. On the other hand, addition of 1mM ATP to the reaction mixture not only inhibited the enzyme further, but enhanced the allosteric response of the enzyme at the low MgCl₂ levels and shifted the MgCl₂ optimum to 10mM presumed by combining with the free magnesium. 0.1mM FDP together with 1mM ATP activated the enzyme and shifted the MgCl₂ optimum to 6mM. This activation increased with increased MgCl₂ and only above 5mM MgCl₂ did this activation surpass the activity of the control (no effector). However, the maximum velocity obtained at the optimum MgCl₂ levels, i.e., 5-10mM was less than that obtained in the presence
Fig. 14. Effect of magnesium (mM) concentration on the total radioactive concentration (mM).
Fig. 4.17. Effect of variation in the total magnesium concentration \([\text{MgCl}_2]\) on the initial velocity of normal human adult liver type L pyruvate kinase in the presence of various effectors. \text{o---o} Control, \text{x---x} 0.1mM FDP, \text{A---A} 1mM ATP, \text{A---A} 4mM ATP, and \text{o---o} 4mM ATP + 0.1mM FDP. Reaction mixtures contained 50mM triethanolamine-HCl buffer, pH 7.4, 12.5 mM EDTA, 0.1M KCl, 1mM ADP, 1mM PEP, 0.15mM NADH and 4U lactate dehydrogenase in addition to \text{MgCl}_2, effector and enzyme.
of 0.1mM FDP alone. Although the kinetics obtained in the presence of
0.1mM FDP and 1mM ATP were still sigmoidal, the sigmoidicity was less
than that obtained in the presence of 1mM ATP.

(b) **Effect of ATP and FDP on the MgCl₂ requirements of hepatoma
type M pyruvate kinase**

Fig. 4.18 shows the results obtained when hepatoma type M pyruvate
kinase was estimated as a function of the MgCl₂ level in the presence of
0.09mM FDP and 1mM ATP as well as in the presence of FDP, ATP or FDP +
ATP.

From Fig. 4.18, it can be seen that in the absence of effector,
maximum activity was obtained at MgCl₂ levels above 2mM. 0.1mM FDP had
no effect on the enzyme or on the MgCl₂ level required for optimum
activity. On the other hand, 1mM ATP inhibited the enzyme allosterically
and shifted the MgCl₂ optimum to 5mM. Addition of 0.1mM FDP to the
reaction mixture containing 1mM ATP, eliminated the allosteric inhibition
of the enzyme obtained in the presence of ATP at the low MgCl₂ levels but
had no effect at the high MgCl₂ levels.

(c) **Effect of ATP and FDP on the MgCl₂ requirements of fetal
type M pyruvate kinase**

The results obtained when fetal type M pyruvate kinase activity
was estimated under the same conditions as hepatoma type M pyruvate kinase
are shown in Fig. 4.19, from which it can be seen that in the absence of
effector, maximum activity was obtained at 5mM MgCl₂. Unlike the results
obtained with the hepatoma isoenzyme, it was found that 0.1mM FDP activated
this isoenzyme at levels of MgCl₂ below 5mM. However, 0.1mM FDP had no
effect on the enzyme above 5mM MgCl₂. FDP also shifted the MgCl₂ optimum
Fig. 4.17. Effect of MgCl₂ on the initial velocity of the enzyme in the presence of various effectors. —— Control; —— ATP + 0.1 mM FDP; —— ATP and a —— APN. ATP and a —— APN were used.

Fig. 4.30. Effect of MgCl₂ on the initial velocity of the enzyme in the presence of various effectors. —— Control; —— ATP + 0.1 mM FDP; —— ATP and a —— APN. ATP and a —— APN were used.
Effect of variation in the total magnesium concentration on the initial velocity of human liver type II pyruvate kinase in the presence of effectors. °°° control, ↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓->
slightly to it. On the other hand, 1mM ATP inhibited the enzyme allo-
sterically and shifted the MgCl₂ optimum to 6mM. As with hepatoma type
2 pyruvate kinase, this allosteric inhibition at low MgCl₂ levels could
be eliminated by addition of 0.1mM FDP to the reaction mixture containing
1mM ATP. Again, FDP was found to activate the enzyme that was inhibited
by ATP at low MgCl₂ levels, but to have no affect at the high MgCl₂ levels.

D. Michaelis constants

(a) Normal adult liver type L pyruvate kinase

i. Michaelis constants for ADP and FDP in the absence of FDP

Lineweaver-Burk plots of reciprocal velocity versus reciprocal ADP
concentration at various constant levels of FDP (Fig. 4.20) gave a series
of straight lines that turned up at the high ADP levels, indicating
inhibition at high ADP levels. This inhibition was found to be dependent
on the FDP concentration, being higher at low FDP levels and eliminated at
the higher concentrations of FDP. When the linear portion of these plots
was extrapolated backwards, a series of lines were obtained that cut the
abscissa at different points, showing that the apparent Michaelis constant
for ADP of normal adult liver type L pyruvate kinase varied with the FDP
concentration.

When the data in Fig. 4.20 were replotted as the reciprocal
velocity versus the reciprocal FDP concentration at various constant
levels of ADP, the lines obtained, curved upwards at the low FDP levels
reflecting allosteric kinetics (Fig. 4.21).

The Michaelis constants were obtained from secondary plots of the
Fig. 4.20. Double reciprocal plots of initial velocity vs. ADP concentration at various fixed levels of PEP for normal human adult liver type I pyruvate kinase. Reaction mixtures contained 50mM triethanolamine-HCl buffer, pH 7.4, 10mM 
MgCl₂, 5mM EDTA and 0.1M KCl in 1.0 ml in addition to PEP, ADP, 0.15mM NADH, 
40¹LDH and enzyme. Velocity is expressed as change of OD 240 per minute at 
30°C. The lowest line is the extrapolated line at infinite concentration of PEP.
Double reciprocal plots of initial velocity vs. PEP concentration at various fixed levels of ADP for normal human adult liver type L pyruvate kinase. Conditions were as in Fig. 4.20. The lowest line is the extrapolated line at infinite ADP concentration.

Fig. 4.21.
data where intercepts on the ordinate were plotted against the reciprocal level of the varied substrate according to the method of Florini and Westling (1957). The results obtained from such secondary plots are shown as dotted lines in Figs. 4.20 and 4.21. In order to determine the Michaelis constant for ADP, the intercept on the ordinate (i.e. at infinite FEP levels) were obtained by extrapolating the curves obtained in Fig. 4.21 backwards to cut the ordinate. Similarly, the Michaelis constant for FEP was determined by extrapolating the curve obtained from the secondary plot of the data (i.e. infinite ADP levels) to cut the abscissa. From such replots, Michaelis constants of approximately 0.56mM for ADP and 0.8mM for FEP were obtained.

ii. Michaelis constants for ADP and FEP in the presence of 0.1mM FDP

As FDP shifts the kinetics from sigmoidal to hyperbolic or Michaelis-Menten, the Michaelis constants for ADP and FEP were estimated in the presence of 0.1mM FDP in order to determine how FDP would affect them.

However, as can be seen from Fig. 4.22a, estimation of the pyruvate kinase at various ADP concentrations and different constant FEP levels in the presence of 0.1mM FDP gave rise to non-linear plots. From the Lineweaver-Burk plots of Fig. 4.22a, it can be seen that a series of lines was obtained that curved down at high ADP and FEP levels, reflecting activation. If these lines were extrapolated backwards (these are shown as dotted lines in Fig. 4.22a) they cut the abscissa at different points. At high ADP levels and at concentrations of FEP below 0.11mM, these lines curved upwards indicating inhibition at high ADP levels.

If the linear portion of these lines was extrapolated backwards, these all met at a point on the abscissa except for the line obtained with
Fig. 4.22a. Double-reciprocal plots of initial velocity vs. ADP concentration at various fixed levels of PEP in the presence of 0.1 M FDP for normal adult human liver type I pyruvate kinase. Conditions were as in Fig. 4.20, with the addition of 0.1 mM FDP.

Fig. 4.22b. Replots of vertical intercepts vs. the reciprocal of the PEP concentration.
0.075M and 0.05M PEP. This cut the abscissa at a different point. These results indicate that the apparent Michaelis constant for ADP in the presence of 0.1mM PDP was independent of the PEP levels above 0.11mM PEP and dependent on the PEP concentration below 0.11mM PEP. An apparent Michaelis constant of 0.125mM was obtained for ADP in the presence of 0.1mM PDP and PEP levels above 0.11mM. At 0.075mM and 0.05mM PEP, the apparent Michaelis constant for ADP in the presence of 0.1mM PDP decreased to 0.105mM.

From Fig. 4.22a, it can be seen that similar results were obtained in the presence of 0.075mM and 0.05mM PEP.

Fig. 4.22b shows a replot of the data as intercept on ordinate (obtained from the linear portion of the line) versus the reciprocal PEP level. From this secondary plot, it can be seen that the sigmoidal kinetics displayed by normal liver type L pyruvate kinase with respect to PEP (see Fig. 4.21) were eliminated. Furthermore, a Michaelis constant for PEP of 0.075mM was obtained in the presence of 0.1mM PDP. This is considerably lower than the value of 0.3mM found in the absence of PDP.

(b) Hepatoma type M pyruvate kinase

From Fig. 4.23 it can be seen that Lineweaver-Burk plots of the reciprocal velocity versus the reciprocal ADP concentrations at various constant levels of PEP gave a series of straight lines that met at a point on the abscissa indicating that the Michaelis constant for ADP was independent of the PEP level. From this plot, the Michaelis constant for ADP was found to be 0.26mM.

The replot of the data, as reciprocal velocity versus the reciprocal PEP concentration at various constant levels of ADP, gave a series of
Fig. 4.23. Double reciprocal plots of initial velocity vs. ADP concentration at various fixed levels of PEP for hepatoma type II pyruvate kinase. Conditions were as in Fig. 4.20.
non-linear plots (Fig. 1.26). As no reliable Michaelis constant could be obtained from these results, the data in Fig. 1.25 were replotted as log \((v/V_{max}-v)\) versus log of the substrate concentration. As can be seen in Fig. 1.25 (inset), the Hill plot of these data gave an U-shaped curve where the \(n_H\) value changed from 2 at the low PEP levels to 0.6 and 1.3 at the high PEP levels. A \(K_2\) value of 0.13mM was obtained for PEP from such a plot. Similar \(K_2\) and \(n_H\) values were obtained with l-alanine 0.15mM and 0.25mM ADP.

(c) Postal type H pyruvate kinase

Postal type H pyruvate kinase (that was isolated in a similar manner to the hepatic enzyme) gave straightforward Michaelis-Menten kinetics (Figs. 1.26 and 1.27).

From Fig. 1.26 and Fig. 1.27, it can be seen that the Lineweaver-Burk plots of reciprocal velocity versus the reciprocal ADP concentration at different constant levels of PEP, and the replotted reciprocal velocity versus the PEP concentration at different constant levels of ADP both gave a series of straight lines that met at a point on the abscissa, indicating that the Michaelis constants of either of the two substrates were independent of the level of the other. The Michaelis constants obtained from these plots were 0.055mM and 0.265mM for PEP and ADP respectively. Lines extrapolated to infinite concentration of the second substrate are shown as dotted lines.

2. Product inhibitor studies

The mode of reaction can be determined by studying the effect of...
Fig. 4.24. Double reciprocal plots of initial velocity vs. PEP concentration at various fixed levels of ADP for hepatoma type M pyruvate kinase. Conditions were as in Fig. 4.20. The lowest line is extrapolated to infinite ADP concentration.
Fig. 4.25. Double reciprocal plots of initial velocity vs. PEP concentration at various fixed levels of ADP for hepatoma type II pyruvate kinase. Conditions were as in Fig. 4.20.

Inset: Hill plot of data.
Fig. 4.26. Double reciprocal plots of initial velocity vs. ADP concentration at various fixed levels of PEP for foetal liver type M pyruvate kinase. Conditions were as in Fig. 4.20.
Fig. 4.27. Double reciprocal plots of initial velocity vs. PEP concentration at various fixed levels of ADP for foetal liver type M pyruvate kinase. Conditions were as in Fig. 4.20.
both products (i.e., ATP and pyruvate) on the velocity of the reaction
when one of the substrates is varied while the other is kept at constant
and saturating levels. The effect of pyruvate could however not be
studied with the assay system employed, for the pyruvate kinase activity
was estimated by reducing the pyruvate formed from the pyruvate kinase
reaction to lactate.

Most product inhibitor studies thus far carried out do not take
into account the chelation of free Mg$^+$ by ATP. As the stability con-
stant for MgATP is high, ATP will readily form MgATP complexes in the
presence of Mg$^+$. Therefore if ATP is merely added to the reaction mix-
ture used for the estimation of Michaelis constants, inhibition not only
due to ATP but also due to the chelation of free magnesium by ATP will
be obtained. On the other hand, if one assumes that MgATP will be
obtained if equimolar quantities of Mg$^+$ and ATP are first mixed, then
addition of this solution to the reaction mixture already containing Mg$^+$
will result in inhibition due to MgATP.

In case the mode of ATP inhibition was altered, and in order to
see whether the results obtained in the present study with human pyruvate
kinase were comparable to those obtained from other sources, most of the
product inhibitor studies were carried out by using both the abo-

d

(a) Normal liver type I pyruvate kinase

The results obtained from plots of reciprocal velocity versus the
reciprocal ADP concentration at constant 5mM FEP and various fixed
MgATP levels are shown in Fig. 4.28.

From Fig. 4.28, it can be seen that a series of straight lines
was obtained that cut the ordinate at different points, indicating that
the inhibition between ADP and MgATP was non-competitive. These lines
cut the abscissa at different points and met at a point below the abscissa.
Above 8mM MgATP no further inhibition was obtained. Because of this the
replot of the data as intercept on the ordinate versus the MgATP concen-
tration gave hyperbolic inhibition (Fig. 4.28, inset).

The results obtained when the reciprocal velocity was plotted
against the reciprocal ADP concentration at 1mM FEP and various constant
levels of ATP (Fig. 4.29) were similar to those obtained with MgATP. The
lines obtained cut the ordinate and abscissa at different points and met
at a point below the abscissa. As 1mM FEP was used, the inhibition of
the enzyme at high levels of ADP became apparent. As shown in the inset
of Fig. 4.29, the replot of the data as intercept on the ordinate versus
the ATP concentration gave non-linear inhibition due to the chelation of
the free Mg by ATP.

Fig. 4.30 shows the results obtained when the reciprocal velocity
was plotted against the reciprocal FEP concentration in the presence of
0.1mM ADP and various constant MgATP levels (0.1mM ADP was used as high
ADP levels are inhibitory).

From Fig. 4.30, it can be seen that due to the allosteric prop-
ties of the enzyme with respect to FEP, a series of concave curves were
obtained. These cut the ordinate at a point when extrapolated backward,
indicating that the inhibition between FEP and MgATP was competitive.
Fig. 4.28. Double reciprocal plots of initial velocity vs. ADP concentration at various fixed levels of MgATP for normal adult human liver type I pyruvate kinase. The reaction mixture contained 50 mM triethanolamine-HCl buffer pH 7.4, 5 mM EDTA, 10 mM MgCl₂, 0.1 M KCl and 5 mM PEP in addition to ADP, MgATP, 0.1 mM NADH, 4 U lactate dehydrogenase and enzyme preparation in 1.0 ml. Velocity is expressed as the change of OD₃₄₀ per minute.

Inset. Replot of vertical intercepts vs. MgATP concentration.
Fig. 4.29. Double reciprocal plots of initial velocity vs. ADP concentration at various fixed levels of ATP for normal adult human liver type I pyruvate kinase. Conditions as in Fig. 4.28 except that 1mM PEP and ATP were used.

Inset. Replot of vertical intercepts vs. ATP concentration.
Fig. 4.30. Double reciprocal plots of initial velocity vs. PEP concentration at various fixed levels of MgATP for normal human adult liver pyruvate kinase. The MgATP concentrations were: ○—○ 0, ○—○ 1.0mM and □—□ 2.5mM. The reaction mixture contained 50mM triethanolamine-HCl buffer, pH 7.4, 5mM EDTA, 10mM MgCl₂, 0.1M KCl, and 0.4mM ADP in addition to PEP, MgATP, 0.15mM NADH, 4U LDH and enzyme preparation in 1.0ml. Velocity is expressed as the change of OD 340 per minute.

Inset. Replot of initial velocity vs. PEP concentration.
Furthermore, from Fig. 1.30 as well as the inset, where initial velocity was plotted against the PEP concentration, it can be seen that MgATP enhanced the allosteric interaction of the enzyme with respect to PEP.

(b) Hepatoma type M pyruvate kinase

Fig. 1.31 shows the results obtained when reciprocal velocity was plotted against the reciprocal ADP concentration in the presence of 1mM PEP and various constant levels of MgATP when using hepatoma type M pyruvate kinase. A series of straight lines was obtained that cut the ordinate at different points and not at a point on the abscissa, showing that the inhibition of ADP and MgATP was non-competitive and that the effect is only on the maximum velocity of the reaction. The apparent Michaelis constant for ADP is unaffected by the presence of MgATP. A replot of the data obtained in Fig. 1.30 as intercept on the ordinate versus the MgATP level (inset, Fig. 1.31) gave a straight line that cut the abscissa at a Kₐ value of 36.6mM.

When the reciprocal velocity was plotted against reciprocal ADP concentration at 1mM PEP and various fixed levels of ATP (Fig. 1.32) non-competitive inhibition was obtained. The apparent Michaelis constant was affected by the addition of various fixed levels of ATP to the reaction mixture. A replot of the data as intercept on the ordinate versus ATP gave parabolic inhibition (inset, Fig. 1.32).

Plots of reciprocal velocity versus the reciprocal PEP concentration at 1mM ADP and various constant ATP levels (Fig. 1.33) gave non-linear plots. From the inset, it can be seen that a replot of the data as initial velocity versus the PEP concentration (only results below 0.33 mM PEP shown) gave a series of hyperbolas with "kinks". This deviation
Fig. 4.31. Double reciprocal plots of initial velocity vs. ADP concentration at various fixed levels of MgATP for hepatoma type M pyruvate kinase. Conditions were as in Fig. 4.28 except that 1mM PEP was used.

Inset: Replot of vertical intercepts vs. MgATP concentration.
Fig. 4.32. Double reciprocal plots of initial velocity vs. ADP concentration at various fixed levels of ATP for hepatic type II pyruvate kinase. Conditions as in Fig. 4.28 except that 1 mM PEP, and ATP were used.

Inset. Replot of vertical intercepts vs. ATP concentrations.
Fig. 4.33. Double reciprocal plots of initial velocity vs. PEP concentration at various fixed levels of ATP for hepatoma type N pyruvate kinase. The ATP concentrations were: o—o 0; x—x 1mM o—o 2mM, Δ—Δ 3mM and □—□ 4mM. Conditions as in Fig. 4.30 except that 1mM ADP was used. 
Inset. Replot of data as initial velocity vs. PEP concentration.
from an ordinary Michaelis-Menten hyperbola became more pronounced with increased ATP levels.

Insufficient fontal enzyme was available to carry out analogous experiments.

P. Isotope exchange rates

The results of isotope exchange studies at equilibrium are shown in Figs. h.34 – h.36.

(a) $^{14}$C-pyruvate to PEP exchange rates

From the plots of reciprocal exchange rate versus the reciprocal substrate concentration and from the plots of exchange rate versus the logarithm of the substrate concentration, it can be seen that with both normal liver type I pyruvate kinase (Fig. h.34a) and hepatoma type II pyruvate kinase (Fig. h.34b), the rate of exchange between $^{14}$C-pyruvate and PEP could be eliminated at the high MgATP/ADP levels when raising the concentration of MgATP and ADP in a constant ratio (100) and keeping equilibrium conditions.

(b) ATP-$^{32}$P to PEP exchange rates

Again from plots of reciprocal exchange rate versus the reciprocal substrate concentration and from plots of exchange rate versus the logarithm concentration of substrate, it can be seen that with both normal adult human liver type I pyruvate kinase (Fig. h.35a) and hepatoma type II pyruvate kinase (Fig. h.35b), the exchange rate between ATP-$^{32}$P and PEP decreased at the high pyruvate-ADP levels when the pyruvate/ADP concentrations were increased in a constant ratio of 3:1. On the other hand, when the MgATP/PEP levels were raised in a constant ratio of 2:1, the rate of exchange was enhanced with both the normal type I and hepatoma type II pyruvate kinases (Figs. h.35a and b).
Fig. 4.34. Isotope exchange rates of $^{14}\text{C}$-pyruvate into PEP when varying MgATP/ADP. The reaction mixture contained 50mM glycine-NaOH buffer, pH 9.0, 12.5mM MgCl$_2$, 0.025mM EDTA, 7.4mM pyruvate, 2mM PEP and MgATP/ADP in a 100:1 ratio in addition to the enzyme preparation.

(a) Normal adult human liver type I pyruvate kinase.
(b) Hepatoma type II pyruvate kinase.
Isotope exchange rates of ATP-$\gamma$-32P into PEP when varying pyr/ADP. The reaction mixture contained 50mM glycine-NaOH buffer, pH 9.0; 12.5mM MgCl$_2$, 0.25mM EDTA, 2mM ATP, 2mM PEP and pyr/ADP in a ratio of 370:1 in addition to the enzyme preparation.

(a) Normal human adult liver type K pyruvate kinase.
(b) Hepatoma type M pyruvate kinase.
Fig. 4.36. Isotope exchange rates of ATP-$\gamma$-$^{32}$P into PEP when varying MgATP/PEP. The reaction mixture contained 50mM glycine-NaOH buffer pH 9.0, 12.5mM MgCl$_2$, 0.25mM EDTA, 74mM pyruvate, 0.4mM ADP and MgATP/PEP in a ratio of 2:1 in addition to the enzyme preparation.

(a) Normal adult human liver type L pyruvate kinase.
(b) Hepatoma type M pyruvate kinase.
Discussion

Enzyme purification

Type L pyruvate kinase was partially purified from normal adult human liver. The initial specific activity of 0.06 U/mg protein obtained with crude extracts of normal adult human liver was found to be 10 times lower than that of rat liver (Tanaka et al., 1967a).

The first few steps used in the purification of type L pyruvate kinase from normal adult human liver viz. the ammonium sulphate and acetone fractionation steps were similar to those described by Tanaka et al. (1967a) for rat liver type L pyruvate kinase. The types L and M pyruvate kinases present in liver were then separated by cutting finer ammonium sulphate fractions as described by Passarone et al. (1967). The type L was precipitated between 0-37% ammonium sulphate. This method of separating the two isoenzymes was preferred to the DEAE chromatographic one which is generally used for separating these two isoenzymes. It was found to be quicker. It eliminated the step of having to concentrate the enzyme after elution and the difficulty often encountered, namely the loss of enzyme activity with dilution of the enzyme. This method gave as good a separation of the isoenzymes, purification and yield as the DEAE-cellulose method used by Tanaka et al. (1967a), Susor and Rutter, (1968), Carminatti et al. (1968) and Campos et al. (1965). Furthermore, in the present study, it was found that with DEAE chromatography of liver pyruvate kinase, the main fraction that contained a high percentage of pyruvate kinase activity was not retained on the column. Electrophoresis of this fraction revealed the presence of both types M and L pyruvate kinases. The failure to retain
type L pyruvate kinase on the DEAE-Sephadex column could be due to the isoelectric point of human type L pyruvate kinase being different to that of rat. On the other hand, it could be due to insufficient dialysis, although the first ammonium sulphate fraction that was used for this step was dialyzed for 3 hours. Furthermore, samples that were dialyzed for 3 hours were also used for chromatography on the other ion exchangers onto which the enzyme did adsorb. The last step used in the purification of human adult liver type L pyruvate kinase was chromatography on Sephadex G-200 that was equilibrated with ammonium sulphate as described by Taylor et al. (1969).

Type II pyruvate kinase was partially purified from the cancerous portion of human liver obtained from human casualties. The initial specific activity of 0.8 U/mg protein obtained with crude extracts of hepatoma pyruvate kinase was found to be considerably higher than that obtained with crude extracts of normal adult human liver pyruvate kinase. Unlike the pyruvate kinase isolated from normal adult liver, which could be precipitated between 25 - 37% ammonium sulphate, the tumour enzyme was only precipitated between 50 - 70% ammonium sulphate. In this respect the enzyme isolated from hepatoma resembles that purified by Taylor et al. (1969) from rat 392H hepatoma. The pyruvate kinase isolated from these primary human hepatomas also resembles muscle (Tanaka et al., 1967a), leucocyte (Koller et al., 1958) and adipose tissue (Fugon, 1968) pyruvate kinase with regard to its solubility in ammonium sulphate. Both normal adult liver type L and hepatoma types II pyruvate kinases were isolated in the same fraction, after elution from Sephadex G-200. It would thus appear that the size of these two isoenzymes is similar.
Although tumour tissue has often been considered to be similar to foetal tissue, only 21% of the total pyruvate kinase activity was recovered in the 50 - 70% ammonium sulphate fraction of crude extracts of human foetal liver. It would thus appear that, unlike hepatoma pyruvate kinase, this isoenzyme does not constitute the major proportion of the pyruvate kinase activity in these 5 months old human fetuses. Furthermore, Llorente et al. (1970) have shown that type 1 pyruvate kinase develops in rat foetal liver before gluconeogenesis.

The percentage contribution by enolase, adenylate kinase and aldolase of these three partially purified pyruvate kinases was found to be low.

2. **Stability of liver pyruvate kinase**

Normal human liver pyruvate kinase, like rat liver pyruvate kinase, could be stabilized with 0.5M sucrose and with 0.5M ammonium sulphate on Sephadex G-200 (cf. Susor and Rutter, 1966 and Taylor et al., 1969). Low levels of sucrose, MgCl$_2$ or ADP failed to protect the enzyme against inactivation. This is contrary to the findings of Tanaka et al. (1967a) who found that inactivation of rat liver pyruvate kinase could be checked with 5mM MgCl$_2$ and 5mM ADP. Ibsen et al. (1967a) have demonstrated that magnesium, mercaptoethanol and inorganic phosphate have an additive effect in stabilizing erythrocyte pyruvate kinase. They also showed DTPA to be a more effective stabilizer than either ADP or inorganic phosphate.

The present results would suggest that both sucrose and ammonium sulphate favour the more stable form of the enzyme, either by preventing
dissociation of the enzyme into inactive subunits or by preventing conformational changes. This could be affected by high ammonium sulphate levels producing a high ionic environment, or by sucrose serving as a support or matrix in keeping the enzyme in its stable conformation.

The possibility of normal human liver pyruvate kinase inactivation being due to oxidation of sulphhydryl groups, or caused by the digestion of pyruvate kinase by proteolytic enzymes can probably be excluded for DNP in the presence of low levels of sucrose and L-α-amino-N-caproic acid respectively, both failed to stabilize the enzyme. Although Ibsen et al. (1968) also failed to stabilize erythrocyte pyruvate kinase with L-α-amino-N-caproic acid, these authors as well as Rosenkurt et al. (1969) found that sulphhydryl compounds such as mercaptoethanol or DMT did afford some protection against inactivation of erythrocyte and rat liver pyruvate kinase.

3. Kinetic parameters

(a) Factors affecting the binding of FDP

The present results show that human liver type L pyruvate kinase is a regulatory enzyme. This is evidenced by the finding that the enzyme displays allosteric properties with respect to its substrate FDP, feed-back inhibition by allosteric inhibitors such as ATP and alanine, and feed-forward activation by FDP. Pyruvate kinases from some other sources have also been found to display sigmoidal kinetics that could be affected by ATP, alanine and FDP. These include those isolated from rat liver (Tanaka et al., 1973b, Llorente et al., 1970, Susan and Butter...
1968, Rosemurgy et al., 1969 and Carminatti et al., 1968), yeast (Bess et al., 1966 and Heickel et al., 1968), rat adipose tissue (Pagana, 1969 and Marco et al., 1971) and human erythrocytes (Staal et al., 1971).

FDP activates the enzyme by changing the kinetic pattern from sigmoidal to hyperbolic and lowering the \( K_m \) for FEP from 0.79mM to 0.11mM (Fig. i.6 and i.5). Hence the enzyme can function at its maximal capacity at a lower FEP level in the presence of FDP. FDP also appears to have an effect on the kinetic parameters of the other substrate of the reaction, ADP. The substrate inhibition found at high levels of ADP for concentrations of FEP between 0.2 and 1mM (Fig. k.20a) is eliminated by FDP (Fig. k.22a). At these FEP concentrations, FDP reduces the affinity of the enzyme for ADP, as shown by higher apparent \( K_m \) values for ADP (dotted lines in Fig. k.22a). At the same time, the maximum velocity of the reaction is increased under these circumstances, showing activation by FDP under conditions of high ADP and high FEP concentrations.

In addition to FDP, other hexose phosphates such as fructose-6-phosphate, fructose-1-phosphate, glucose-1-phosphate and 2-deoxyglucose-6-phosphate were also found to activate this isoenzyme. These were not as potent as FDP. However, the first three were found to activate the enzyme to the same maximal level as FDP and not less, as reported by Eggelston and Woods (1970) for rat liver pyruvate kinase. The finding that glucose-1-phosphate was less effective than the other hexose phosphates in activating this isoenzyme is in agreement with the findings for rat liver pyruvate kinase (Eggelston and Woods, 1970). Rat liver pyruvate kinase has also been found to be activated by
fructose-1,6-diphosphate, fructose-6-phosphate, glucose-6-phosphate, fructose-1-phosphate and glucose-1-phosphate (Taylor and Bailey, 1967 and Eggleston and Woods, 1970), whereas yeast pyruvate kinase is activated only by fructose-1,6-diphosphate and glucose-1-phosphate (Haacke et al., 1968). Glucose-6-phosphate and fructose-1-phosphate have also been shown to activate human erythrocyte pyruvate kinase (Staal et al., 1971). The finding that glucose, fructose and galactose-6-phosphate had no effect on this isoenzyme agrees with the findings for yeast pyruvate kinase (Haacke et al., 1968).

Other precursors of FDP in the glycolytic pathway such as glyceraldehyde-3-phosphate, dihydroxyacetone phosphate and glycerate-2-phosphate were also found to activate this isoenzyme. Of these, dihydroxyacetone phosphate was the most potent. However, only low levels of this effector could be used, for it was found that above 1.2 mM dihydroxyacetone phosphate there was a steady increase in the activation with time. Other triose phosphates such as glycerol-1-phosphate and glycerate-3-phosphate had no effect on the enzyme. The activation obtained in the presence of glycerate-2-phosphate cannot be attributed to enolase activity, for a blank containing all the reagents except FDP was included. Weber (1969) has also indicated that glyceraldehyde-3-phosphate may act as a modulator molecule by activating pyruvate kinase. However, Taylor and Bailey (1967) found no activation of rat liver pyruvate kinase by glycerate-2-phosphate or 2,3-diphosphoglycerate.

The human liver type L isoenzyme was inhibited allosterically by ATP, alanine, AMF and citrate. DL-Alanine (K_i = 0.62 mM) and ATP (K_i = 2.63 mM) were found to be more potent inhibitors than citrate.
(Kₐ = 3.2mM) whereas AMP (Kₐ = 72mM) was the least effective.

FDP partially reversed the inhibition by ATP, AMP and citrate and increased the Kₐ values for these compounds, so that they became similar to those of foetal and hepatoma types II pyruvate kinases. However, the alanine inhibition was completely reversed by FDP. Weber (1969), has shown that rat liver pyruvate kinase is not inhibited by L-alanine. If the human type L pyruvate kinase resembles that of the rat in this respect and, as DL-alanine was used in the present study, then this isoenzyme would be more sensitive to alanine rather than ATP inhibition. This and the postulate made above, namely that these inhibitors act at different sites (Fig.4.10) could then explain why the ATP- and alanine-inhibited enzymes responded differently to FDP, though their Kₐ values were found to be similar (Fig.4.11a and b.11a). As in the present study, the ATP inhibition of rat liver pyruvate kinase is also partially overcome by FDP (Nozamurt et al., 1969 and Llorante et al., 1970).

Type L pyruvate kinase predominates in liver, where not only glycolysis but also gluconeogenesis occurs. Since the enzyme is under the control of various metabolites, the amount of FDP being utilized via these two pathways can be closely regulated. Under gluconeogenic conditions, the FDP levels are depressed due to enhanced fructose-1,6-diphosphatase and reduced phosphofructokinase activities. Because of this, the isoenzyme would no longer be activated by FDP. It would assume the conformation that displays sigmoidal kinetics, has a higher Kₐ for FDP, and hence requires more FDP to saturate it. It would also become more susceptible to feed-back inhibition by ATP, citrate and alanine. As a result of all these effects, less FDP would
be utilized via the pyruvate kinase reaction, thus making more available for the gluconeogenic pathway. On the other hand, under glycolytic conditions, the PEP levels would be elevated. As a result of this, the enzyme would be activated, the \( k_2 \) for PEP would be lowered, and the susceptibility of the enzyme to feedback inhibition by ATP, and citrate would be lowered. Hence more PEP can be utilized by pyruvate kinase, making less available for gluconeogenesis.

The properties of the hepatoma and foetal type \( H \) pyruvate kinases differ from those of the \( L \) type pyruvate kinase. Unlike the \( L \) type pyruvate kinase, the \( H \) type isoenzymes have properties that indicate that they are not regulatory. This is suggested by the finding that the activities of both these \( H \) types pyruvate kinases were not affected by the presence of various hexose phosphates or triose phosphates, showing that these \( H \) type isoenzymes are not subject to feed-forward regulation by PEP precursors. In this respect, these \( H \) type isoenzymes resemble the type \( H \) pyruvate kinases isolated by Jiménez De Anda et al. (1971b) from rat liver and muscle, which were unaffected by glucose-1-phosphate, fructose-1,6-diphosphate and glyceraldehyde-3-phosphate.

On the other hand, these \( H \) type isoenzymes were inhibited by the same inhibitors as the \( L \) isoenzyme, i.e. ATP, AMP, citrate and alanine. These \( H \) type isoenzymes were most sensitive to alanine inhibition.

The \( k_2 \) value for L-alanine for the hepatoma enzyme was 0.52mM and for the foetal enzyme 0.12mM which is similar to the value of 0.63mM found for the liver type \( L \) isoenzyme. They showed less susceptibility to ATP with \( k_2 \) values for the hepatoma enzyme of 5.2mM and for the foetal enzyme of 5.6mM, and to citrate inhibition where \( k_2 \) values of 11.5mM.
and 12.9% for the hepatoma and fetal enzymes respectively were found. These values are higher than those for the liver isoenzyme. The H isoenzymes were least susceptible to AMP inhibition. The $K_a$ values for $\text{AMP}$ of 55 and 52X for the hepatoma and fetal type H enzymes resemble the values for the L isoenzyme.

As in the present study, the L type isoenzyme isolated from rat livers has also been found to be more sensitive to ATP inhibition than the muscle type H isoenzyme (Ponsen et al., 1967a and b, Sauer and Matter, 1968 and Weber, 1969). Although Taylor et al. (1959) also found rat muscle pyruvate kinase to be less susceptible to ATP inhibition ($K_a = 1.7 \times 10^{-2} M$) than the liver type L isoenzyme ($K_a = 5.6 \times 10^{-3} M$), they found the rat hepatoma isoenzyme ($K_a = 4.3 \times 10^{-3} M$) to resemble that isolated from liver. As the sensitivity of pyruvate kinase to these inhibitors could affect the rate of utilization of FDP, the lower susceptibility of the human hepatoma isoenzyme to ATP inhibition as compared to the liver type L isoenzyme would be more in line with the reputed high glycolytic capacity of some cancer cells.

Unlike the findings with normal liver type L pyruvate kinase, the ATP- and AMP-inhibited H isoenzymes were unaffected by the presence of FDP. FDP did however partially reverse the citrate inhibition at high levels of citrate. This again indicates that the controlling mechanism which determines the flux of FDP is altered as compared to that of the liver type L isoenzyme. However, as is shown in Chapter II and as is generally reported (see introduction) gluconeogenesis is low or absent in hepatomas and fetal livers. Hence controlling mechanisms that are required to divert FDP into gluconeogenesis would
not be as important in these tissues as they are in gluconeogenic tissues such as normal adult liver.

The alanine inhibition of these type M pyruvate kinases differed from that obtained with the other inhibitors mentioned in that all three isoenzymes tested were equally susceptible to this inhibitor. Furthermore, the alanine inhibition of the type M isoenzymes could be reversed by FDP. Contrary to the findings in the present study, Taylor et al. (1969) found pyruvate kinase from 392A rat hepatoma to be more susceptible to alanine inhibition ($K_I = 3.0 \times 10^{-4} M$) than either the muscle ($K_I = 2 \times 10^{-2} M$) or liver ($K_I = 2.5 \times 10^{-3} M$) pyruvate kinases. As in the present study these authors found that the tumour and liver pyruvate kinases could be protected against alanine inhibition. However, they found that FDP had no effect on the alanine-inhibited muscle pyruvate kinase.

The properties of the hepatoma and foetal types M pyruvate kinases were similar in many respects but not identical. For instance, when the pyruvate kinase activity was estimated as a function of the FDP level, the foetal but not the hepatoma type M isoenzyme could be activated at low FDP levels by FDP (Fig. 6.6 and 6.7). Also, when the pyruvate kinase activity was determined as a function of the ATP concentration, FDP was found to reverse the inhibition partially at high ATP levels of the foetal type M isoenzyme but not of the hepatoma enzyme (Fig. 6.12b and c). This suggests that these two type M isoenzymes might be slightly different.

These results are in general consistent with the Monod-Vyman-Changeux (1965) model. According to this theory, the protein exists in two interconvertible forms, namely an active and an inactive form.
The equilibrium between these two states may be shifted by ligand binding preferentially to a particular form. Thus, the nature of the saturation curve for the ligand will depend on the relative proportion of these two forms.

The present results show that normal liver type I pyruvate kinase exists mainly in the inactive conformation. This is indicated by the finding that this enzyme shows co-operative interaction with respect to its substrates, FDP. An \( n_H \) value of 2 was obtained when the pyruvate kinase activity was estimated as a function of the FDP level, indicating that at least 2 moles of FDP must bind per mole of enzyme for full activity (Fig. 4.b, inset and Fig. 4.5b). Inhibitors such as ATP and alanine shift the equilibrium more towards the inactive conformation, as shown by the finding that these compounds increase the allosteric interaction of the enzyme with respect to FDP. In the presence of ATP or DL-alanine, \( n_H \) values of approximately 3 were obtained, indicating that as many as 3 moles of FDP per mole of enzyme must be bound in order to retain the enzyme in its active conformation (Fig. 4.b, inset and Fig. 4.5b). Only 1 mole of inhibitor per mole of enzyme need be bound, for \( n_H \) values of approximately 1 were obtained when the pyruvate kinase activity was estimated as a function of the inhibitor level (insets, Figs. 4.11a - 4.11b). On the other hand, the allosteric effector FDP shifts the equilibrium to the active form. Only 1 mole of FDP per mole of enzyme is necessary to convert the enzyme to its active conformation, for \( n_H \) values of 0.5 were obtained when the activity of the enzyme was estimated as a function of the FDP level (inset, Fig. 4.10). When in this active form, only 1 mole of FDP per mole of enzyme is sufficient to give full enzyme activity (\( n_H = 0.8 \)).
(inset, Fig. 4.4). However, in order to shift the equilibrium back to the inactive conformation when FDP is present, 2-3 moles of inhibitor (ATP, AMP, alanine or citrate) must be bound. This is indicated by the finding of $n_M$ values of 2.0 - 2.5 when the pyruvate kinase activity was estimated as a function of the inhibitor concentration (inset, Figs. 4.11a - 4.11a). Conversely, in the presence of these inhibitors, the equilibrium can be shifted back to the active conformation by the enzyme binding 2-3 moles of FDP per mole of enzyme. $n_M$ values between 1.5 and 2.5 were obtained when the pyruvate kinase activity was estimated as a function of the FDP level in the presence of inhibitors (inset, Fig. 4.10).

The kinetics of the $M$ type isoenzyme resemble those of the activated type $L$ isoenzyme. Like the activated type $L$ isoenzyme, they have a low $K_M$ for FDP and a similar sensitivity to ATP, AMP, and citrate inhibition. Furthermore, Hill numbers greater than 1 were obtained when the activity of these $M$ type pyruvate kinases were estimated as a function of the inhibitor levels (Figs. 4.11 - 4.11b and c). Hill numbers greater than 1 were also obtained when the normal adult liver type $L$ pyruvate kinase was estimated under similar conditions but in the presence of FDP. These factors, together with the finding that FDP has no effect on the hepatoma and fetal types $M$ pyruvate kinases suggest that these enzymes normally occur in the active conformation. However, inhibitors such as ATP, AMP, citrate and alanine can still shift the equilibrium to the inactive form, though higher concentrations of ATP and citrate are required to inhibit the $M$ isoenzymes than the $L$ isoenzyme.

The Monod-Wyman-Changeux model represents a special case of the
more general theory proposed by Koshland et al. (1965) for mechanisms of allosteric interaction of enzymes. According to the Monod-Hyman-Changoux model, all subunits have equal affinity for a particular ligand irrespective of how many molecules are bound. On the other hand, the Koshland theory postulates that interaction of a molecule of ligand with one subunit of enzyme results in a conformational change as a result of which further ligands may bind more readily (positive co-operativity) or less easily (negative co-operativity). The Hill plots of changing slopes obtained in some cases and the non-linear Lineweaver-Burk plots of reciprocal velocity versus the reciprocal FDP concentration (inset, Fig. 1.25) could indicate that there are different binding constants at different substrate concentrations. However, detailed binding studies would be required to distinguish between these models. Such studies could also give further information as to whether each effector has a separate binding site or whether some of these are shared. The results of Fig. 1.10 do however show that ATP and alanine occupy separate binding sites. Furthermore, the finding that the H type isoenzymes are inhibited by the same inhibitors as the L isoenzyme, would suggest that these could have the same inhibitor binding sites as the L isoenzyme. Moreover, the finding that the alanine inhibition of both the hepatoma and fasted types H isoenzymes could be completely reversed by FDP (Fig. 1.11b and c) indicates the presence of an FDP binding site in these as well. The presence of such a site is also indicated by the finding that the citrate inhibition of both these H type pyruvate kinases could be reversed by FDP at high citrate levels (Fig. 1.11b and c). It is also indicated by the finding that the ATP inhibition of the fasted type H pyruvate kinase could be reversed at high ATP levels (Fig. 1.11c).
(b) The effect of magnesium concentration

From the results it is seen that normal liver type I, hepatoma and foetal types III pyruvate kinases all have an absolute requirement for magnesium. Furthermore, they were all found to display optimum activity in the presence of 1–10 mM MgCl₂. As the magnesium level was raised above 10 mM, the activity of liver type I pyruvate kinase was found to decrease in the presence of 0.1, 0.5 or 1 mM ADP (Fig. 4.15). This decrease in the pyruvate kinase activity is presumably due to inhibition of the enzyme by excess magnesium and not solely to ionic strength effects as suggested by Holmsen and Storm (1969) for rabbit muscle pyruvate kinase, since the activity of this enzyme was estimated at 0.14 KCl which already has a high ionic strength of 0.2.

The present results do not show clearly whether free Mg²⁺, ADP or MgADP are the substrates. When the activity of the liver type I isoenzyme was estimated at various MgCl₂ levels in the presence of 0.15 mM and 3.0 mM ADP respectively, a shift of the MgCl₂ optimum from 1 mM to 5.5 mM was observed (Fig. 4.16), indicating that MgADP may be the favoured substrate. Melchior (1965) had earlier concluded from kinetic studies with rabbit muscle pyruvate kinase that the magnesium nucleotide is the favoured substrate, but omitting to calculate the concentration of MgPSP in his mixture, considering only free Mg²⁺ and MgADP. On the other hand, Kildjian and Cohen (1966 and 1970) have shown by nuclear magnetic resonance studies that both free Mn²⁺ and ADP, and the MgADP complex can bind to the enzyme which therefore differs from creatine kinase where magnesium nucleotide is an obligatory substrate. Because of this, and as it is difficult to evaluate the contribution of other species such a KDP, MgPSP, HEP and free K⁺. . . in this system,
it was considered advisable to use total concentrations of ADP, Mg$^+$
and PEP in the evaluation of kinetic parameters. Moreover, as dis-
cussed later (page 167), the kinetic and isotope exchange data suggest
an ordered reaction mechanism with PEP binding before ADP. Since Mg$^+$
is also required to bind PEP as shown in the mechanism proposed by
Mildvan and Cohn (1956), it would seem reasonable to assume that Mg$^+$
could bind to the enzyme before ADP. Furthermore, estimation of the
Mg$^+$ and nucleotide levels in vivo also indicate that the free nucleotides
could act as natural substrates for the enzyme under certain conditions.
Although the physiological level of Mg$^+$ is about 0.3 mM (Kerson et al.,
1967), Günther (1967) has estimated the free intracellular Mg$^+$ level
to be as low as 1.5 mM in rat liver and ascites tumours. As the
physiological levels of ADP vary from 0.2 mM - 3 mM, and the ATP levels
from 0.5 mM - 10 mM (Kerson et al., 1967), and as there are a large number
of other sites that bind Mg$^+$, it is obvious at high nucleotide low Mg$^+$
levels, these nucleotides could well exist in their free forms in vivo.

The magnesium requirements of the normal adult human type L,
hepatoma and fetal types H pyruvate kinases were similarly affected by
the presence of ATP. When the activity of all these three isoenzymes
was estimated as a function of the MgCl$_2$ concentration in the presence
of ATP (Fig. 4.17 - 4.19), it was found that ATP increased the magnesium
level required for maximum activity presumably by binding the magnesium.
ATP also increased the allosteric interaction of these isoenzymes with
respect to magnesium, indicating that in the presence of ATP more
magnesium must be bound per mole of enzyme in order to obtain full
activity of these isoenzymes. ATP also decreased the maximum velocity
in all cases. As the maximum velocity was lower in the presence of
ATF than in its absence, and as all the ATP at the high magnesium levels is in the form of MgATP, these results suggest inhibition of these isoenzymes by MgATP. This would agree with the findings of Holmsten and Storm (1969) and Boyer (1969) who also showed MgATP inhibition of rabbit muscle pyruvate kinase. However, this suggestion is contrary to the findings of Woods (1968) who proposed that earlier published results showing ATP inhibition represented an artifact due to the removal of Mg⁺ by ATP.

FDP affected the magnesium levels required for maximum activity of the liver type L and hepatoma and fetal type H isoenzymes differently. FDP not only activated the L isoenzyme but decreased the magnesium level required for maximum activity as well as the allosteric interaction of the isoenzyme with respect to MgCl₂, both in the presence and absence of ATP (Fig. 1.17). The present results would suggest that in the presence of FDP, this isoenzyme would be able to function at its maximal capacity at lower magnesium levels in vivo.

Unlike the liver type L isoenzyme, the kinetics of the hepatoma type H isoenzyme with respect to magnesium were not affected by the presence of FDP (Fig. 1.18). FDP did however, eliminate of sigmoidal response of this isoenzyme to MgCl₂ in presence of ATP. These findings again suggest the presence of an allosteric effector site on this isoenzyme. They also show that the allosteric response of this isoenzyme to magnesium in the presence of ATP cannot be entirely attributed to removal of magnesium by ATP, for FDP does not alter the magnesium requirements of this isoenzyme in the absence of ATP.

It was found that the magnesium levels required for maximal
activity of the foetal type \( M \) isoenzyme were decreased in the presence of FDP. In this respect, this isoenzyme resembles adult liver type \( L \) pyruvate kinase, but differs from the hepatoma type \( M \) isoenzyme which was not affected by the presence of FDP. This again indicates that the hepatoma and foetal isoenzymes are not identical. The foetal type \( M \) isoenzyme did however resemble the hepatoma type \( M \) isoenzyme in the respect that the allosteric interaction displayed by this isoenzyme with respect to magnesium in the presence of ATP could be eliminated by FDP.

Hence from the above it can be seen that the activity of these isoenzymes could be altered by ATP and FDP in vivo partly by the ability of these substances to alter the magnesium requirements of these isoenzymes.

(c) Michaelis constants

The kinetics and Michaelis constants of all three preparations were found to differ. Although all three preparations displayed Michaelis-Menten kinetics with respect to ADP, only normal liver type \( L \) pyruvate kinase was inhibited at the high ADP levels and had Michaelis constants for ADP which varied with the PEP level (Fig. I.20). Similar results have been reported for human erythrocyte pyruvate kinase by Stea al. et al. (1971) as well as for rat liver type \( L \) pyruvate kinase by Tanaka et al. (1967a). The limiting Michaelis constant for ADP of normal adult liver type \( L \) pyruvate kinase was found to be higher (0.56mM) than that of the hepatoma (0.26mM) and foetal (0.28mM) types \( M \) pyruvate kinases. It was also higher than the apparent Michaelis constant for ADP (0.1mM) obtained by Tanaka et al. (1967a) for rat liver type \( L \) pyruvate kinase. However, it was similar to that obtained by Stea et
al. (1971) for human erythrocyte pyruvate kinase and by Pogson (1968) for rat adipose tissue PyK B (0.67 mM). On the other hand, in the presence of 0.1 mM FDP, the Michaelis constant for ADP of normal adult liver type I pyruvate kinase decreased to 0.1 mM, thus resembling that of rat liver type I pyruvate kinase in the absence of FDP (Tanaka et al., 1967a), but being lower than that obtained for the hepatoma and foetal types II pyruvate kinases. The Michaelis constants obtained with the hepatoma and foetal types II pyruvate kinase were similar to those of rat muscle (Tanaka et al., 1967a), rat adipose tissue PyK B (Pogson, 1968), rabbit muscle (Heynald et al., 1961, Mldvan and Cohn, 1966 and Holmsen and Storm, 1969), and that found for human erythrocytes by Tsen et al. (1968).

Although the hepatoma type II pyruvate kinase resembled the foetal isoenzyme with respect to its Michaelis constant for ADP, their kinetics with respect to FEP as well as those of human adult liver type I pyruvate kinase all differed.

The Michaelis constant for FEP, viz. 0.8 mM, of normal adult human liver type I pyruvate kinase was higher than the Michaelis constant for FEP (0.058 mM) of foetal type II pyruvate kinase, as well as the Ki for FEP of 0.11 mM obtained with the hepatoma preparation. However, in the presence of 0.1 mM FDP, the Michaelis constant for FEP of normal human adult type I pyruvate kinase decreased to 0.038 mM, thus resembling that obtained for hepatoma and foetal types II pyruvate kinases closely.

Of the three pyruvate kinase preparations tested in the present study, only the foetal type II pyruvate kinase displayed simple Michaelis-Menten kinetics with respect to FEP (Fig. 1.27). This, together with
the finding of a Michaelis constant of 0.05mM for PEP as well as the finding that the Michaelis constant was independent of the ADP level agrees with the findings in rat and rabbit muscle (Tanaka et al., 1967a, Tietz and Dobos 1959, Taylor et al., 1969 and Raymond et al., 1961). Similar kinetics with respect to PEP have also been reported for human leucocyte pyruvate kinase by Campos et al. (1963), erythrocyte pyruvate kinase by Ibsen et al. (1968), hepatoma pyruvate kinase by Taylor et al. (1969) and adipose tissue PyK B by Pogson (1968).

On the other hand, normal adult human liver type I pyruvate kinase displayed sigmoidal kinetics with respect to PEP (Fig. 4.21). This, together with the finding of a high Michaelis constant (0.8mM) for PEP is consistent with the findings in human erythrocyte by Staal et al. (1971), adipose tissue PyK A (Pogson, 1968) and rat liver pyruvate kinase (Tanaka et al., 1967a and b, Susan and Rutter, 1968, Rosengart et al., 1969, Carminatti et al., 1968 and Taylor et al., 1969).

The kinetics of hepatoma type N pyruvate kinase not only differed from the foetal type N and normal adult liver type I pyruvate kinase, but were also different to those described by Taylor et al. (1969) for pyruvate kinase isolated from 3924A rat hepatoma. Whereas in the present study, non-Michaelis Menten kinetics were obtained (Fig. 4.21), these authors found typical hyperbolic kinetics. Both the results obtained from the Lineweaver-Burk plots and the Hill plots (Fig. 4.25 and inset) point to the presence of more than one binding site for PEP. One cannot, however, distinguish from these data whether these are separate allosteric and catalytic sites or merely catalytic sites whose affinity for PEP is altered due to conformational changes occurring in the protein. It could be argued that the presence of two isoenzymes with different affinities for PEP would also give similar results. However, in the
present study, the L isoenzyme was separated from the M in the ammonium sulphate fractionation step. Furthermore, type M migrated as a single protein under the electrophoretic conditions used in the present study. Hence the only possibility of this preparation not being homogenous (due to the presence of different pyruvate kinase isoenzymes) would be, if two very similar type M pyruvate kinases were present that would require more sensitive techniques to separate. The finding that the $p_H$ value changes from 0.6 and 1.3 at the high FEP levels to 2 at the low FEP level could indicate that this enzyme also shows mixed cooperativity with respect to FEP. The presence of both negative and positive cooperativity is not uncommon, and has been reported for UTP-synthetase by Levitzki and Koshland (1970).

Hence, although tumour tissue has often been associated with a reversion to a more immature state, from the present results it would appear that it does not simply revert to the foetal type. The complex kinetics displayed by the enzyme with respect to FEP would probably ensure the efficient utilisation of both high and low levels of FEP in the glycolytic pathway, and thus limit its utilisation via gluconeogenesis. From these in vitro studies, it can be seen that the low Michaelis constant for FEP of hepatoma type M pyruvate kinase would enable the enzyme to function efficiently at the low FEP levels, whereas at the high FEP levels, the enzyme would be activated by its substrate.

(d) Product inhibitor studies

Both normal adult liver type L and hepatoma type M pyruvate kinases displayed a parabolic variation of the intercepts versus the ATP
concentration (inset Fig. 4.29 and 4.30), which indicates a mixed inhibition due to the binding of magnesium by ATP. By plotting the intercepts as a function of the MgATP concentration (inset Fig. 4.31) this parabolic variation of the intercept versus the ATP concentration could be transformed to a linear variation in the case of hepatoma type K pyruvate kinase. Such a plot however, gave a hyperbolic variation in the case of normal adult liver type I pyruvate kinase (inset Fig. 4.28). Such a hyperbolic variation might be attributed to MgATP acting at two sites. As normal liver type I pyruvate kinase displays allosteric kinetics with respect to FEP, it could be that MgATP saturates the allosteric site but has a lower affinity for the catalytic one.

With both the normal human adult liver type I and hepatoma type K pyruvate kinases, the MgATP inhibition was non-competitive against ADP. Furthermore, although parabolic inhibition was obtained with both the aforementioned pyruvate kinase preparations when assaying in the presence of limiting amounts of magnesium, the mode of inhibition still remained non-competitive with respect to ADP. These findings are similar to those of Holmean and Storm (1969) for the enzyme from rabbit muscle, but differ from those of Reynard et al. (1961). These latter mentioned authors found ATP to be competitive with respect to ADP and no mixed inhibition due to the binding of Mg⁺ and ATP when assaying with 1mM MgCl₂ and levels of ATP up to 7.5mM. However, Holmean and Storm (1969) did not obtain strictly competitive inhibition for rabbit muscle pyruvate when using a similar assay system to that described by Reynard et al. (1961).

Although MgATP was found to be competitive with respect to FEP
with normal human adult liver type L pyruvate kinase, the mode of ATP inhibition against FEP could not be determined with hepatoma type H pyruvate kinase. The finding that the MgATP inhibition against FEP was competitive in the case of normal adult liver type L pyruvate kinase agrees with the findings for rabbit muscle (Raynard et al., 1961) and erythrocyte pyruvate kinase (Campos et al., 1965). Boyer (1969) found this inhibition to be of a mixed type but mainly of a competitive nature.

Both the human liver type L pyruvate kinase as well as the hepatoma isoenzyme differed from all the aforementioned pyruvate kinases in that they were allosterically inhibited by MgATP and ATP respectively.

(a) Mechanism of reaction

Although differences were found in the allosteric properties and kinetic constants, the overall kinetics, particularly the isotope exchange results, indicate that the mechanism of reaction of these two isoenzymes might be the same. The findings that all the lines in the initial rate studies met at a point, indicate that the normal liver type L and hepatoma and foetal types H pyruvate kinases have a sequential mechanism, whereby both substrates must add to the enzyme before either product is released. A ping-pong mechanism (i.e. first product is released before the second substrate combines to the enzyme) would be indicated if parallel lines were obtained (Cleland, 1963).

The finding that MgATP or ATP are non-competitive inhibitors with respect to AMP with both normal adult human type L and hepatoma type H pyruvate kinases, (Figs. 4.28, 4.29, 4.31 and 4.32) and inhibit competitively versus FEP with normal adult human type L pyruvate kinase.
(Fig. 14) might indicate an ordered sequential mechanism with PEP being the first substrate to bind to the enzyme, and ATP, the last product to be released as shown schematically below:

\[
\begin{array}{cccc}
\text{PEP} & \text{ADP} & \text{FDP} & \text{ATP} \\
\downarrow & \downarrow & \uparrow & \uparrow \\
E_{\text{PEP}} & E_{\text{ADP, FDP}} & E_{\text{ATP, FDP}} & E \\
\end{array}
\]

This scheme does not take into account the various metal ions such as Mg\(^{2+}\) and F\(^{3-}\) for their exact role in this mechanism is not clear from the available data.

This mechanism is contrary to the conclusions of Midtvedt and Collin (1966) and Reynard et al. (1961), who proposed a random sequential mechanism for rat muscle pyruvate kinase. A rapid equilibrium random sequential mechanism would be indicated if MgATP were competitive against PEP and ADP. The present results would not be inconsistent with a rapid random mechanism if the enzyme could form a dead-end E-ADP-ATP complex (Cleland, 1963). However, in view of the large size of ATP and ADP, and of the fact that they probably occupy the same site on the enzyme, such a mechanism seems very unlikely.

Further evidence that the mechanism of normal adult human liver type L and hepatoma types K pyruvate kinases is probably ordered, comes from the isotope exchange results. The finding that \(^{14}C\)-pyruvate to PEP exchange could be eliminated at high MgATP/ADP levels (Fig. 14.3a), would suggest that either one or both of the varied reactants add between the labelled reactant. Similarly, the ATP-\(^{32}P\) to PEP exchange could be eliminated at high pyruvate/ADP levels (Fig. 14.35).
Although these later findings could be attributed to dead-end E-ADP-pyruvate forma
tions, this would require one to postulate an E-ADP-ATP complex, which is unlikely. Elimination of exchange could however also be obtained if the substrate of the varied pair inhibited the enzyme (Morrison and Uelman, 1966). Although no substrate inhibition was obtained with hepatoma type II pyruvate kinase, inhibition by high levels of ADP were found with normal adult liver type I pyruvate kinase in the present study. Similarly, rat liver and human erythrocyte pyruvate kinases have been found to be inhibited by high levels of ADP (Tanaka et al., 1967a and Staal et al., 1971). However, as the isotope exchange pattern of normal liver type I and hepatoma types II pyruvate kinases were so similar, and as no substrate inhibition was obtained with the hepatoma type II pyruvate kinase, it seems unlikely that the elimination of \(^{14}C\)-pyruvate to PEP exchange on increasing the MgATP/ADP levels can solely be attributed to substrate inhibition.

In conclusion, it appears that the reaction mechanisms of both liver type I and type II are identical. The ordered mechanism shown schematically above fits all available data, but a rapid equilibrium random mechanism cannot be entirely excluded.
Summary

1. (a) The levels of some glucose metabolizing enzymes were estimated in 30,000g supernatants of extracts of host and cancerous tissues of primary human hepatomas and in two normal adult and fetal livers.

(b) When the enzyme levels were estimated in different samples of the same normal adult liver as well as in different samples of host tissue, some variation was found. In spite of this, significant differences were found between the levels of some of the glucose metabolizing enzymes of host and cancerous tissue.

(c) A comparison of the enzyme levels in cancerous tissue of different hepatomas with those of their respective host tissue showed that the phosphoglucomutase, fructose-1,6-diphosphatase, α-glycerophosphate dehydrogenase, lactate dehydrogenase and malate dehydrogenase levels were consistently lower, whereas the FDP/FR aldolase activity ratios were elevated in cancerous tissue. When the data obtained with different host and cancerous tissues were pooled and statistically evaluated, these differences were found to be significant. Although the triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and phosphoglycerate mutase levels showed no consistent change, these levels were significantly lower in cancerous tissue than compared to host tissue. Similarly, the pyruvate kinase levels showed no consistent differences between respective host and cancerous tissue, but overall proved to be significantly elevated in cancerous tissue. No significant differences were found in the hexokinase, phosphoglucomutase, glucose-6-phosphate dehydrogenase and
ancase activities, as well as the aldolase activity with FDP.

(d) A comparison of the glucose metabolizing enzymes in extracts of human adult and foetal livers showed the phosphoglucone mutase, fructose-1, 6-diphosphatase and α-glycerolphosphate dehydrogenase levels to be lower in foetal livers.

2 (a) The isoenzymes of various glucose metabolizing enzymes present in 36,000g supernatants of normal adult human liver, and host and cancercous tissue of primary human hepatomas were separated by starch gel electrophoresis.

(b) The hexokinase and pyruvate kinase isoenzyme patterns were found to differ in the different tissue types tested. Those of cancercous tissue resembled those of fast-growing rat hepatomas.

(c) No differences were found in the lactate dehydrogenase, α-glyceroolphosphate dehydrogenase, malate dehydrogenase and triosephosphate isomerase isoenzyme patterns of normal adult human liver, host and cancercous tissue. It was thus concluded that the level differences found in these enzymes were not due to isoenzyme pattern changes.

3 (a) Type L pyruvate kinase was partially purified from normal human adult liver and type N from hepatoma and foetal livers. Purification procedures included ammonium sulphate fractionation, acetone fractionation and molecular sieving on a Sephadex G-200 column. The L isoenzyme was precipitated in the 25-37% ammonium sulphate fraction, whereas the N isoenzyme was located in the 50-70% ammonium sulphate fraction.

By using this procedure, a 35.3-fold purification and 19% yield was obtained with the liver type L isoenzyme, a 62.5-fold purification and
35% yield with the hepatoma type M pyruvate kinase and a 13.5-fold purification and 5.1% yield was obtained with the foetal type M isoenzymes.

(b) Enzymic studies using these three preparations revealed striking differences.

(c) Liver type L pyruvate kinase displayed allosteric kinetics with respect to PEP. These could be enhanced by allosteric inhibitors, via DL-alanine and ATP and eliminated by fructose-1,6-diphosphate. Fructose-1,6-diphosphate activated this isoenzyme, decreased the $K_v$ for PEP from 0.79mM to 0.11mM and changed the kinetics from sigmoidal to hyperbolic. This isoenzyme could also be activated by various other precursors of PEP such as fructose-6-phosphate, fructose-1-phosphate, glucose-1-phosphate, 2-dexoxyglucose-6-phosphate, dihydroxyacetone phosphate, glyceraldehyde-3-phosphate and 2-phosphoglycerate. Of all these activators tested, fructose-1,6-diphosphate was the most potent with a $K_v$ value of 0.35 - 0.5 µM.

(d) The kinetics of the hepatoma and foetal isoenzymes with respect to PEP were not affected by any of the afore-mentioned activators.

(e) All three isoenzymes were allosterically inhibited by ATP, DL-alanine, citrate and AMP. Normal liver type L pyruvate kinase was more sensitive to ATP and citrate inhibition, but less sensitive to AMP inhibition than either of the type M isoenzymes. All three isoenzymes tested were however equally susceptible to DL-alanine inhibition.

(f) The inhibition of the L isoenzyme by these compounds could be relieved by fructose-1,6-diphosphate. On the other hand, fructose-1,6-diphosphate could only reverse the alanine inhibition of the hepatoma.
and fetal types M pyruvate kinases. It did however, also partially reverse the citrate inhibition of both M type isoenzymes at high citrate levels as well as the ATP inhibition of the fetal isoenzyme.

(g) From these results it was concluded that the properties displayed by liver type L pyruvate kinase make it best suited for control of both gluconeogenesis and glycolysis.

(h) The MgCl₂ requirements of all three preparations were found to be similar and to be altered in the presence of ATP and PEP.

(i) Lineweaver-Burk plots showed substrate inhibition by ADP of the normal liver type L isoenzyme, and reflected the allosteric interaction of this isoenzyme with respect to PEP. They showed no inhibition by high ADP levels of the hepatoma type M isoenzyme, but gave non-linear plots when the reciprocal velocity of this isoenzyme was estimated as a function of the reciprocal PEP concentration. On the other hand, the fetal isoenzyme showed simple Michaelis-Menten kinetics with respect to both ADP and PEP, thus indicating that the properties of the hepatoma and fetal type M isoenzymes are different.

(j) The Michaelis constants obtained for ADP of normal liver type L pyruvate kinase (0.56mM) was higher than that of the hepatoma (0.26mM) and fetal type M (0.28mM) isoenzymes. The Michaelis constant for PEP of normal liver type L pyruvate kinase was also higher (0.6mM) than that obtained for the fetal type M isoenzyme (0.058mM) as well as the Kₚ for PEP of the hepatoma type M isoenzyme (0.11mM).

(k) Product inhibitor studies showed non-competitive inhibition of ADP by MgATP for both the liver type L and hepatoma type M isoenzymes.
On the other hand, the inhibition of MgATP versus FEP was competitive with the normal liver type L isoenzyme.

(1) Isotope exchange studies with the liver type L and hepatoma type M isoenzymes showed that the $^{14}$C-pyruvate to PEP exchange could be eliminated at high MgATP/ADP levels. Also, the ATP-$^{32}$P to PEP exchange could be eliminated at high pyruvate/ADP levels. However, the ATP-$^{32}$P to PEP exchange was enhanced on raising the MgATP/PEP levels.

(m) From the initial velocity, product inhibitor and isotope exchange studies, it was concluded that the mechanism of reaction is similar for liver type L and hepatoma type M pyruvate kinases. The results are consistent with a sequential ordered mechanism with PEP adding on to the enzyme first and MgATP coming off last.
Abbreviations

**ADP**
adenosine-5'-diphosphate

**AK**
adenylate kinase \(\text{ADP} \rightarrow \text{ATP} + \text{AMP}\) phosphotransferase \(\text{EC 2.7.5.3}\)

**Ald**
alcohol \(\text{Fructose-1,6-diphosphate aldolase} \ (\text{EC 4.1.3.7})\)

**AMP**
adensine-5'-monophosphate

**ATP**
adensine-5'-triphosphate

**C3P**
cytosine-5'-triphosphate

**DHAP**
dihydroxyacetone phosphate

**DIT**
dithiothreitol

**Eno**
emulsase \(\text{D-2-phosphoglycerate hydro-lyase} \ (\text{EC 4.2.2.11})\)

**F-1-P**
fructose-1-phosphate

**F-6-P**
fructose-6-phosphate

**FDP**
fructose-1,6-diphosphate

**F6Pase**
fructose-1,6-diphosphatase \(\text{6-fructose-1,6-diphosphate 1-phosphatase} \ (\text{EC 3.1.3.1})\)

**Gal-6-P**
galactose-6-phosphate

**DAP**
glyceraldehyde-3-phosphoric acid

**GAPDH**
glyceraldehyde-3-phosphate dehydrogenase \(\text{D-glyceraldehyde-3-phosphate} + \text{NAD} \rightarrow \text{NADH} + \text{3-phosphoglycerate} \ (\text{EC 1.2.1.12})\)

**a-GDR**
a-glycerolphosphate dehydrogenase \(\text{D-glycerol-3-phosphate} + \text{NAD} \rightarrow \text{D-glycerate} + \text{NADH} \) (EC 1.1.1.13)
glucokinase/ATP:D-glucose-6-phosphotransferase (EC 2.7.1.2)

G-1-P glucose-1-phosphate

G-6-P glucose-6-phosphate

2-deoxy G6P 2-deoxyglucose-6-phosphate

G6PDH glucose-6-phosphate dehydrogenase

GDP guanosine-5'-diphosphate

GTP guanosine-5'-triphosphate

HK hexokinase/ATP:D-hexose-6-phosphotransferase (EC 2.7.1.1)

INT 2-p-(iodophenyl)-3-(p-nitrophenyl)-5-phenyl-1,2-tetrazolium salt

ITP inosine-5'-triphosphate

LDH lactate dehydrogenase/L-lactate:NAD oxidoreductase (EC 1.1.1.27)

MDH malate dehydrogenase/L-malate:NAD oxidoreductase (EC 1.1.1.37)

NADH nicotinamide adenine dinucleotide, reduced form

NADP+ nicotinamide adenine dinucleotide phosphate

PEP phosphoenolpyruvate

2PGA glycerate-2-phosphate

3PGA glycerate-3-phosphate
1,3 d1-PGA 1,3-diphosphoglycerate

PGI phosphoglucone isomerase /D-glucose-6-phosphate
        keto-isomerase (E C 5.3.1.9)

PGLyK phosphoglycerate kinase /ATP:3 phospho-D-glycerate
        L-phosphotransferase (E C 2.7.2.3)

PGlyM phosphoglycerate mutase /2,3 diphospho-D-glycerate:
        2 phospho-D-glycerate phosphotransferase (E C 2.7.5.3)

PGM phosphoglucomutase /D-glucose-1,6 diphosphate: D-,
        D-glucose-L-phosphotransferase (E C 2.7.5.1)

PK pyruvate kinase /ATP:pyruvate phosphotransferase
        (E C 2.7.1.40)

PKS phenazine methosulphate

TPI triosephosphate isomerase /D-glyceraldehyde-3-phosphate-
        isomerase (E C 5.3.1.1)

TTP thymidine-5'-triphosphate

UTP Uridine-5'-triphosphate
References


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**References**

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