PYRUVATE METABOLISM AND TRANSPORT IN INTESTINAL EPITHELIUM

PROEFSCHRIFT

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Voor Tilly en Frouke

Aan mijn Ouders

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CHAPTER I

FACTORS INFLUENCING THE STORES OF ENERGY-RICH PHOSPHATE IN RAT SMALL INTESTINE AND ITS RELATIONSHIP TO GLUCOSE AND FRUCTOSE METABOLISM

Introduction

The small intestinal mucosa is known to have a high rate of aerobic and anaerobic glycolysis[1-3]. Oxidative phosphorylation, although not tightly coupled, has been demonstrated using mitochondrial preparations from small intestinal mucosa of guinea-pig[4] and rat[5,6]. Recent work from our laboratory[7] showed that the high Mg^{2+} stimulated ATPase activity of the isolated mitochondrial preparations contributed to the absence of tight coupling. A "loose" coupling of oxidative phosphorylation, or the presence of considerable ATPase activity could explain the absence of a clearcut Pasteur effect in small intestine of various species[1-3]. The metabolism of glucose and fructose is of particular importance in relation to their absorption (reviews: 11,12). The amount of the lactate formed within intestinal mucosa is discharged unidirectionally into the serosal fluid (unpublished observations and refs. 13-15). It was postulated by Porteous[13] that the preponderance of lactate dehydrogenase over pyruvate dehydrogenase (the activity ratio was 300) as well as the regulatory properties of a "muscle type" lactate dehydrogenase ensure the high rate of lactate production even at high O_2 concentrations. However, in our studies with isolated epithelial cells (Paper III) almost equal rates of pyruvate decarboxylation and pyruvate conversion to lactate were observed. A balance study of glucose transport *in vitro* (1-10 mM) showed a considerable conversion to lactate (27-41%) of the glucose absorbed[13]. However, comparable studies *in vivo*, carried out by Kiyasu et al.[16], clearly showed a different pattern: ^{14}C-labelled glucose (2.5-100 mM) was introduced into loops of small intestine of rats and practically all of the ^{14}C in portal plasma was shown to be present as glucose (90-92%) and only 4-9% as lactate. In vitro studies with rat "everted intestine" have left no doubt that the intestine can transfer hexoses, aminoacids and short-chain fatty acids, against concentration gradients, (review: 17). For these transfer mechanisms to function at maximum rates the presence of a metabolic fuel, such as glucose, is required.

Determinations of in vivo levels of glycolytic intermediates in a variety of tissues during increased glycolytic flux indicate that phosphofructokinase plays a central role in the regulation of glycolysis and the operation of the Pasteur effect (reviews: 18-20). Low concentrations of ATP relative to ADP and AMP are favourable conditions for high phosphofructokinase activity. Indeed, Iemhoff et al.[5] found these conditions to be present in everted sacs of rat small intestine. We therefore reasoned: perhaps the O_2 availability in these non-vascularly perfused everted sac preparations is limited, leading to suboptimal rates of ADP phosphorylation by limited mitochondrial activity and hence to excessive rates of glycolysis. The study of a better oxygenation of the small intestine by in vitro vascular perfusion was therefore the start of the present investigation. In this connection it may be of interest to note that Pietra and Cappelli[21] found that indeed in everted sac preparations O_2 availability is limited.

Recent studies firmly establish that fructose, a major ingredient in the human diet, is actively transported by the small intestine of the rat[22,23]. Several reports claim that fructose is converted to glucose during absorption, e.g. in guinea-pig and human intestine[24-26]. In the rat, however, this conversion is unimportant[26]. In vivo experiments showed that a considerable proportion of fructose (12-46%) appears in the portal vein as lactic acid[26]. This then differs from the results obtained on glucose absorption. This led us to study also the effect of intraluminal fructose administration on the in vivo levels of energy-rich phosphate compounds in the small intestine. In the meantime it had been observed by others[27,28], and confirmed by us (Paper II), that the intravenous administration of fructose brings about a depletion of ATP in liver. The high rate of lactate production could also be due to the utilization of fructose via fructose-1-P to triose-P, as was shown in liver, kidney cortex and gut mucosa (review: 29). This pathway is not subject to the regulatory controls of hexokinase or phosphofructokinase, since these enzymes are not involved in the conversion of fructose to pyruvate. Possibly then in vivo, hexokinase and phosphofructokinase are involved in the control of the conversion of glucose to pyruvate, since glucose resorption does not lead to excessive lactate production, as has been mentioned above.

Methodological remarks

The small intestine has been studied in the past by a variety of in vitro techniques, the most popular being the everted gut sac preparation, introduced by Wilson and Wiseman[30]. Other popular in vitro preparations are rings of whole small intestine, scrapings of mucosa and isolated villi. More recently, however, evidence has become available that the morphological integrity of the everted sac preparation is short lived, 50-75% of normal epithelium disappearing during 30 min incubation in oxygenated buffer at 37°C[31]. Parsons[32] already concluded that the most important drawback of the in vitro preparation is insufficient oxygenation. Oxygenation of the mucosal cell has been carried out by perfusion of oxygenated fluid through the intestinal lumen. Vascular perfusion on the other hand does not introduce unphysiological barriers and might ensure a greater efficiency of oxygenation. In vitro vascular perfusion, however, has not been very popular in gastrointestinal research (review: 33). From 1968 on, however, considerable work has been carried out to cope with problems like vasoconstriction, hypermotility and progressive destruction of mucosal epithelium (review: 34). Measurements of survival time, oxygen consumption, flow rate and perfusion pressure[33, 35-38] were done.However, none of these tests is sufficiently critical and more stringent biochemical parameters of intestinal function need to be established. Glucose consumption, active hexose-and aminoacid transport, lipoprotein synthesis, ion absorption have been studied by some workers in this field[33,38] and could be adapted to appropriate function tests. A variety of methods has been applied to overcome the problems of hypermotility, vasoconstriction and excessive secretion by addition of drugs to the medium. In the perfusion studies done in our laboratory[39] (compare Paper I) promethazin and papaverine were used as antihistaminic and vasodilatory agents respectively, as described by Forth[36]. There are objections to this approach, because the reagents added might have metabolic effects, e.g. promethazin is said to be an inhibitor of (Na^+,K^+)ATPase[40] and papaverine is a strong inhibitor of phosphodiesterase[41]. Perfusion studies carried out by Windmueller et al.[38,42] suggested that the above mentioned difficulties were related to the loss of central sympathetic innervation. They demonstrated that this loss can be adequately compensated by the continuous infusion of small amounts of norepinephrine into the recycling perfusate, provided it contained sufficient levels of a glucocorticoid.

Most of the perfusion studies of liver and small intestine, described in the literature, were performed with either an erythrocyte-free medium or with a buffer enriched with erythrocytes. The non-erythrocyte medium has a low oxygen carrying capacity and therefore requires a high flow rate to prevent organ hypoxia (in small intestine: 15-20 ml/min; Paper I), when compared with an erythrocyte supplemented medium (4-8 ml/min; ref. 39). However, a problem with an erythrocyte-containing medium, is lysis of the red cells on the one hand and their ability to consume glucose on the other hand. The latter problem was avoided in studies of Hülsmann[39] by using aged human erythrocytes[43]. In these studies the final hematocrit was about 20%, since at significantly higher cellular concentration vascular obstruction often occurred. When the small intestine was damaged, the first measurable changes were tissue edema, excessive accumulation of fluid intraluminally and reduced flow at constant pressure. In order to avoid tissue hypoxia in the present studies, use was made of liquid fluorocarbon to transport oxygen and carbondioxide. Fluorocarbon FC-75 (the usual trade name is FX 80) is a biologically inert liquid[44]. Geyer et al.[45] have shown that emulsions of liquid fluorocarbon can be used to replace almost entirely the blood of intact rats and that these animals react well when kept in an oxygen atmosphere. Fluorocarbons were later used also in the study of isolated tissue preparations, such as brain[46], liver[47-51] and heart[52]. Their suitability for the maintenance of organ function has now been demonstrated (review: 53). The solubilities of oxygen and carbondioxide in pure FC-75 at 37°C amounted to 0.41 and 1.42 ml of gas/ml FC-75 respectively. Compare solubilities of 0.024 ml and 0.67 ml of gas/ml isotonic saline respectively[50,53]. Water and polar substances, such as glucose and salts, are virtually insoluble in FC-75[46]. The high molecular weight non-ionic detergent F 68 was used as emulsifier of the fluorocarbon in our experiments (Paper I) and the emulsification was accomplished by ultrasonic treatment of the mixture. The dispersed material could be sedimented by centrifugation and the sedimented material was easily washed with buffer to remove excess F 68 present in the waterphase. After resuspension by sonification, the emulsion was checked under the phase contrast microscope. This preparation was already successfully used in vascular perfusion of rat liver[54].

Regulation of glycolysis in rat small intestine

There are numerous reports on the glycolytic activity of the small intestine[1-5,8-13] studied as everted sacs, scrapings or isolated epithelial cell preparations. The pattern of the glycolytic enzymes in the mucosa of the small intestine differs from that observed in other mammalian tissues[55]. The main difference is the low activity of fructose-1,6-P_2 aldolase (in the 105,000 g sup. of mucosal cell homogenates: 5-25 mU/mg protein; unpublished own observations and refs. 55,56), while the cytosolic activities of phosphofructokinase, hexokinase and pyruvatekinase, measured under optimal conditions, amount to 0.16, 0.03 and 2.5 U/mg protein respectively[55,57-59]. The maximal activities of the other glycolytic enzymes equalled or exceeded that of pyruvatekinase. The initial approach to the problem of identifying the rate-limiting and controlling step in glycolysis was the determination of maximal catalytic activities of the component enzymes. Hexokinase was found to be the rate-limiting enzyme when the rate of formation of lactate from glucose was compared with that from glucose-6-P in studies with subcellular preparations of the intestinal mucosa of the cat or rat[60]. It was also shown that lactate production resided predominantly in the particle-free supernatant and that this activity decreased during starvation. This decrease was mainly due to the diminished hexokinase activity by binding of the enzyme to subcellular particulate structures. Recently Jones and Mayer[61] presented a fractionation study in rat small intestinal epithelial cells showing predominant association of hexokinase with mitochondria. Fractionation studies of Van den Berg et al.[58] confirmed the mitochondrial localization of particulate hexokinase. In vivo as well as in vitro experiments indicated a decrease of particle-bound hexokinase activity when glucose or 3-O-methylglucose was applied to the lumen of the gut[62], but recent studies in our laboratory have shown[58] that extensive redistribution occurs during the time of preparation. From in vivo freeze-clamped small intestine of rats refed with glucose after 50 h starvation a strong decrease of soluble hexokinase was concluded[58]. The meaning of this redistribution is now being studied. The use of a low maximal capacity of an enzyme in vitro as an indicator for a rate-limiting step in vivo may be misleading, as well as the use of the rate of a biochemical pathway in cell homogenates. To estimate actual enzymatic rates in vivo, additional information will be required, which includes the levels, and intracellular distribution of the substrates, and knowledge of the kinetic parameters of the enzymes in the tissue and species under study. Some data on the kinetics and allosteric properties of phosphofructokinase[57,63,64] and on the kinetics of pyruvatekinase in rat small intestine[59] were recently presented. We reported substrate levels of intermediates in glucose and fructose metabolism in freeze-clamped small intestine after intraluminal hexose loading in vivo (Paper II). After glucose loading no significant changes in the levels of glycolytic intermediates were observed. Therefore the irreversible steps of glycolysis must have been adapted. Hexokinase isoenzymes of small intestine are subject to glucose-6-P inhibition. Phosphofructokinase may still be the rate-limiting step since its inhibition will cause hexosemonophosphate accumulation, which subsequently inhibits hexokinase, preventing sequestration of P_i during glucose loading. During fructose loading the glycolytic rate increases which is not in contradiction with the conclusion on the rate-limiting step(s) of glycolysis just reached, since fructokinase- and the subsequent fructose-1-P aldolase reactions catalyze the formation of triose-phosphates while circumventing hexokinase, phosphofructokinase and fructose-1,6-P_2 aldolase.

Examination of factors which may control the glycolytic flux in a particular tissue requires the knowledge of levels of metabolic effectors, which influence the activity of the enzymes of these pathways. The inhibition of phosphofructokinase from skeletal muscle by high concentrations of ATP was first shown by Lardy and Parks[65]. The reversal (deinhibition) of ATP inhibition by ADP and AMP was shown by Passoneau and Lowry[66]. Later work of the same authors[67] added fructose-6-P, P_i, NH_4^+ and K^+ as activators of the enzyme. These effects were subsequently confirmed in studies on phosphofructokinase from different other sources (reviews: 18,19). Since adenine nucleotides are the universal coupling agents between anabolic and catabolic segments of metabolism and since many enzymes are regulated by these nucleotides (e.g. pyruvatedehydrogenase, Chapter II), Atkinson[68] advanced the adenylate control hypothesis. This states that the concentrations of adenine nucleotides in a cell are important parameters in the regulation of sequences that leads to the regeneration of ATP or to production of storage compounds. Atkinson introduced the term "adenylate energy charge" defined as: $[ATP] + \frac{1}{2}[ADP] / [ATP] + [ADP] + [AMP]$. The highest possible ratio is unity, when all adenine nucleotides in the cell are present as ATP. Phosphofructokinase, being generally a rate-limiting enzyme of glycolysis, responds to changes of the energy charge[68]. Thus low concentrations of ATP relative to ADP and AMP (to be expected to occur under anaerobic conditions) will tend to activate the enzyme, thus explaining the increased rate of glycolysis during this condition. Alternatively excess ATP, as can be expected only when the mitochondrial respiratory chain is operative, thus in the presence of O_2, the rate of glycolysis will be inhibited (Pasteur effect). Studies on the effectors of phosphofructokinase in rat small intestine indicated that they are essentially the same as those of other mammalian phosphofructokinases[57,63]. The lack of Pasteur effect in small intestinal mucosa (see Introduction) may therefore be related to the favourable ratio of activators to inhibitors of the enzyme, as will occur during tissue ischemia when the "energy charge" is low. The concentrations of ATP, ADP, AMP, P_i and fructose-6-P were therefore determined in jejunum of the rat in vivo (Papers I and II) and revealed relatively low levels of ADP, AMP and P_i. The energy charge was appreciably higher than that reported in the literature[18,64]. In liver, as in most other tissues studied, the energy charge has been shown to be maintained at a value of approximately 0.9[69]. The lower value, reported by Tejwani et al.[64], might be due to the use of a slow "freeze stop" method. The same authors, however, found that the ATP concentration was not critical to influence the enzyme activity owing to the synergic activation by NH_4^+, AMP, ADP and P_i (compare also refs. 18,70,71). The most important activator, NH_4^+, may indeed have a high concentration in view of the presence of urease and the high adenosinedeaminase activity in intestinal mucosa as argued by Tejwani[64]. We consider the lower energy charge rather unlikely as long as excess of creatinephosphate (and creatinekinase) exists in the cell. We were first to show the presence of creatinephosphate (CP) in the intestinal mucosa (Paper II). Creatinephosphate is also a potent inhibitor of brain phosphofructokinase[72]. It would be of interest to see whether this also holds for phosphofructokinase from small intestine. Furthermore no data were given about the effect of pH on the allosteric properties of the enzyme. Mansour found that the effect of pH is so critical that a change of 0.2 pH units around the neutral point could cause a considerable change in sensitivity to inhibition by ATP[19,71].

Metabolic characteristics of the vascular perfused small intestine

The effect of increasing the energy charge from 0.81 to 0.91 on the rate of glucose consumption was studied in the vascularly perfused intestine (Paper I). A value of 0.81 could be calculated from the data of TABLE I of Paper I, when Krebs-Henseleit bicarbonate was perfused at a rate of 4.8 ml/min. When an erythrocyte-containing medium was perfused at a rate of 4-8 ml/min (not shown) an identical energy charge was obtained. Under these conditions the rates of glucose utilization (100-200 µmoles/h) were relatively high when compared with values (50-80 µmoles/h) obtained in perfusions with media containing fluorocarbon particles (flow rate 11 ml/min). Freeze-clamping a part of the jejunum under the latter conditions after 10 min perfusion, showed an increase in energy charge (0.91), approximating that of the rat small intestine freeze-clamped *in situ*. In the presence of nitrogen instead of oxygen, the rates of glucose utilization (and lactate production) were much higher (Paper I), suggesting a clearcut Pasteur effect. The oxygen consumption in the aerobically perfused intestine increased from 4-9 to 14-23 µmoles O_2/min when perfusion with Krebs-Henseleit bicarbonate medium at a flow rate of 16 ml/min was replaced by perfusion with fluorocarbon emulsions at a flow rate of 11 ml/min. When the uncoupler 2,4-dinitrophenol (DNP) was added, the oxygen consumption was also stimulated as was the conversion of $[6-^{14}C]$-glucose to $^{14}CO_2$ (Fig. 1). Under this condition the rate of lactate

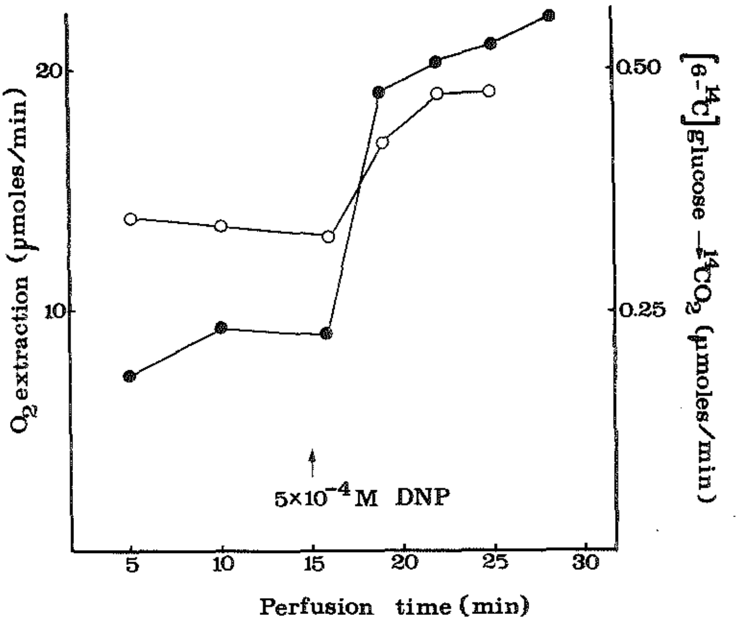

Fig. 1. Effect of DNP on oxygen extraction and [6-^{14}C]-glucose to $^{14}CO_2$ conversion in vascularly perfused rat small intestine. Perfusions were carried out with medium II (see Table I, Paper I). Oxygen consumption was calculated from the measured oxygen tensions at the arterial and venous sides of the perfused preparation (determined with Clark electodes). $^{14}CO_2$ produced was trapped in hyamine contained in a plastic cup present in a vessel covered with a rubber cap. Arterial and venous samples by the addition of $HClO_4$ were injected through the rubber cap, followed by the addition of $HClO_4$ so that the gas CO_2 evolved could be taken up by the hyamine. ●—● [6-^{14}C]-glucose to $^{14}CO_2$ conversion; ○—○ O_2 consumption.

production was also strongly stimulated (Paper I). This indicates "tight coupling" of oxidative phosphorylation in the absence of added uncoupler. It also indicates that the glycolysis rate is controlled by the supply of ATP, generated during oxidative phosphorylation. During 10 min anaerobiosis the energy charge had decreased from 0.91 to

0.62 and the creatinephosphate was lowered to 1.4% of the control value. Furthermore a decrease of the rate of lactate production was observed after 10 min anaerobiosis, which was parallelled by an increase of release of the nucleosides adenosine, inosine and hypoxanthine. The sum of the released nucleosides between 0 and 10 min (aerobic) was 0.026 μmoles, between 10 and 20 min (anaerobic) 0.048 μmoles and between 20 and 30 min (anaerobic) 0.442 μmoles. These values were obtained in separate perfusion studies (not shown) and determined as indicated in ref. 73.

These results then suggest a strong increase in the rate of AMP degradation, since the nucleosides mentioned are products of AMP breakdown. Under aerobic conditions,about half of the glucose utilized is converted to lactate and another important part completely oxidized. The latter requires approximately 3-4 μmoles O_2/min. This is less than the observed rate of oxygen consumption (Fig. 1). Therefore it must be assumed that a considerable fraction of the oxidative metabolism can be attributed to endogenous metabolism. The measurements of the conversion of [6-^{14}C]-glucose to $^{14}CO_2$, however, indicates (Fig. 1) that only a very small amount of glucose was oxidized, although the amount of label dilution could not be estimated. Our work has been critisized by Ramaiah[18] while Lohman et al.[2] observed a Pasteur effect in mouse jejunum when the temperature was lowered to 20-30°C. They found that the ATP concentration was three times higher at this temperature than at 38°C. On the one hand this condition may increase the oxygen availability and on the other hand may decrease ATP utilization. Ramaiah[18] and Krebs[20] both mention in recent reviews on the Pasteur effect that the high aerobic rate of lactate formation by the intestinal mucosa is confirmed by <u>in vivo</u> studies[16,74]. However, when calculations from their absolute lactate production rates in portal blood during the absorption of 2.5-100 mM glucose were made, extremely low production rates are obtained. Moreover Windmueller[38] found already that the small intestine,vascularly perfused in vitro with an erythrocyte-containing medium showed very low rates of lactate production, even during glucose transport.

Fructose metabolism in rat small intestine

Fructose is mainly converted to glucose and lactate in man[25,75], guinea-pig and rat[26,76]. Conversion of fructose to glucose in rat is relatively sluggish due to the absence of glucose-6-phosphatase[76]. Once in the epithelial cell, fructose is rapidly transformed into either fructose-6-P or fructose-1-P by action of hexokinase[77] or fructokinase[76,78,79]. In the presence of both fructose and glucose as will occur after intraluminal supply of sucrose or glucose continuous supplied from the blood side, fructose is preferentially phosphorylated by fructokinase. The K_m's of hexokinase for fructose and glucose in rat small intestine are approximately 4 and 0.2 mM respectively[77]. Fructokinase has been reported to occur not only in small intestine, but also in liver and in kidney[79,80]. Fructose-1-P is converted to dihydroxyacetone-P and glyceraldehyde by fructose-1-P aldolase. It is likely that both fructose-6-P and dihydroxyacetone-P are further metabolized in the glycolytic pathway or to glucose by combined actions of fructose-1,6-P_2 phosphatase and glucose-6-phosphatase. The fructose-1-P aldolase activity is probably contained in the same enzyme molecule that has fructose-1,6-P_2 aldolase activity, since at least purification of the liver enzyme has not led to separation of the two activities. The ratio of the two activities differs in each organ. About unity in liver, kidney and small intestine; relatively low fructose-1-P aldolase activities are found in brain, lung, heart, cardiac and skeletal muscle[81]. In mammalian species three types of aldolase are defined: aldolase A (muscle type), aldolase B (liver type) and aldolase C (brain type). By analogy with liver, it is assumed that in intestine glyceraldehyde is phosphorylated by triosekinase[29]. Thus the rate of fructose catabolism in the intestine is not influenced by phosphofructokinase as has been mentioned earlier.

After the intravenous injection of fructose in rats, the ATP and P_i levels in the liver are known to decrease while the fructose-1-P level strongly increases[27,28] (Paper II). This sequestration of P_i results in a sluggish rephosphorylation of AMP and ADP, so that increased rates of AMP dephosphorylation and deamination occur[28], leading to the formation of inosine, hypoxanthine and uric acid, at the expense of the adenine nucleotide pool. In jejunum, after intraluminal or intravenous fructose administration, fructose-1-P levels also increase (Paper II). Also in jejunum the P_i-concentration was strongly lowered. However, the ATP level was relatively constant, perhaps due to the presence of creatinephosphate and creatinekinase in the epithelial cells. Indeed, the creatinephosphate level declined after fructose loading (Paper II) to compensate ATP breakdown. Intraluminal loading with fructose in vivo had no effects on liver nucleotide levels. This was recently[82] confirmed. The explanation of the accumulation of fructose-1-P in small intestine was based on the examination of the reported V_{max} and K_m values of fructokinase and fructose-1-P aldolase (Paper II). Fructose metabolism in vitro (lactate and glucose formation) measured recently by Mavrias and Mayer[83,84] in guinea-pig and rat epithelium was appreciably lower than the maximal activities of fructokinase and fructose-1-P aldolase, as measured by the same authors. This indicates that these enzymes may be rate-limiting in vitro. The extreme low energy charge in the preparation used, as measured by Iemhoff et al. earlier[5], could explain these results, since fructokinase is strongly inhibited by ADP (competitive with ATP[80]) and IMP, the degradation product of ATP, powerfully inhibits fructose-1-P aldolase[28]. Therefore the interpretation of fructose metabolism in a preparation with a low energy charge may be difficult, which may also hold for glucose metabolism. The effect of intraluminal fructose loading on the glycolytic metabolite levels was virtually nil. Only fructose-1-P accumulation was large, while some of its conversion products may have somewhat increased, probably related to the presence of fructose-1,6-P_2 phosphatase[85,86] and the absence of glucose-6-phosphatase[76,87] in rat small intestine.

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CHAPTER II

PYRUVATE METABOLISM IN ISOLATED EPITHELIAL CELLS FROM JEJUNUM OF RAT AND GUINEA PIG

Introduction

It has been demonstrated[1-3] that in muscle fatty acids and ketone bodies are a preferred substrate when compared with glucose. It has been shown that in this tissue during fatty acid and ketone body oxydation two key enzymes in glucose metabolism, phosphofructokinase[4,5] and pyruvatedehydrogenase[6-8], are inhibited. In an earlier study from this laboratory[9], it was shown that octanoate and oleate both inhibit glucose oxidation in *in vitro* vascularly perfused intestine. Under the conditions employed, glycolysis was not inhibited, but rather slightly stimulated. In Chapter I it was suggested that a somewhat decreased energy charge, when compared with *in vivo* values, may result in activation of phosphofructokinase and therefore to an increased rate of glucose uptake. The study of the mechanisms involved in the inhibition of the pyruvatedehydrogenase complex by fatty acids in intestine is the purpose of the present work. Particular attention was devoted to the phosphorylation-dephosphorylation cycle shown by others to influence the activity of the pyruvatedehydrogenase complex in heart muscle[10], liver[11], brain[12], kidney[13] and adipose tissue[14,15]. It has been demonstrated that the phosphorylation state of this enzyme is under metabolic and hormonal control. We will briefly review some of the reported data.

In our studies on pyruvate metabolism in rat small intestine it was found that the $[1\text{-}^{14}C]$-pyruvate to $^{14}CO_2$ conversion could be inhibited 92% by arsenite (Paper III). The uninhibited reaction may be due to the action of pyruvatecarboxylase followed by the decarboxylation of the dicarboxylic acid products formed. Pyruvatecarboxylase occurs predominantly in the mitochondrial fraction in guinea-pig and rat jejunum. Anderson[16] demonstrated relatively high activities of pyruvatecarboxylase and phosphoenolpyruvate carboxykinase in intestinal mucosa of guinea-pig when compared with those observed in rat[17]. They extended these observations to measurements of glucose-6-P phosphatase and fructose -1,6-P_2 phosphatase activities[16]. Hitherto intestinal mucosa was not considered to be a gluconeogenic tissue. Starvation, however, was found to increase the total activities of pyruvatecarboxylase, phosphoenolpyruvate carboxykinase[16,18] and fructose-1,6-P_2 phosphatase[19,20]. On the other hand diabetes was found to be accompanied by reduction of gluconeogenic enzyme activities in small intestine[21]. Their observation[16] that homogenates of jejunal mucosa from 72 h starved guinea-pigs catalyze glucose formation from lactate, malate or pyruvate at rates between 3 and 5 μmoles/h/g wet weight was of sufficient importance, not only as a possible contribution to overall gluconeogenesis, but also to pyruvate metabolism in general, that we decided to look also into this aspect of pyruvate metabolism in small intestine.

Properties and regulation of the pyruvatedehydrogenase complex

The oxidative decarboxylation of pyruvate to acetylcoenzyme A occurs according to the following reaction sequence (the proteins of the involved enzymes are omitted):

Pyruvate + thiamine-PP (= TPP) → 1-hydroxyethyl-TPP + CO_2
1-hydroxyethyl-TPP + lip-S_2 → acetyl-S-lip-SH + TPP
acetyl-S-lip-SH + HS-CoA → acetyl-S-CoA + HS-lip-SH
HS-lip-SH + NAD^+ → lip-S_2 + NADH + H^+
Sum: pyruvate + HS-CoA + NAD^+ → acetyl-S-CoA + CO_2 + NADH + H^+

The complex has been resolved into three separate enzymes: pyruvatedecarboxylase (reaction 1), lipoatereductase-transacetylase (reactions 2 and 3) and dihydrolipoatedehydrogenase (reaction 4). From biochemical studies it is well established that pyruvatedehydrogenase (PDH) is localized exclusively in the mitochondria. A recent electronmicroscopical study[22], using the pyruvate-dependent reduction of ferricyanide as indicator for enzyme activity, only revealed reaction product in the innermembrane-intracristae-and intramembrane-spaces of mitochondria. In addition to the above mentioned component enzymes, the complex contains two enzymes involved in the interconversion of active- and inactive forms of the dehydrogenase complex. Pyruvatedehydrogenase kinase (PDH-kinase) inactivates the pyruvatedehydrogenase complex (PDC) by phosphorylation of a seryl residue in one of the subunits of the complex[23-25] with MgATP as the second substrate. It was observed that thiamine-PP reduces the inactivation (i.e. phosphorylation) of PDC by PDH-kinase. Its effect is apparently exerted on PDH rather than on the kinase. Phosphorylation of PDH decreases its ability to bind thiamine-PP[26,27]. Roche and Reed[27] consider the possibility that the binding of thiamine-PP to PDH may contribute to the regulation of the phosphorylation reaction. Phosphopyruvatedehydrogenase-phosphatase (PDH-P phosphatase) reactivates the enzyme by removing covalently bound phosphate[24,25,28]. PDH-P phosphatase requires both magnesium[24,29-31] and calcium[32-34] for activity. Pettit et al.[32] showed that Ca^{2+} acts by binding the PDH-P phosphatase to the dihydrolipoyltransacetylase component of the complex, thus lowering the apparent K_m of the phosphatase for the phosphorylated PDC about 20-fold. The interconversion found <u>in vitro</u> probably also occurs <u>in vivo</u>, as was shown in studies on heart[31,35], liver[36], kidney[31], brain[12], adipose tissue[37] and the small intestine (Paper III). The pyruvatedehydrogenase reaction occupies a strategic position in the metabolic crossroads, involving pyruvate as the substrate. In muscle, brain and small intestine pyruvate is probably of major importance as the substrate for ATP production; in liver and kidney pyruvate (or its precursors lactate and alanine) is the major substrate for gluconeogenesis, while in adipose tissue pyruvate is the main carbon source for fatty acid synthesis. Control of enzyme activity by phosphorylation and dephosphorylation is well established in the case of glycogen synthetase and glycogen phosphorylase. This system is under the control of cyclic-AMP. By analogy one could wonder whether PDH-phosphorylation and dephosphorylation are possibly also under the control of cyclic-AMP. However, it has no direct effect on the activity of any component of the multi enzyme complex (Hucho; ref. 38).

Notes on the methods followed

The usual method for determining the active and inactive forms of PDH (PDH_a and PDH_b respectively) in tissue homogenates is based on the fact that the PDH-P phosphatase converts the inactive PDH_b to PDH_a in the presence of high concentrations of free Mg^{2+} (10 mM). The activity of the samples incubated without Mg^{2+} is taken to present the a-form of the complex[28,31]. The increase in activity by incubating the homogenate with Mg^{2+} represents the amount of b-form present. A number of investigators[38-41] add a small aliquot of purified PDH-P phosphatase, isolated from heart tissue according to Siess and Wieland[42], to accelerate the activation of the complex. The low endogenous phosphatase activity could be due to its poor accessibility to the phosphorylated multi enzyme complex and this may originate from its loose association of this enzyme component to the complex[23,29]. PDH-P phosphatase activity was also found to be strongly dependent on environmental parameters, such as the pH, ionic strength, osmolarity and the buffer chosen[26,38,39]. In our studies on intestinal epithelial cell extracts (Paper III) the activation was very slow in dilute homogenates, which may be due to the dissociation of PDH-P phosphatase from the complex. Furthermore, the interference of PDH-kinase activity was excluded by the addition of glucose and hexokinase to the extracts to maintain a low ATP level. In the second place it was found that the activation of PDC by incubation with Mg^{2+} was suboptimal, possibly due to lability of the enzyme at 30°C. This was also described for extracts of liver mitochondria[43] and isolated kidney tubules[40]. The inactivation could largely be prevented by the addition of a clear 100,000 g liver supernatant. The irreversible PDC inactivation in liver mitochondrial extracts is due to partial proteolysis by lysosomal enzyme(s)[44], leading to disaggregation of the multi enzyme complex. The validity of the described procedure for measuring the two forms of PDC from kidney, heart and brain tissue has been substantiated by experiments in which ^{32}P incorporation and release were measured together with the change in enzyme activity of PDC[12,13,28,29], although the final proof will require the isolation of $[^{32}P]$-labelled PDH protein following the addition of $^{32}P_i$ to respiring mitochondria.

Originally, highly purified PDC was assayed spectrofotometrically at 340 nm, measuring the reduction of NAD^+ by pyruvate as the substrate, as described by Reed and Wilms[45] and Wieland et al.[46]. Lower purity of the enzyme did not permit this direct optical assay[46] of the enzyme complex. Therefore high concentrations of lactate and exogenous lactatedehydrogenase were added to provide the substrate pyruvate, and the reaction was started with CoASH. The disadvantage of this method was that NADH already in micromolar amounts inhibited PDC[46,47]. Before the raised interest in the determination of PDC activity in crude homogenates, Wieland et al.[35] described a method, in which PDC activity was determined by coupling the reaction with arylaminoacetyltransferase, using p-nitroaniline as acetyl acceptor. This assay procedure is now generally accepted[30,37-39,41]. The decrease of absorbance of p-nitroaniline could be recorded continuously at 405 nm, as well as the possible interconversion of PDH during the assay. The inhibitory products acetyl-Coenzyme A and NADH were rapidly removed in the arylaminotransferase and lactatedehydrogenase reactions respectively. Another assay, increasingly being used, is the measurement of $^{14}CO_2$ evolution from $[1-^{14}C]$-pyruvate[15,48-54]. This assay was also employed in our studies (Paper III). Drawbacks of this procedure are the discontinuity of the assay, the high blank values, originating from non-enzymatic decomposition of $[1-^{14}C]$-pyruvate and from an exchange reaction between the labelled carboxyl group and CO_2, possibly involving dicarboxylic acids, as mentioned earlier and first suggested by Kneer and Ball[55] (see also Paper III). However, low levels of HCO_3^-, acetyl-SCoA and ATP in the assay make the carboxylation of pyruvate and hence the contribution of the latter exchange reaction unlikely. To avoid overestimation of PDC activity, we used arsenite in the blank, a specific inhibitor of pyruvatedehydrogenase by binding covalently to dihydrolipoate in PDC[56].

Regulation of PDH-kinase and PDH-P phosphatase

According to the data available from the literature, the following general conclusions can be presented: PDH-kinase utilizes MgATP as substrate with an apparent K_m of 4-20 µM[10,26,38,57]. ADP is a competitive inhibitor with respect to ATP[11,14,23,26] with a K_i-value of 30-100 µM, depending on the K^+ and NH_4^+ concentrations[26,57]. Pyruvate protects PDC against inactivation and appears to exert this effect by inhibition of PDH-kinase, with apparent K_i-values of about 0.2 mM for the heart complex and 1.4 mM for the kidney complex[26]. PDH-P phosphatase requires both Mg^{2+} [10,14,24,26,28-30,34,38,42] (K_m is 0.5-3 mM) and Ca^{2+} [32-34] for activity. The K_m of PDH-P phosphatase from adipose tissue for Ca^{2+} is 1 μM[34]. The overall activity of PDC is determined by the relative activities of PDH kinase and PDH-P phosphatase.The available evidence indicates that the maximum activity of both enzymes is of the same order of magnitude[26]. If both enzymes were to be maximally active a wasteful ATP hydrolyzing cycle would exist, while pyruvatedehydrogenase would be partially active[38]. Since ADP inhibition of PDH-kinase is competitive with ATP and since ADP is only a weak chelator of Mg^{2+} when compared with ATP, it has been proposed that changes of the intramitochondrial $^{ATP}/ADP$ concentration ratio may regulate the overall activity in two ways. The $^{ATP}/ADP$ ratio may have a direct effect on the PDH-kinase and the ratio may also contribute to the regulation of PDH-P phosphatase by determining the "free" Mg^{2+} concentration. This mode of regulation has been first suggested by Linn and Reed [13,58], and in experiments on intact rabbit liver mitochondria. Schuster and Olson[59,60] showed a dependence of pyruvate oxidation both on the levels of intramitochondrial ATP and "free" Mg^{2+}. Additionally, also many recent reports [14,36,41,61-64] now provide strong support for the view that the intramitochondrial $^{ATP}/ADP$ ratio is an important determinant of PDH_a activity. Therefore, an elevated energy charge, as defined by Atkinson[65] (see also Chapter I), resulting in a decreased "free" Mg^{2+} to bound Mg^{2+} ratio, as discussed by Hucho[38], will result not only in an inhibition of the respiratory chain, but also in inhibition of pyruvatedehydrogenase. Some evidence has also been provided that the intramitochondrial Ca^{2+} concentration may regulate PDH-P phosphatase activity <u>in vitro</u>[14,34,41,61] in adipose tissue and liver mitochondria. Therefore, the action of insulin on pyruvate metabolism may partly be due to changes of intracellular Ca^{2+} fluxes[34,39]. Changes in the activity of PDC in adipose tissue by insulin, could also be correlated with an increase of the intracellular pyruvate concentration[39,52,66]. Recently it was suggested that glucagon may stimulate pyruvate entry into mitochondria[53]. However, Halestrap[67] could not demonstrate any increase in the rate of pyruvate transport by mitochondria prepared from livers of rats pretreated with glucagon. The inhibition of the mitochondrial pyruvate carrier by palmitoylcarnitine, described by Mowbray[68,69], could contribute to the inhibition of PDC by long-chain fatty acids. Thus, the accessibility of pyruvate to the mitochondrial matrix may also contribute to regulation of PDH-kinase.

Sparing action of fatty acids on glucose- and pyruvate oxidation

Fatty acids have been shown to inhibit the oxidation of pyruvate in various mammalian tissues: heart[1-4,8,70], diaphragm[8,70], liver[71], kidney[72,73], small intestine[9] (Papers III and V), adipose tissue[49] as well as in isolated mitochondria from heart[59,74], liver[53,74,75] and kidney[76]. In addition, it has been shown that the oxidation of ketone bodies also reduced the rate of oxidative decarboxylation of pyruvate in different organs. Originally Pearson et al.[77] and Villee and Hastings[78] showed that the oxidation of pyruvate by rat heart and diaphragm muscle was impaired in alloxan diabetes. The fundamental mechanism appears now to be that fatty acid supply determines the rate of oxidation of fatty acids in the diabetic muscle. Similar effects were found in starved rats[79]. In both starved and diabetic rats the plasma concentrations of nonesterified fatty acids are increased[35,80]. The role of fatty acid oxidation in gluconeogenesis by preventing pyruvate, formed from lactate or alanine, from oxidative decarboxylation has been proposed[81-85]. The data on liver were in agreement with reports on kidney that free fatty acids can replace glucogenic substrate as metabolic fuel, thus sparing substrate for gluconeogenesis[73,86]. In adipose tissue, some 95% of the acetylcoenzyme A generated by PDC may be converted to fatty acids[87]. The fact that insulin favours the conversion of PDH_b to PDH_a, as shown by several groups (review: 39) aids to explain that insulin in this tissue stimulates de novo fatty acid synthesis from glucose.

In an earlier study from our laboratory[9] it was shown that octanoate and oleate both inhibited glucose oxidation in vascularly perfused intestine. An inhibition at the level of PDC was suggested by the increase of the percentage of glucose converted to lactate. Under the conditions mentioned, glycolysis was not inhibited, while fatty acids were oxidized. The slightly increased rate of glycolysis, suggesting a lowered energy charge (see Introduction). No conclusions could be drawn in these studies about the mechanism(s) involved in the inhibition of PDC in rat small intestine. The present investigations were undertaken to study the regulation of this enzyme complex in isolated epithelial cells from rat small intestine. It had already been shown that long- and medium-chain fatty acids as well as β-hydroxybutyrate may be well oxidized in vascularly perfused intestine and in the isolated intestinal epithelial cell preparation[9,88]. Cells were incubated at 30°C (TABLE I) with 5 mM glucose as a substrate and only octanoate or oleate clearly affected the relative rate of conversion of glucose to lactate. This is in agreement with earlier studies[9]. Also an increase of the absolute amount of glucose utilization can be observed, indicating again the effect of fatty acids on the glycolytic flux rate. When fatty acids are present, β-oxidation may be expected to cover some of the ATP expenditure, the rest being provided by glucose breakdown. If cellular economy is not influenced by the fatty acid addition, the amount of carbohydrate utilized may be expected to decline. If lactate is considered as "half glucose", indeed octanoate and 3-hydroxybutyrate spare glucose from being combusted. Oleate, in addition, however may stimulate total glucose utiliza-

TABLE I

EFFECT OF FATTY ACIDS AND KETONE BODIES ON GLUCOSE METABOLISM IN ISOLATED EPITHELIAL CELLS FROM RAT SMALL INTESTINE

Substrate added to the incubation medium	(n)	− Δ glucose	+ Δ lactate	PDH_a	glutamate dehydrogenase
		in nmoles/min/mg protein		in mU/mg protein	
glucose 5 mM	(5)	4.11±0.28	5.57±0.71	4.43±0.30	57.1±3.2
glucose 5 mM, octanoate 5 mM	(5)	5.03±0.32	9.40±0.33	3.78±0.20	55.4±2.4
glucose 5 mM, D,L-3-hydroxybutyrate 5 mM	(4)	4.01±0.24	6.23±0.38	-	-
glucose 5 mM, oleate 1.8 mM*	(4)	5.47±0.43	8.11±0.28	-	-

* complexed with 0.26 mM bovine serumalbumin.

Small intestinal epithelial cells were isolated as described in Paper III. Incubations were carried out in 1 ml Krebs Ringer bicarbonate (8-10 mg protein/ml, 30°C, pH 7.4), containing 1.4 mM $CaCl_2$, 0.6 mM $MgSO_4$ and 0.1% albumin and substrates indicated. For assay of lactate, glucose see ref. 9. Pyruvatedehydrogenase (TABLE II of Paper III) and glutamatedehydrogenase activities[8 9] were determined as indicated.

tion, possible due to triglyceride synthesis and interference with oxidative phosphorylation. The stimulation of lactate formation by the additions made may be related to decreased PDH_a activity and the expected increase of "reductive power" ($NADH/NAD^+$ ratio, see refs. 8,84 and 90) by the additions made. As for the PDH_a activity measurements, the enzyme was extracted by homogenization in hypotonic P_i buffer, containing 1 mM EDTA. As a control for the extraction efficiency we measured at the same time another mitochondrial matrix enzyme, glutamatedehydrogenase. A high degree of reproducibility is suggested. Measurement of the total activity of PDH by incubation of the homogenate with Mg^{2+} was hindered by the more pronounced lability of the enzyme at 30°C (Paper III). A small but significant decrease of PDH_a was found during the presence of 5 mM octanoate. This finding is in agreement with the small decrease found <u>in vivo</u> after the intraluminal administration of fatty acid (Paper III). In those studies no conclusion could be drawn about the additional mechanism(s), possibly involved in the inhibition of PDC in rat small intestine.

The rate at which pyruvate is oxidized by PDC in intact cell preparations can be followed by measuring the evolution of $^{14}CO_2$ from $[1-^{14}C]$-pyruvate. Exchange reactions, possibly contributing to the evolution of $^{14}CO_2$ (Paper III), could be excluded by registering only the arsenite-sensitive release of $^{14}CO_2$ from $[1-^{14}C]$ pyruvate. The rate of $^{14}CO_2$ production was strongly decreased (58%) by octanoate (TABLE II; Paper III). A less pronounced effect was found with 5 mM hydroxybutyrate (Paper III). The inhibition of pyruvate oxidation found in these studies is large compared with the small decrease in PDH_a determined under the same condition. The phosphorylation-dephosphorylation cycle is operating also in the small intestine, but possibly determines only to a minor extent the preferential fatty acid oxidation observed. This conclusion was also reached by Wieland et al.[28] in studies on the vascularly perfused heart. Randle et al.[8,79] concluded from their studies on pyruvate metabolism in heart and diaphragm muscle that the inhibition of the PDH complex may be caused by high acetyl-SCoA/CoASH and NADH/NAD$^+$ ratios during fatty acid oxidation. Recently Taylor et al.[91] showed indeed that octanoate also inhibited pyruvate oxidation in isolated rat liver mitochondria where PDH$_a$ activity was only slightly altered and proposed also product inhibition as the responsible factor. Therefore, we measured under essentially the same condition the acetyl-SCoA/CoASH ratio in epithelial cells (TABLE II), indicating an increase during medium- and long-chain fatty acid oxidation. As an additional evidence for this hypothesis could be used the observation that propionate had no effect on pyruvate oxidation (TABLE I of Paper III). Propionate, when activated to propionyl-SCoA may enter the citric acid cycle at the level of succinyl-SCoA, which may result in a decrease of acetyl-SCoA/CoASH ratio rather than an increase, although propionate activation is a slower process compared with octanoate activation in rat small intestine (H.R. Scholte, personal communication). The rate of lactate production from pyruvate was not affected by fatty acids (Paper III), although the reduction of the rate of pyruvate decarboxylation and the increase of the NADH/NAD$^+$ ratio might have expected increased lactate production. Interference of fatty acids with pyruvate transport across cellular membrane was already suggested by Randle et al.[79] for explaining similar results. The K_m of octanoate activation in rat small intestine is lower than 1 mM (P.H.E. Groot, personal communication). The strong concentration-dependency of the effect of octanoate on pyruvate metabolism between 1 and 5 mM did not suggest involvement of octanoate activation.

TABLE II

EFFECT OF FATTY ACIDS ON THE LEVELS OF CoASH AND ACETYL-SCoA DURING PYRUVATE OXIDATION IN EPITHELIAL CELLS FROM RAT SMALL INTESTINE.

Substrate added to the incubation medium	(n)	% inhibition of pyruvate oxidation	CoASH (nmoles/mg protein)	Acetyl-SCoA (nmoles/mg protein)	Acetyl-SCoA/CoASH ratio
2 mM pyruvate	(4)	-	0.340±0.056	0.054±0.007	0.169±0.029
2 mM pyruvate, 1 mM octanoate	(3)	20.0±6.0	0.295±0.046	0.068±0.009	0.265±0.054
2 mM pyruvate, 5 mM octanoate	(4)	58.1±2.2	0.325±0.056	0.091±0.008	0.312±0.063
2 mM pyruvate, 0.75 mM oleate*	(4)	39.6	0.335±0.045	0.079±0.004	0.248±0.019

* complexed with 0.13 mM bovine serumalbumin

Small intestinal epithelial cells were isolated as described in Paper III. Incubations were carried out in 1 ml Krebs-Henseleit bicarbonate (4-8 mg protein, pH 7.4), containing 1.4 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.1% albumin and substrates as indicated. For the assay of CoASH and Acetyl-SCoA, cells (incubated for 5 min at 30°C) were rapidly separated from the incubation medium by centrifugation through silicon oil (0.45 ml) into 5% perchloric acid (0.10 ml), according to the procedures described in Paper V. Aliquots of 0.08 ml of the perchloric acid extracts were aspirated with a Hamilton syringe and neutralized with $KHCO_3$. A recycling assay system was used, utilizing citrate synthase, phosphotransacetylase and malate-dehydrogenase[92]. Metabolism of $[1-^{14}C]$-pyruvate was measured as described in Paper III.

Chapter III will deal with the possible contribution of inhibition of pyruvate transfer to the overall inhibition of pyruvate metabolism observed. The mechanism by which fatty acids act on the overall control of PDC interconversion then is still not clear. It is possible that still other factors are involved. Recently, Wieland et al.[39,61] proposed that *in vivo* an increased supply of free fatty acids might lead to an elevation of the mitochondrial ATP/ADP ratio, whereby inhibition of the adenine nucleotide translocator by long-chain fatty acyl-SCoA may be involved. Inhibition of the adenine nucleotide translocator in small intestine by CoA-esters of oleate and ricinoleate has been described by Gaginella et al.[93] and may be correlated indeed with the almost complete inhibition of pyruvate oxidation by 0.5 mM ricinoleate (Paper V). On the other hand the possible role of fatty acyl-SCoA as an inhibitor of the monocarboxylate transporter in mitochondria, as found by Mowbray[68,69], should be investigated also.

Absence of significant rates of gluconeogenesis from pyruvate, alanine and lactate in intestinal mucosa of starved guinea-pigs

Only in liver and kidney that catalyze significant rates of gluconeogenesis from various precursors (e.g. alanine, pyruvate, malate, fructose and glycerol) gluconeogenic enzymes have high activities. These key-enzymes then have low activities in brain, heart and skeletal muscle (review: 94). White muscle, however, has appreciable fructose-1,6-P_2 phosphatase activity[95]. Although the latter organ contains some phosphoenolpyruvate carboxykinase activity no three-carbon precursors for glucose synthesis could be used, due to the absence of pyruvate carboxylase. For the operation of gluconeogenesis in a tissue not only the presence of these irreversible gluconeogenic enzyme reactions is required, but also the ability to inhibit the irreversible steps of glycolysis, since simultaneous full activity of both gluconeogenic and irreversible glycolytic reactions would lead to undesired wasteful cycling, costing ATP. Recently, an investigation on the kinetic properties of pyruvate kinase of rat small intestine was reported[96]. The enzyme is relatively insensitive to ATP inhibition but its aminoacid sensitivity indicate that glycolysis could be inhibited in the presence of certain aminoacids (e.g. alanine). As mentioned in the Introduction of this chapter, significant activities of the gluconeogenic enzymes pyruvatecarboxylase, phosphoenolpyruvate carboxykinase, fructose-1,6-P_2 phosphatase[16] and glucose-6-P-phosphatase[16,20,97] were found in small intestinal mucosa of the guinea-pig. Furthermore, starvation increased the activities of these enzymes in small intestine[16,18-20]. The rate of glucose production, as observed by Anderson and Rosendall[16] in mucosal homogenates of 72 h starved guinea-pig, was approximately 10% of the rate found for liver. From these results it was concluded that intestinal mucosa could contribute importantly to the glucose requirement of the fasting guinea-pig. The contribution of glycogenolysis to the observed rate of glucose production was minimal. Although glycogen is present in mucosal scrapings from the small intestine of rat and guinea-pig (unpublished observations and Paper IV), a recent electron microscopical study reported only glycogen particles in goblet- and in submucosal cells of normal fed rats[98]. Although we were able to confirm glucose production in homogenates of starved guinea-pigs (Paper IV), we found no significant incorporation of the labelled gluconeogenic precursors used (pyruvate, alanine and lactate) into glucose. Subsequent results led to the conclusion that glucose was formed from ribose-5-P, a degradation product of nucleotides, added to the homogenates to obtain optimal glucose production rates. It was found that glucose, glucose-

6-P and fructose-6-P were readily formed from ribose-5-P, in agreement with reports on liver homogenates[99,100]. Ribose-5-P may be converted to triose-P and hexose-P in the non-oxidative part of the pentose cycle and subsequently to glucose by the combined actions of fructose-1,6-P_2 phosphatase and glucose-6-P phosphatase. The rate of hexose plus hexose-P production was appreciably lower in mucosal homogenates of starved rats (unpublished observations), probably due to the absence of glucose-6-P phosphatase in rat small intestine[97]. The physiological role of the rapid conversion of triose-P to glucose, found in mucosal homogenates of the starved guinea-pig, may be the conversion of fructose to glucose, observed _in vitro_[101] and _in vivo_[102].

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CHAPTER III

THE EFFECT OF FATTY ACIDS ON PYRUVATE TRANSPORT ACROSS THE MEMBRANE OF EPITHELIAL CELLS FROM RAT SMALL INTESTINE; EVIDENCE FOR A CARRIER-MEDIATED FLUX OF MONOCARBOXYLATE ANIONS

Introduction

In the previous chapter the possible inhibitory action of fatty acids on the transport of pyruvate was discussed. Therefore the aim of the present study was to investigate the mechanism of translocation of pyruvate or other monocarboxylic acids across the epithelial cell membrane. The studies with isolated epithelial cell sheets do not allow conclusions to be made about the localization of the translocation sites, since both brushborders and basolateral membranes are exposed to the incubation fluid simultaneously. With the epithelial cell preparation, the kinetics and mechanism of the translocation across the membrane can be studied more easily than with the everted intestine, a preparation used by many authors interested in weak electrolyte transport[1-7]. Firstly, the absorptive surface of the intact small intestine is composed of a complex series of macro- and microfolds giving rise to the presence of an appreciable unstirred layer[8], which may interfere with the kinetic measurements. In fact, 6 min may even be required to allow complete equilibration of the extracellular volume marker, such as $[^3H]$-inulin, with the unstirred layer[9], whereas less than one min suffices for the isolated cell preparation (Fig. 1 of Paper V). Secondly, the initial uptake rates measured may be a summation of transport process both in the epithelial cells as well as the cells of the underlying connective tissue and smooth muscle. Thirdly, loss of the labelled probe molecule into the serosal compartment may result in underestimation of "unidirectional" flux rates. However, comparison of results obtained with the everted intestine preparation on mucosal to serosal flux, with our studies on uptake rates in isolated epithelial cells may be very useful to find out the nature of the rapid epithelial transport of weak electrolytes *in vivo*.

Methodological remarks

Epithelial cells from the jejunum of the rat were harvested according to the vibration procedure first described by Harrison and Webster[10,11]. These cells appeared morphologically intact[2]. Furthermore, the data in Fig. 2 show that the cells accumulate glucose against a concentration gradient at 30°C already after 30 sec incubation. This process is dependent on the presence of Na^+ in the medium. In the absence of Na^+ we found no accumulation against a gradient, in agreement with studies of Crane[13]. For the rapid separation of the cells from the incubation medium, the centrifugal filtration through silicon oil was applied in our laboratory. The following comments should be made regarding the procedure: 15 sec of centrifugation were found to be sufficient for the sedimentation of the cells and it was found that the amount of adherent buffer accounted for a large percentage (60-70%) of the total water space found in the underphase as determined from the distribution of ^3H_2O and $[^3H]$-inuline. It was found in a later stage of the investigations that the volume of adherent fluid could be somewhat overestimated when $[^3H]$-inuline was used as the extracellular marker by doing parallel studies with $[^{14}C]$-polyethyleneglycol instead (Paper VI). For estimation of very small uptake rates a more suitable $[^3H]$-labelled marker for adherent buffer should be used. A recent study showed that during the passage of cells through the oil cellular damage was minimal[14], which could be understood if cells or

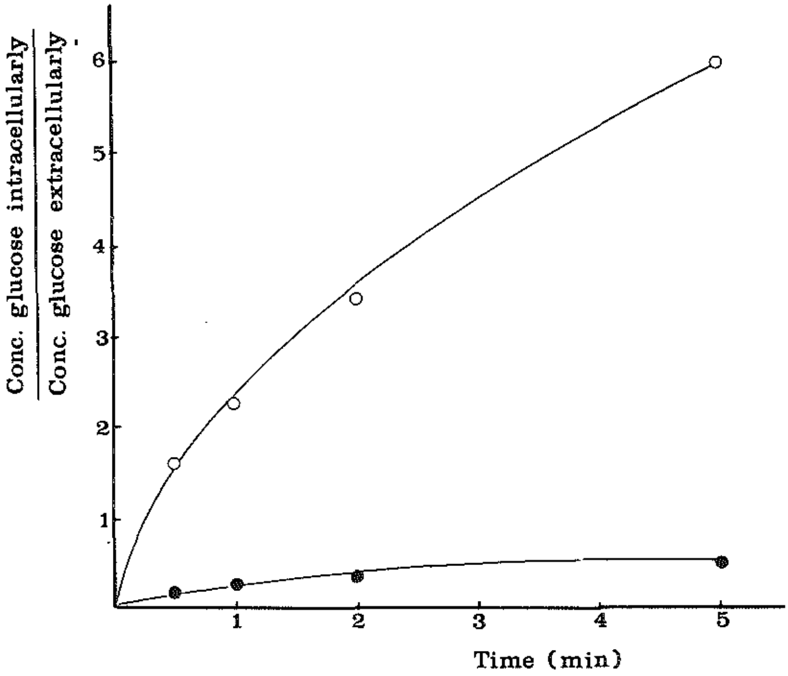

Fig. 2. Na^+ dependency of glucose accumulation in isolated epithelial cells from rat small intestine. Epithelial cells were incubated at 30°C in Krebs-phosphate buffer (gassed with O_2) containing 0.4 mM $[^3H]$-inulin and 0.1% albumin. The measurement of the intracellular glucose concentration by silicon oil filtration is described in Paper V. Intracellular ^3H_2O was determined by parallel incubation with ^3H_2O and $[U-^{14}C]$-sorbitol. In Na^+-free buffer the Na^+ was replaced by $choline^+$. ○—○ In the presence of Na^+, ●—● Na^+ was replaced by $choline^+$.

cell aggregates were indeed to be surrounded by a protective layer of incubation medium during the passage through the silicon oil. In our studies (unpublished) the leakage of lactate dehydrogenase (estimated in the upperphase) during this passage was found to be minimal. The present method has been found to be useful for the study of transport of monocarboxylic acids in intestinal epithelial cells. The time-dependency of pyruvate and propionate transport at 0°C showed a biphasic pattern (Fig. 1 of Paper V): an initial rapid phase (0-15 sec) and thereafter a phase with a slower rate. We tried to investigate the kinetics during very short incubation periods (30 sec), but it was found that the amount of adherent substrate accounted for a large percentage of the total substrate found in the sample, so that a large error in the uptake rate measurements was introduced. Therefore, kinetic studies were carried out by measurements over 5 min. The initial phase of uptake could be a non-specific binding of monocarboxylic acids to the membrane or binding of anions to polar groups of membrane proteins. An aspecific binding cannot explain the countertransport found, because preloading the cells for 5 min with unlabelled substrate stimulated the influx of another labelled substrate (TABLE II of Paper VI).

Effect of fatty acids on in- and outflux of pyruvate

Measurement of pyruvate transport in epithelial cells at 0°C showed that short- and medium-chain fatty acids as well as ricinoleate inhibit this transport (Paper V). This result may partially explain the observed inhibition of fatty acids on both pyruvate oxidation and conversion to lactate. Propionate inhibits pyruvate transport by another mechanism than octanoate and ricinoleate. The competitive inhibition by short-chain fatty acids (Fig. 3 of Paper V) suggested a carrier-mediated transport of monocarboxylic acids. Gardner[15] suggested that the characteristics of nonionic diffusion need not necessarily differ from those of carrier-mediated transport. In the nonionic diffusion model only the undissociated molecule can cross the membrane. Intracellular dissociation may occur, the extent of which will depend on the pH of the cytosol and the pK_a value of the substrate. When the intracellular fatty acid concentration is raised, the increased flux of associated protons might reduce the intracellular pH and therefore limit the degree of dissociation and in this way also limit the rate of transport. Even on this basis mutual competition could be explained. If for instance pyruvate as well as propionate are present outside the cell, propionate could inhibit the pyruvate flux by possibly competing for the available H^+ in the unstirred layer, or by changing the intracellular pH by acting as a proton vehicle. Therefore, the influence of the extracellular pH on both pyruvate and propionate influx rates was studied The influx of pyruvate was not dependent on the pH between 6.0 and 7.9. Strong accumulation against a concentration gradient was observed at the lower pH values (5.9-7.0) for propionate and octanoate (Figs. 4 and 5 of Paper V). The absence of an effect of pH lowering on the influx rate of pyruvate tends support to the conclusion that the ionic form is the only species transported. In efflux studies, countertransport of intracellular pyruvate for extracellular propionate, butyrate, acetate or bicarbonate could be shown. No effects were seen with octanoate, hexanoate or ricinoleate. On the other hand the influence of the pH on the exchange suggested an exchange between anions. The contribution of nonionic diffusion to overall transport is determined by the pK_a value and the lipophilicity of the monocarboxylic acid. Propionate efflux may also be stimulated by fatty acids and also by pyruvate, but in that case the stimulations found for pyruvate, hexanoate or octanoate were similar (TABLE III of Paper V). The possible mechanism of this exchange might depend on the generation of protons at the inside of the membrane by the influx of undissociated fatty acids, as described by Bakker and van Dam[16] for liposomes. Our studies on monocarboxylic acid transfer were solely based on measurements at 0°C because of the rapid rate of transmembrane fluxes in small intestinal epithelial cells and the possible interference of metabolism of the acids studied at higher temperatures. Propionate metabolism is relatively sluggish[7,17] when compared with that of pyruvate in rat small intestine (Paper III). TABLE I of Paper VI shows the inhibition of propionate uptake at 30°C by pyruvate, octanoate or ricinoleate in epithelial cells. The inhibition by pyruvate was largely abolished at the higher propionate concentrations, suggesting a competitive nature of the inhibition. These data agree with the conclusions reached in the pyruvate transport studies, carried out at 0°C.

Properties of the carrier-mediated flux of monocarboxylate anions

At first it seemed very likely that pyruvic acid transport involved only a carrier-mediated flux, but in competition experiments with propionate (Fig. 1 of Paper VI) the maximal inhibition differed from 100%, indicating a contribution of ionic diffusion to pyruvate permeation. Indeed, some deviation from linearity in a Lineweaver-Burk plot of the rate of pyruvate transport as a function of the pyruvate concentration was observed at high concentrations of pyruvate (unpublished). Non-linear relationships were also found in studies on propionate transport (TABLE I of Paper VI), indicating also for propionate a contribution of both nonionic and ionic diffusion to the overall transfer process. By plotting the ratios of uninhibited influx to inhibited influx versus the inverse inhibitor concentration, affinity constants of 1.7 and 1.2

mM could be calculated for pyruvate and propionate in their carrier-mediated fluxes respectively.

The kinetics of the exchange diffusion between pyruvate and short-chain fatty acids were also studied (Paper VI). Small aliquots of $[1-^{14}C]$-pyruvate loaded epithelial cells were transferred to a number of different media, containing various concentrations of unlabelled countersubstrate and the stimulate efflux was recorded (TABLE IV and Fig. 2 of Paper VI). The efflux stimulation appeared to be a saturable function of the external counteranion concentration. The V_{max} of stimulated pyruvate efflux was similar whether acetate, propionate or butyrate were employed as countersubstrates. The affinity constant for propionate, obtained in this study (K_m=1.0 mM), almost equalled the value obtained in the propionate transport studies.

Temperature-dependencies of the transfer of pyruvate and propionate obtained from "unidirectional" efflux experiments are shown in Paper VI. The Arrhenius plots revealed a linear relationship between 5 and 15°C, from which activation energies could be calculated (19.6 and 12.6 kcal/mole respectively). The lower activation energy for the propionate efflux may have its origin in a partial contribution of nonionic diffusion to propionate overall permeation. Deviation from linearity appeared in the Arrhenius diagram between 0 and 5°C. This may be due to a phase transition in the membrane structure at the lower temperatures. A high activation energy seems to be a common property of anion transfer across membranes[18-20].

A possible function of carrier-mediated anion flux in the small intestine

The moderately high affinity of short-chain fatty acids for the monocarboxylate anion carrier described for rat jejunal epithelial cells in the present study (Paper VI), suggests that this translocation system might have a function in the transepithelial transport of short-chain fatty acids. A specific transfer mechanism for the transport of short-chain fatty acids in rat small intestine has been demonstrated earlier[3,7,17, 21-23]. *In vitro* preparations of jejunum were found to build up a concentration gradient of the acids from mucosa to serosa. These studies furthermore revealed properties such as saturation kinetics, dependency on metabolic activity and sensitivity to the Na^+ concentration at the mucosal side. However, other reports indicate that absorption of short-chain fatty acids is a passive process[4,6,24,25] and suggest the importance of the lipophilicity of these acids in determining the rate of uptake. The transport of certain drugs and of short-chain fatty acids was also studied recently by Jackson et al.[1,2]. These authors discuss the hypothesis of Hogben et al.[6] that the luminal secretion of H^+ ions results in the formation of a region of low pH adjacent to the epithelial surface and that the pH of this "microclimate" is an important determinant of weak electrolyte transport in the intestine. Jackson and Morgan[1] confirmed previous work [26,27] that showed that during glucose metabolism metabolic anions, such as lactate or HCO_3^-, were preferentially discharged at the serosal side, whereas H^+ ions were secreted at the lumen side. The studies of Jackson et al. [1,2] suggest that the mechanism for jejunal transport of weak electrolytes may be described by a three compartment model system. In the ill-defined intermediate compartment the pH is higher than in both other compartments. Such an intermediate compartment could be present within the intestinal wall. In order to account for the observed net mucosal to serosal flux of weak electrolytes they suggest that the barrier, separating the intermediate compartment from the serosal bulk phase, is permeable to both the ionized as well as the non-ionized form of the electrolyte. The barrier separating the intermediate compartment from the mucosal bulk phase, however, could only be permeable to the non-ionized form of the acids. The mutual inhibition of weak acids could readily be explained in terms of a system of the mentioned compartments, in which the intermediate compartment should have a relatively high pH[1].

The monocarboxylate carrier, described in the present study, may be localized at the basolateral membrane of the epithelial cells to accelerate the movement of anions from the "intermediate" compartment to the serosal bulk phase. This localization is compatible with the carriers proposed in heart-, brain- and erythrocyte membranes[28-31]. Naupert reported in her thesis[7] on short-chain fatty acid transport in rat small intestine _in vitro_ high K_m values (50-100 mM) for the fatty acids studied (propionate, butyrate) and probably high K_i values in competition experiments. A carrier-mediated transport, however, was not excluded in her study. Preliminary competition studies were also carried out in our laboratory in the everted sac preparation (unpublished). Propionate (1 mM) uptake at 30°C appeared to be linear up to 20 min. It could be inhibited (15-20%) by octanoate (5 mM), ricinoleate (0.5 mM) and pyruvate (10 mM). In other experiments, everted sacs were preloaded for 20 min with labelled propionate (1 mM) and the efflux rate of propionate to the lumen was studied. No acceleration by the addition of unlabelled pyruvate to the mucosal fluid was found (compare Paper V). This may indicate that exchange of anions is absent at the brush-border side of the epithelial cell. Parsons[32] proposed earlier that H^+ secretion accompanied HCO_3^- absorption in rat jejunum and speculated that H^+ secretion is partly associated with Na^+ absorption. Experiments, carried out by Turnberg et al.[33], also suggested that in human jejunum HCO_3^- absorption is initiated by exchange of H^+ for Na^+ ions. The H^+ ions react with HCO_3^- ions to form carbondioxide which then diffuses from the lumen. Recently the same characteristics were found in rat jejunum, but a part of the HCO_3^- absorption was not Na^+-dependent[34]. Because short-chain fatty acid absorption is partly sensitive to Na^+, it is possible that the exchange of H^+ for Na^+ at the brushborder side of the epithelial cell is involved to some extent (compare also ref. 1).

Based on the observations mentioned, it is suggested that part of short-chain fatty acid- and bicarbonate transport is mediated by the operation of a Na^+/H^+ exchange at the brushborder and a carrier-facilitated flux at the basolateral side of the epithelial cell (Fig. 3).

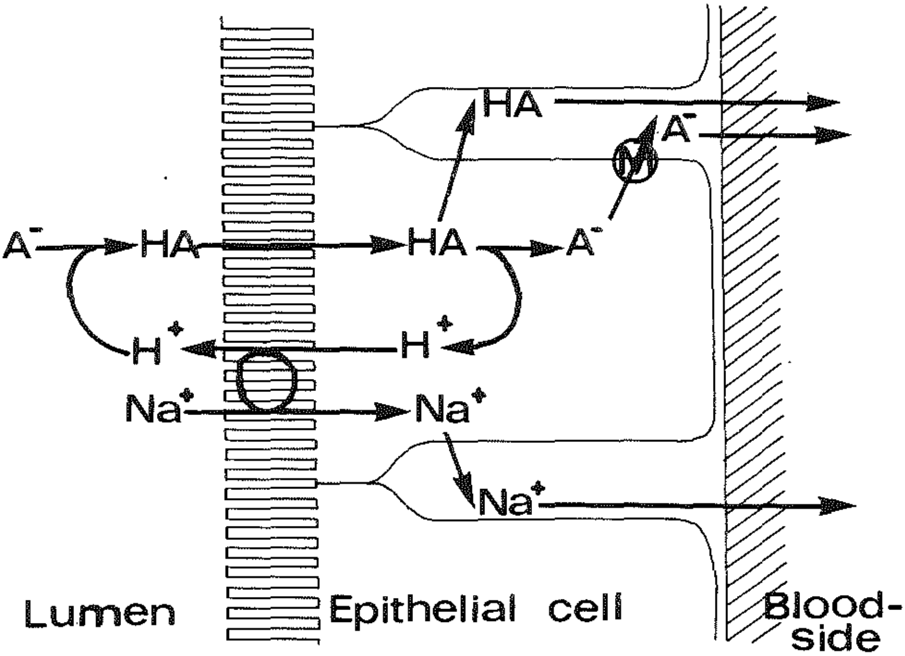

Fig. 3. A hypothetical model for the absorption of weak electrolytes in jejunum. (M = monocarboxylate carrier, A^- = weak electrolyte anion)

For the rat these short-chain fatty acids are quantitatively not important as metabolic fuel. However, in some animal species (guinea-pig and ruminants) large amounts of volatile fatty acids are produced by the bacterial flora of the intestine[35-37] and may contribute to energy metabolism to a significant extent[38] . In order to avoid accumulation of these acids in the intestine and to guarantee rapid uptake by the body, the presence of monocarboxylate carrier(s) may be important.

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SUMMARY

The small intestinal mucosa is known to have a high rate of aerobic glycolysis. The absence of a Pasteur effect in the small intestine is related to this observation. It was questioned whether this is an artefact. The knowledge of the rate-limiting factors of glycolysis is therefore important. The possibility that hexokinase or phosphofructokinase is rate-limiting is discussed. For phosphofructokinase activity, the energy charge is of critical importance. The energy charge in the usual *in vitro* preparations, such as the everted sac or isolated epithelial cells, is very low. Oxygen availability *in vitro* might be limited. Therefore the vascularly perfused small intestine was chosen as a model system. It was found that increasing the oxygen availability by the addition of fluorocarbons to the perfusion medium, raised the energy charge up to *in vivo* values. Under these conditions a clearcut Pasteur effect could be demonstrated. When glucose was replaced by fructose as the substrate, a high rate of lactate production was also observed. This may be due to the circumvention of the rate-limiting step(s) of glycolysis or by alteration of the energy charge. The latter was verified by loading fructose instead of glucose *in vivo*. The most striking alterations after fructose loading were the accumulation of fructose-1-P and the decrease of the concentrations of P_i and creatinephosphate. As long as the latter compound had not been removed considerably, adenine nucleotide levels remained virtually the same. It is therefore likely that at least in the early phase after fructose loading, the high rate of lactate production is due to circumvention of the rate-limiting step(s) of glycolysis:hexokinase and/or phosphofructokinase.

Another aspect of energy metabolism in small intestinal epithelium: the regulation of pyruvate metabolism,

was also studied. It had been found earlier that fatty acids inhibit the oxidative decarboxylation of pyruvate and this thesis demonstrates that this inhibition may partially be due to the conversion of the active form to the, phosphorylated, inactive form of the pyruvatedehydrogenase complex, as has been demonstrated in other tissues by other investigators. However, the effect was rather small, so that other possible regulatory effects were also investigated. Indeed, it was found that fatty acid addition to isolated epithelial cells increased the levels of the products of the pyruvatedehydrogenase reaction. The relative contribution of both regulatory mechanisms could not be established. During these investigations it was found that the decrease of the rate of pyruvatedecarboxylation was not compensated by an increased rate of lactate formation. Hence it was investigated whether fatty acids have an effect on pyruvate transport across the cellular membrane. For this study a method was applied, in which rapid anion fluxes could be examined: centrifugal filtration of isolated cell sheets through silicon oil. Pyruvate influx was indeed inhibited by the addition of fatty acids to the incubation medium. Since the small intestine of fasted guinea-pigs, as reported in literature, showed considerable rates of net glucose production and since in other tissues fatty acids influence the rate of gluconeogenesis from pyruvate, it was also studied whether in small intestine gluconeogenesis really occurs. It was found, however, that this process is virtually absent in small intestine of both guinea-pig and rat.

Finally, the mechanism by which fatty acids inhibit pyruvate flux across the cellular membrane, was studied in detail. On the basis of the findings of saturation kinetics, competitive inhibition by short-chain fatty acids and counteranion transport, a carrier-mediated monocarboxylate anion transport was proposed. Arguments were presented that the localization of the carrier is likely to be present at the basolateral part of the epithelial cellular membrane.

SAMENVATTING

Het is uit de literatuur bekend dat de dunne darm een hoge aerobe glycolysesnelheid bezit en dat het Pasteur effect in het slijmvlies van de dunne darm afwezig is. Door ons werd de mogelijkheid overwogen of deze gegevens op een artefact berusten. In dit verband is het van belang te weten welke factoren de snelheid van de glycolyse bepalen. De mogelijkheid dat hexokinase of fosfofructokinase snelheidsbepalend zijn wordt besproken. De fosfofructokinase activiteit correleert nauw met de fosforyleringspotentiaal. De fosforyleringspotentiaal in de meest gebruikte in vitro preparaten, de omgedraaide darm en geïsoleerde epitheelcellen blijkt echter erg laag te zijn. Dit is wellicht te wijten aan een gebrekkige zuurstofvoorziening in deze preparaten. Daarom werd als modelsysteem voor de bestudering van factoren die de glycolyse snelheid beïnvloeden de vasculaire perfusie van de dunne darm in vitro gekozen. Het bleek dat door toevoegen van fluorocarbons aan het perfusiemedium de zuurstofvoorziening verder kon worden verbeterd ten gevolge waarvan de fosforyleringspotentiaal verder steeg tot waarden die ook in vivo worden gevonden. Wanneer fructose in plaats van glucose werd gebruikt als substraat werd een hoge melkzuurproductie geregistreerd. Dit kan erop wijzen dat de omzetting van fructose tot triose fosfaat de snelheidsbepalende stappen van de glycolyse omzeilt doordat andere enzymatische stappen worden gebruikt dan die welke glucose in triosefosfaat omzetten. Het is echter ook mogelijk dat de hexosen ten dele gelijkelijk worden afgebroken maar dat fructose de fosforyleringspotentiaal doet dalen. Dit laatste werd nagegaan door de dunne darm in vivo met fructose in plaats van glucose te belasten. De veranderingen die hierbij het meest opvielen waren de ophoping van fructose-1-P en de daling van de P_i en de creatinefosfaat concentraties. Zolang de creatinefosfaat concentratie niet in belangrijke mate verandert, blijven de adenine nucleotide niveau's practisch constant. Dus is het mogelijk dat ten minste in een vroege fase na fructosebelasting de hoge snelheid van melkzuurproductie te wijten is aan het omzeilen van de snelheidsbepalende reactie(s) van de glycolyse: hexokinase en/of fosfofructokinase.

Een ander aspect van de energiehuishouding in het dunne darm epitheel werd tevens bestudeerd: de regulatie van het pyruvaatmetabolisme. Reeds eerder was gevonden dat vetzuren de oxydatieve decarboxylering van pyruvaat remmen. Dit proefschrift laat zien dat deze remming ten dele verklaard kan worden met een omzetting van de actieve naar de gefosforyleerde inactieve vorm van het pyruvaatdehydrogenase complex, zoals reeds voor andere weefsels was beschreven. Echter het remmende effect was gering, zodat ook andere mogelijk regulerende effecten werden onderzocht, zoals productremming. Toevoeging van vetzuur aan het incubatiemedium van geïsoleerde epitheelcellen gaf inderdaad een toename te zien van de producten van de pyruvaatdehydrogenase reactie: NADH en actief acetaat. De relatieve bijdrage van beide regulatiemechanismen kon niet worden vastgesteld. Verder werd gevonden dat de afname van de snelheid van pyruvaat decarboxylering door vetzuren niet gecompenseerd wordt door een toegenomen melkzuurproductie. Daarom werd nader onderzocht of vetzuren misschien een remmende werking hebben op het transport van pyruvaat door het celmembraan. Voor dit onderzoek werd een methode toegepast waarmee snelle anionfluxen kunnen worden bestudeerd: scheiding van geïsoleerde epitheelcel aggregaten van het incubatiemedium door centrifugering door siliconenolie. Inderdaad bleek de pyruvaatinflux door toevoeging van vetzuren aan het incubatiemedium te worden geremd. In de literatuur was eerder beschreven dat de dunne darm van de gevaste cavia een hoge snelheid van glucoseproductie kan vertonen en aangezien in andere weefsels vetzuren de gluconeogenesesnelheid beïnvloeden werd nagegaan of deze glucoseproductie werkelijk de novo synthese van glucose voorstelt. In de dunne darm echter van zowel de cavia als de rat, bleek dit proces afwezig te zijn.

Tenslotte werd het mechanisme waarop de remming van de pyruvaatflux langs het celmembraan door vetzuren berust, nader onderzocht. Op basis van de gevonden verzadigingskinetiek, competitieve remming door kort-keten vetzuren en counteraniontransport werd een "carrier"-versneld monocarboxylaat anionentransport verondersteld. Argumenten werden aangevoerd dat deze carrier waarschijnlijk in het basolaterale deel van het epitheelcelmembraan is gelocaliseerd.

CURRICULUM VITAE

De schrijver van dit proefschrift behaalde in 1964 het diploma HBS-B aan het Bisschoppelijk College te Roermond. In hetzelfde jaar begon hij zijn scheikunde studie aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen werd afgelegd in 1967, terwijl het doctoraalexamen werd behaald in 1971 (hoofdvak: Biochemie, bijvak: Farmacologie). Van september 1968 tot september 1969 was hij verbonden als leraar scheikunde aan het Thomas à Kempis College te Arnhem en van 1969 tot 1971 aan het lyceum Mater Dei te Nijmegen. In september 1971 volgde een aanstelling als wetenschappelijk medewerker op de afdeling Biochemie I van de Medische Faculteit te Rotterdam, waar dit proefschrift werd bewerkt.

APPENDIX

PAPERS

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BBA Report

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Pasteur effect in the *in vitro* vascularly perfused rat small intestine

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SUMMARY

Isolated small intestine perfused *in vitro* with media with low oxygen concentration was found to contain low levels of ATP when compared with rat small intestine *in vivo*. The addition of fluorocarbon FC 75 to an erythrocyte-free perfusion medium was found to result in a high phosphate potential and a low rate of lactate production from glucose in isolated perfused small intestine, resembling the *in vivo* condition. This allowed the demonstration of a Pasteur effect in that replacement of oxygen by nitrogen (or the addition of 2,4-dinitrophenol) led to a rapid increase of the rate of glycolysis, and a decrease of the ATP concentration in the tissue.

Earlier work from this laboratory[1] showed that mitochondrial preparations, isolated from epithelial cells from rat small intestine, carried out oxidative phosphorylation, although the coupling was not tight. The possibility was considered that the high Mg^{2+}-stimulated ATPase activity of the mitochondrial preparations, contaminated with brush-border fragments, could contribute to the absence of tight coupling. Indeed, more recently we[2] were able to show tight coupling in isolated rat small intestinal epithelium mitochondria by omitting Mg^{2+} from the incubation medium. Earlier, we had given the possibility of "loose coupling" of oxidative phosphorylation serious thought, since in isolated cells and everted jejunum of the rat a clearcut Pasteur effect was found to be absent[3–5]. Similarly, no Pasteur effect was observed in intestine of other species (*cf.* ref. 6). The explanation of this phenomenon could be a low energy charge[7] in rat small intestine, since the phosphofructokinase reaction is also stimulated in small intestine, if the ATP concentration is relatively low and the ADP, AMP and P_i concentrations are relatively high[8,9]. Indeed, in earlier studies we reported[1] relatively low levels of ATP when compared with those of ADP and AMP for isolated rat small epithelial cells, as well as for everted rat small intestine, incubated in a glucose-containing and aerated incubation medium. Also, in studies with *in vitro* (vascular) perfused rat small intestine[10], the high rate of lactate production while the intestinal lumen was empty suggested to us that the energy charge here might also have been low. In the present paper it will be shown that an insufficient oxygen supply might be the basis of these phenomena, since the situation can be altered by increasing the availability of oxygen to the perfused tissue.

TABLE I

RELATIVE ATP, ADP AND AMP CONCENTRATIONS OF JEJUNUM PERFUSED WITH DIFFERENT MEDIA

Perfusion Medium I was Krebs–Henseleit[11] bicarbonate buffer (pH 7.4), containing 1.4 mM $CaCl_2$ and 0.6 mM $MgSO_4$ and in addition 4.7% dextran (mean mol. wt 70 000), 4 mg/100 ml promethazin and 5 mM glucose. Perfusion Medium II contained fluorocarbon FC-75 (3 M Company, St. Paul, Minn.), emulsified in Medium I, in which the dextran was replaced by 3.4% fatty acid-poor bovine serum albumin (Pentex, Kankakee, Ill.). The fluorocarbon emulsion was prepared[12] with the use of a nonionic polyalkylene oxide detergent F-68 (Wyandotte Chem. Corp., Wyandotte, Mich.). 40 ml of fluorocarbon were added to 200 ml dextran-free (perfusion) Medium I, containing 4 g F-68. After sonication (Branson Sonifier, Model S 75) for 4 periods of 5 min at 4.5 A (the temperature was kept below 6 °C), the emulsion was washed 3 times with 200 ml dextran-free Medium I by centrifugation at 9000 × $g_{av.}$. The washed sediment was finally suspended in 200 ml dextran-free Medium I, containing 3.4% albumin and again sonified as above for 4 periods of 3 min. The larger fluorocarbon particles were removed by centrifugation for 5 min at 450 × $g_{av.}$ and the supernatant filtered through Whatman No. 589. Perfusions were carried out as described earlier in ref. 10. Rats were pretreated with intravenous injections of 5 mg heparin and 1 mg papaverin 2 min prior to cannulation. The temperature of the isolated intestine was kept close to 37 °C with an infrared lamp and by rinsing with warm saline. After 15 min perfusion (or when indicated *In situ*) part of the jejunum was freeze clamped[13]. The frozen tissue was pulverized in a mortar while liquid N_2 was continuously added. The powder was mixed with 5% $HClO_4$, thawed, centrifuged and the supernatant filtered through Millipore (pore size 0.4 μm), neutralized with KOH and again centrifuged. ATP[14], ADP[15] and AMP[15] were determined as indicated. The nucleotide concentrations were calculated per ml extract and the sum of [ATP] + [ADP] + [AMP] made 100%. *n* = number of experiments; average values are given.

Perfusion medium	*Flow rate (ml/min)*	*n*	*% of adenine nucleotides* *ATP*	*ADP*	*AMP*
I	15.8	2	78.5	18.8	2.7
I	4.8	3	70.0	22.5	7.5
II	10.8	2	84.0	14.2	1.8
In situ	–	4	82.1	15.9	2.0

It can be seen from Table I that perfusion with a modified Krebs–Henseleit[11] bicarbonate buffer, containing 5 mM glucose and saturated at 37 °C with O_2–CO_2(95:5,v/v), especially when the flow rate is low, leads to a lower energy charge than that of the rat small intestine *in situ* (non-resorptive state; rat under Nembutal narcosis). The situation *in situ* can be approached during perfusion *in vitro* of the isolated small intestine, when a fluorocarbon emulsion is added to the perfusion medium. The fluorocarbon-containing medium carries considerably more oxygen than the medium without fluorocarbon, as can be calculated from the data of Ruigrok and Elbers[12]. Another series of experiments in which, in addition to ATP, ADP and AMP, creatine phosphate was determined and in which the values were expressed in nmoles per mg DNA, is seen in Table II.

TABLE II

CONCENTRATIONS OF ATP, ADP, AMP AND CREATINE PHOSPHATE IN VASCULARLY PERFUSED RAT SMALL INTESTINE UNDER DIFFERENT CONDITIONS

The perfusions were carried out with Medium II (see Table I). Creatine phosphate was determined in the ATP determination[14] after the exhaustion of ATP, by adding 3.6 units creatine kinase. DNA[16] was determined in the residues of $HClO_4$ extracts (*cf.* Table I). The values (nmoles per mg DNA) represent the mean of two separate experiments. The mean flow during vascular perfusions was 10.8 ml/min.

	In situ	*Aerobic perfusion (10 min)*	*Anaerobic perfusion (10 min)*
ATP	216.5	202.6	85.9
ADP	40.8	38.4	51.5
AMP	4.9	4.2	43.9
Creatine phosphate	245.1	276.6	10.6
$\Sigma{\sim}P$	718.9	720.2	233.9

Now that we have a means of increasing the energy charge of the tissue by the addition of fluorocarbon to the aerated perfusion medium, we reinvestigated aerobic glycolysis during perfusions *in vitro* . It can be seen from Table III that a Pasteur effect can be observed: in the presence of oxygen, the rates of glucose utilization and of lactate productions are low; when oxygen is replaced by nitrogen, or when the uncoupler of oxidative phosphorylation 2,4-dinitrophenol is added, the rates of glucose utilization and lactate production are strongly increased. These effects are very rapid, as can be concluded from the experiments shown in Fig. 1. The fact that in the presence of nitrogen the amount of lactate formed is less than twice the amount of glucose utilized (Table III) may be due to incomplete anaerobiosis, since the tissue remains exposed to air.

From these results it may then be concluded that proper oxygenation of the tissue leads to a high energy charge resulting in limitation of the rate of lactate production. The intestine *in vivo*, even during glucose transport, also shows a very low rate of lactate production[17] and so does the rat intestine perfused *in vitro* with a solution containing erythrocytes as well as norepinephrine and dexamethasone that results in an adequate perfusion pressure, as so elegantly worked out by Windmueller and Spaeth[18].

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TABLE III

GLUCOSE UPTAKE AND LACTATE PRODUCTION IN VASCULARLY PERFUSED RAT SMALL INTESTINE UNDER DIFFERENT CONDITIONS

The perfusions were carried out with Medium II (see Table I). Measurements were done over a perfusion time of 30 min. Glucose[9] and lactate[20] were determined in the recirculated medium at zero time and 30 min after centrifugation of the samples.

Perfusion conditions	*Glucose uptake (μmoles/h)*	*Lactate output (μmoles/h*	*Flow (ml/min)*
Aerobic	78	88	7.6
Aerobic	52	41	10.9
Anaerobic	310	545	10.9
Anaerobic	221	414	10.8
Aerobic + 0.5 mM dinitrophenol	228	277	10.7
Aerobic + 0.5 mM dinitrophenol	252	292	11.0

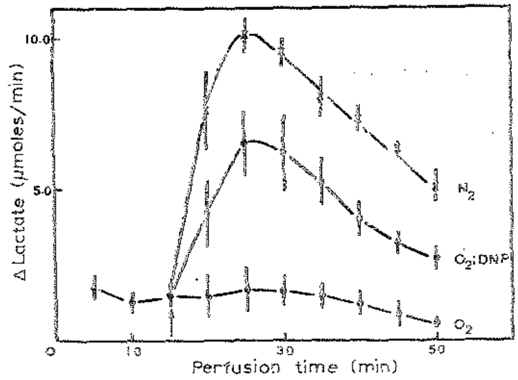

Fig. 1. Pasteur effect in vascularly perfused rat small intestine. The perfusions were carried out with Medium II (see Table I). Arterial and venous samples were taken from the recirculated medium at the times indicated. Mean flow was 10.7 ml/min. Where indicated dinitrophenol (DNP) was added to the incubation medium (final concn of 0.4 mM) or O_2–CO_2(95:5, v/v) was replaced by N_2–CO_2(95:5, v/v) Lactate was determined as described in Table III. The results are expressed as means ± S.E. (n = 4).

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THE EFFECT OF FRUCTOSE ON THE STORES OF ENERGY-RICH PHOSPHATE IN RAT JEJUNUM *IN VIVO*

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SUMMARY

1. The changes of the concentrations of ATP, ADP, AMP, P_i and creatine phosphate in *in vivo* freeze-clamped jejunum of rat were examined after intraluminal loading of fructose, glucose and 3-*O*-methylglucose. The most striking effects were seen after fructose loading. Although the creatine phosphate concentration was lowered to 55 % of the control value, the ATP concentration was relatively constant.

2. The changes of the concentrations of fructose-1-*P*, glycerol-3-*P* and of various glycolytic intermediates were studied 10 min after hexose loading. In the case of intraluminal fructose loading, fructose-1-*P* rapidly accumulated and P_i decreased due to its incorporation in fructose-1-*P*.

3. Ketohexokinase (EC 2.7.1.3) and fructose-1-*P* aldolase (EC 4.1.2.7) were predominantly found in the mucosal layer, which was separated from the muscle layer by scraping. In both samples the same creatine kinase activity (EC 2.7.3.2) was found.

4. 10 min after intraluminal loading of fructose only a small increase in the concentration of fructose-1-*P* and a small decrease in the concentration of ATP in liver was observed, in contrast to the large effects on the levels of these metabolites after vascular fructose perfusion, as described in the literature. We therefore also compared the effect of intravenous hexose loading on the metabolite levels in rat liver and rat small intestine.

INTRODUCTION

It has been shown by several authors[1–4] that intravenous or intraperitoneal injection of large amounts of fructose rapidly depletes liver ATP and P_i in rats and other species. Marked elevations of the plasma levels of uric acid and of allantoin follow these fructose loadings[2], corresponding to an accelerated breakdown of the adenine nucleotides[1,3]. These effects on adenine nucleotide and P_i levels are accompanied by a large accumulation of fructose-1-*P* in the liver. Depletion of the P_i pool by the accumulation of fructose-1-*P*, resulting in a sluggish rephosphorylation of AMP and ADP, is the apparent cause of the lowering of levels of ATP and total adenine nucleotides. After intravenous injection of about 1 mmole fructose in rats, the blood concentration of fructose is high (12 mM) at 2.5 min (ref. 5) and is rapidly lowered to 0.5 mM at 10 min. Such high values are unlikely to be formed under physiological feeding conditions, but high concentrations may be found in the intestinal lumen and therefore in the resorptive epithelial cells. Part of the fructose in these cells may be converted to fructose-1-*P*, since ketohexokinase is also present in rat small intestine[6]. Another, presumably much smaller, part may be converted to fructose-6-*P* by hexokinase[7] (EC 2.7.1.1). Whatever pathway may be operating, the conversion to glucose is extremely slow[8,9], due to the virtual absence of glucose-6-phosphatase (EC 3.1.3.9) activity[10]. The breakdown of fructose-1-*P* is limited by the low fructose-1-*P* aldolase activity[11,12]. For these reasons only a small part of the fructose in the lumen will be involved in endogenous metabolism and the bulk will be actively[13,14] transported. Nevertheless, the consequence of fructose loading on the energy metabolism of the epithelial cells was investigated, because of its possible toxicity to the epithelial cells.

MATERIALS AND METHODS

Reagents

All chemicals were of analytical grade purity. Enzymes and cofactors were obtained from Boehringer and Co., Mannheim, Germany.

Operation procedure

Normal fed male Wistar rats, weighing approximately 200 g, were anesthetized with Nembutal. Through an abdominal incision, the small bowel, cecum and proximal large bowel were gently exteriorized. The duodenum was opened about 5 cm from the stomach, keeping the pancreatic duct intact. The end of the small intestine was ligated and also opened, so that the whole small intestine could be canulated and rinsed with 50 ml warm saline, followed by the passage of air. The abdomen was covered with gauze soaked in warm saline and kept at about 37 °C by warming with an infrared lamp.

Administration of hexose

Hexose solutions were made isotonic with NaCl. Control experiments were done with 0.9 % NaCl. In intraluminal loading experiments,we delivered 3 ml 100 mM hexose *via* the canula in the small intestine. Intravenous loading was carried out by injecting 1 mmole hexose in the left femoral vein. After the time indicated, a part of intestine or liver was freeze-clamped[15]. The frozen tissue was rapidly weighed, pulverized in a mortar, while liquid N_2 was continuously added. The powder was then mixed with 5 % $HClO_4$, thawed, centrifuged and the supernatant filtered through a Millipore (pore size 0.4 μm), neutralized with KOH and again centrifuged. ATP, ADP, AMP and creatine-*P* were determined as described earlier[15]. Freeze-clamping of the right lobe of the liver was performed after elevation by means of a loose ligature conducted through the organ with a needle.

Assays

Fructose-1-*P* was determined by the method of Eggleston[16] with liver aldolase, isolated from rat liver[17] and P_i was determined by the method of Fiske and SubbaRow[18]. Both compounds were measured in a Zeiss P.M.Q. II spectrophotometer. The following hexose and triose derivatives were tested in an Aminco-Chance dual-wavelength spectrophotometer (wavelengths 355 and 380 nm). Fructose-1,6-P_2 was measured together with the total triose-P in a combined assay[19]. Pyruvate, phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate were determined in sequence[20], as well as glycerol-3-P[21] and lactate[22]. Glucose-6-P[23], glucose-1-P[23] and fructose-6-P were measured in the sequence given. The values are expressed as µmoles/g wet weight ± S.E. We also determined the dry weights of the $HClO_4$ extracts, or the DNA[24] content of these extracts with similar final results. The DNA content per g wet weight in jejunum is 3.3 times higher than in liver.

For the enzyme assays the animals used were killed by cervical fracture, and the small intestine was removed. After rinsing the jejunal lumen with 50 ml ice-cold saline, the jejunum was everted and the mucosa removed by gentle scraping with a microscope slide. The mucosal scraping and residual smooth muscle *plus* connective tissue were homogenized with a PT 10 Polytron homogenizer (set at Position 4) for 120 s in 0.05 M triethanolamine buffer, pH 7.4, containing 5 mM EDTA and 0.5 mM dithiothreitol. The homogenate was centrifuged at 105 000 × *g* for 60 min and a sample of the supernatant incubated according to ref. 25. After 10 min incubation at 30 °C, the reaction was stopped with $HClO_4$ (final concentration 5 %) and the extracts neutralized with KOH and centrifuged. In the deproteinized extracts fructose-1-P was determined[16]. An incubation without added fructose was used as the blank. Creatine kinase[26] and fructose-1-P aldolase[25] were measured as indicated. Protein was determined by the biuret procedure[27].

RESULTS AND DISCUSSION

Effects of intraluminal administration of hexoses in rat small intestine

The effect of intraluminal hexose loading on the concentration of energy-rich phosphates in jejunum was studied first. Although the whole small intestine was canulated and in contact with the hexose solution, we only freeze-clamped the upper jejunal portion, since fructokinase is most active in jejunum[7]. In Table I the results are represented. After 10 min fructose loading there was a 29.1 % decrease of the

TABLE I

CONCENTRATIONS OF ADENINE NUCLEOTIDES, CREATINE PHOSPHATE AND INORGANIC PHOSPHATE IN JEJUNUM OF RAT, FREEZE-CLAMPED *IN SITU* AFTER INTRALUMINAL HEXOSE LOADING

10 min after intraluminal loading of 3 ml 0.1 M hexose, freeze-clamping was performed (see Materials and Methods).

Hexose used	*Concentration (µmole/g wet wt)*				
	ATP	*ADP*	*AMP*	*Creatine-P*	P_i
None (*n* = 10)	1.02±0.04	0.19±0.01	0.027±0.002	1.23±0.07	2.95±0.19
Glucose (*n* = 4)	1.14±0.11	0.21±0.02	0.029±0.004	1.08±0.06	2.73±0.06
Fructose (*n* = 4)	1.03±0.05	0.24±0.02	0.031±0.004	0.68±0.04	2.09±0.10
3-*O*-Methyl glucose (*n* = 4)	1.10±0.08	0.23±0.02	0.057±0.022	1.06±0.09	—

concentration of P_i. The levels of ATP, ADP and AMP were unaffected, whereas creatine phosphate was decreased by 44.8 %. Earlier[15] we measured creatine-*P* in vascularly perfused small intestine. The possibility was left open that creatine-*P* is confined to the smooth muscle cells. This now has become improbable, since we have found (Table III and De Jonge, H. R. and Hülsmann, W. C., unpublished) that creatine kinase is very active in intestinal mucosa. The metabolic consequence of fructose loading in liver is a rapid phosphorylation of fructose to fructose-1-*P*, leading to a lowering of the P_i concentration, so that a rapid resynthesis of ATP is not possible[1–4]. As can be concluded from Table I in intestine the ATP level remains constant, but the level of creatine-*P* declines, probably due to its function to compensate for ATP breakdown (compare the data obtained for muscle[28–30]). In order to determine whether the partial depletion of creatine-*P* by fructose loading is a specific effect of fructose, we compared the effects of equivalent loadings of glucose or 3-*O*-methylglucose. It can be seen from Table I that the decrease of the concentration of creatine-*P* is then probably insignificant.

TABLE II

LEVELS OF HEXOSE METABOLITES IN RAT JEJUNUM FREEZE-CLAMPED AFTER INTRALUMINAL HEXOSE LOADING

10 min after intraluminal loading of 3 ml 0.1 M hexose, freeze-clamping was performed (see Materials and Methods).

Metabolite level (μ*mole*/*g wet wt* $\times 10^3$)	*Intraluminal hexose*		
	None ($n = 4$)	*Glucose* ($n = 4$)	*Fructose* ($n = 4$)
Glucose-6-*P*	71 ± 28	92 ± 19	137 ± 10
Glucose-1-*P*	4 ± 1	5 ± 1	7 ± 1
Fructose-1-*P*	74 ± 14	103 ± 11	1565 ± 146
Fructose-6-*P*	14 ± 1	18 ± 2	26 ± 2
Fructose-1,6-P_2	21 ± 2	18 ± 5	34 ± 1
Triose-*P*	20 ± 4	24 ± 5	34 ± 1
Glycerol-3-*P*	107 ± 15	112 ± 6	142 ± 14
3-*P*-Glycerate	20 ± 5	10 ± 5	10 ± 1
2-*P*-Glycerate	5 ± 2	2 ± 1	5 ± 1
Phosphoenolpyruvate	4 ± 1	6 ± 2	3 ± 1
Pyruvate	61 ± 3	78 ± 7	74 ± 8
Lactate	1703 ± 223	1996 ± 123	2096 ± 179

The effect of glucose or fructose loading, resulting in an increased rate of glycolysis, on the levels of various glycolytic and other intermediates of hexose breakdown in rat small intestine were subsequently investigated. It can be seen from Table II that 10 min after hexose loading a major difference between glucose and fructose feeding has arisen in that fructose loading leads to the accumulation of fructose-1-*P*. This then also explains for intestine the lowering of the creatine-*P* level. Furthermore, it can be seen that glucose did not significantly alter the sum of hexose and triose phosphates determined, which explains the absence of a clear effect of glucose on the creatine-*P* level. The effect of fructose loading on the metabolites determined (Table

II) is mainly on fructose-1-*P*, although some conversion products of fructose-1-*P* are also somewhat increased, probably related to the presence of fructose diphosphatase (EC 3.1.3.11)[25] and the absence of glucose-6-phosphatase[10] in rat small intestinal mucosa.

Now that it had been established that fructose has a specific effect on the levels of certain phosphate-containing compounds, we studied the time relationship of intraluminal loading of fructose on the levels of these compounds (Fig. 1). 2 min after loading, the jejunal content of P_i decreased to almost a minimal value. In comparison with this change, the level of creatine-*P* fell more slowly and reached a minimal value after 5 min. If we assume that about half of the creatine-*P* is also localized in the smooth muscle cells, it might be lowered to a very low concentration in the epithelial cells, at which point the ATP level begins to decrease. However, under the conditions of these experiments no significant decrease in the ATP level was observed. One explanation could be that fructose rapidly disappears from the lumen by active transport. However, a second loading of fructose at 10 min did not change the curves of Fig. 1 (results not shown).

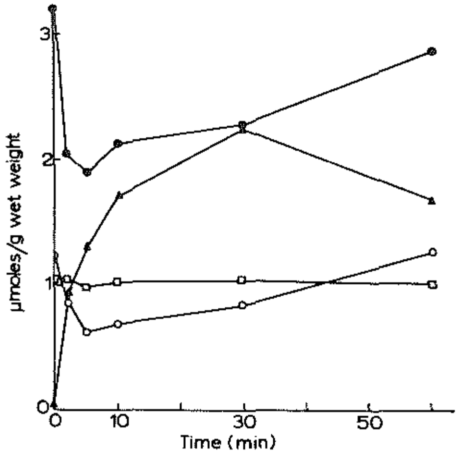

Fig. 1. Time dependence of metabolite levels after intraluminal loading of 3 ml 0.1 M fructose. Each point represents the mean of two experiments. ○–○, Creatine-*P*; □–□, ATP; ▲–▲, fructose-1-*P*; ●–●, P_i.

Preferential localization of fructokinase and fructose-1-P aldolase in the epithelial cells of intestine

That the effects of fructose on rat small intestine, as observed above, are due to its metabolism in the mucosa cells, required the exclusion of major effects on the cells of the underlying smooth muscle. A major effect of fructose on smooth muscle cells does not seem very likely, since in heart and skeletal muscle and adipose tissue no activity was found[6,31]. Indeed, Table III shows that the supernatant from homogenized mucosal tissue scraped from the everted intestine, contributes most to the total fructokinase activity.

The accumulation of fructose-1-*P* after fructose loading in intestinal mucosa must be explained by the different activities of ketohexokinase and fructose-1-*P* aldol-

TABLE III

DISTRIBUTION OF ENZYMES BETWEEN MUCOSA AND RESIDUE OF JEJUNUM

The data were calculated from three separate experiments in which 105 000 × *g* supernatants of the total homogenate, mucosal scraping and the scraping residue were prepared and assayed. The average amount of soluble protein in the three fractions was 100, 61.4 and 38.6 mg, respectively. The data are expressed in total units (±S.E.).

Enzyme	*Total*	*Mucosal scraping*	*Residue*
Fructose-1-*P* aldolase	0.72±0.07	0.85±0.09	0.14±0.04
Fructokinase	0.80±0.15	0.82±0.06	0.17±0.04
Creatine kinase	68.4 ±9.5	31.7 ±3.2	26.7 ±3.0

ase. We have found about the same *V* values for both enzymes (Table III). The K_m for intestinal fructokinase has been reported to be about 0.1 mM[11], whereas the K_m for intestinal fructose-1-*P* aldolase has been found to be 1.1 mM (unpublished observations). These data then provide the explanation of the fructose-1-*P* accumulation, as reported here.

Effects of intravenous administration of hexoses in the rat

Intraluminal administration of fructose had a negligible effect on liver ATP. After 10 min the concentration of ATP had decreased by 11.3 % and the concentra-

TABLE IV

METABOLITE LEVELS IN JEJUNUM AND LIVER AFTER INTRAVENOUS HEXOSE TREATMENT

5 min after intravenous loading of 1 mmole hexose, the organs were freeze-clamped.

Metabolite (μ*mole/g wet wt* $\times 10^3$)	*Jejunum*			*Liver*		
	None ($n = 2$)	*Glucose* ($n = 2$)	*Fructose* ($n = 2$)	*None* ($n = 2$)	*Glucose* ($n = 2$)	*Fructose* ($n = 2$)
ATP	1110	1110	1040	2560	2550	670
ADP	230	210	280	430	480	800
AMP	40	20	50	50	50	180
Creatine-*P*	1320	1290	690	—	—	—
P_i	3100	3380	1520	2730	2660	760
Glucose-6-*P*	75	105	81	173	124	79
Glucose-1-*P*	4	2	8	8	8	3
Fructose-1-*P*	104	94	1623	254	242	6430
Fructose-6-*P*	23	22	19	46	36	27
Fructose-1,6-P_2	23	24	34	47	44	91
Triose-*P*	22	20	35	105	66	115
Glycerol-3-*P*	152	145	167	606	776	478
3-*P*-Glycerate	25	10	6	207	144	41
2-*P*-Glycerate	3	<1	1	30	10	17
Phosphoenolpyruvate	17	6	2	17	78	22
Pyruvate	71	92	60	71	195	44
Lactate	1573	1523	1781	1404	2658	694

tion of fructose-1-*P* had increased by 36 % (data not shown). These results may be expected 5 min after an intravenous dose of 0.3 mmole fructose in adult rats (extrapolated from data given by Raivio *et al.*[2]). Apparently, high concentrations of fructose in the blood are needed to bring about a rapid fall of ATP content in the liver. The effects of intravenous loadings of 1 mmole fructose on the stores of energy-rich phosphates in liver and jejunum are presented in Table IV. Freeze-clamping was carried out after 5 min, because after 2.5 min the fructose concentration in the cells is maximal and decreases thereafter very rapidly[5]. In our experiments we found the same effects of fructose on the metabolite levels in liver as reported by others[1,3,4,5], with the exception of an accumulation of glycerol-3-*P*. Under identical conditions of fructose loading, the creatine-*P* and P_i concentrations in jejunum had decreased by 44.8 % and 50.7 %, respectively, while the fructose-1-*P* concentration was already near maximal.

CONCLUDING REMARK

From the results obtained it may be concluded that due to the presence of creatine-*P* and creatine kinase in small intestinal epithelial cells of the rat, fructose feeding does not affect the phosphate potential of the cells and therefore may not result in serious dysfunction of resorption.

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THE EFFECTS OF FATTY ACIDS ON OXIDATIVE DECARBOXYLATION OF PYRUVATE IN RAT SMALL INTESTINE

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Summary

1. Rat small intestinal epithelial cells were incubated in vitro at 30°C in bicarbonate buffer, pH 7.4, with 2 mM [1-^{14}C]pyruvate. The rate of formation of $^{14}CO_2$ was inhibited by 5 mM octanoate (58%), 5 mM D,L-3-hydroxybutyrate (37%) and 5 mM acetate (17%). No significant inhibition was observed, when 5 mM propionate was used.

2. On incubation with Mg^{2+}—ATP, the pyruvate dehydrogenase complex (EC 1.2.4.1) in homogenates from intestinal epithelial cells was rapidly inactivated. After inactivation, further incubation with 10 mM $MgCl_2$ and an ATP-removing system resulted in a time-dependent reactivation, suggesting a regulation of the pyruvate dehydrogenase complex in intestine by phosphorylation and dephosphorylation of pyruvate dehydrogenase.

3. Measurement of the pyruvate dehydrogenase complex in extracts from cells, which had been incubated under different conditions, provided evidence that interconversion of the pyruvate dehydrogenase complex determines only to a minor extent the rate of pyruvate oxidation. Intraluminal loading in situ with 5 mM octanoate, compared with 5 mM glucose, gave a slight inactivation of the pyruvate dehydrogenase complex estimated in extracts from freeze-clamped tissue.

Introduction

It has repeatedly been demonstrated (cf. refs 1—3), that fatty acids and ketone bodies may inhibit glucose oxidation in muscle. It has been shown, that in this tissue during fatty acid oxidation two key enzymes in glucose metabolism, phosphofructokinase (EC 2.7.1.11) [4,5] and pyruvate dehydrogenase (EC 1.2.4.1) [6—8] are inhibited. Recently much work has been carried out to study the mechanism of regulation of the latter enzyme.

Experiments with vascular perfusion of rat heart [8,9], diaphragm [8] and rat liver [10] showed that during fatty acid oxidation pyruvate decarboxylation is inhibited. Two mechanisms have been proposed in the literature: regulation by enzymic interconversion of pyruvate dehydrogenase complex itself and regulation by cofactors (and their products) involved in the oxidative decarboxylation of pyruvate. Recent studies have revealed that the pyruvate dehydrogenase complex from kidney [11], heart muscle [12—14], liver [13], brain [15] and adipose tissue [16] is subject to regulation by phosphorylation and dephosphorylation. The active form (pyruvate dehydrogenase *a*) is converted to the inactive form (pyruvate dehydrogenase *b*) by a Mg^{2+}—ATP-dependent kinase (pyruvate dehydrogenase *a* kinase). Reactivation, i.e. dephosphorylation, is catalysed by a high free Mg^{2+} concentration requiring phosphatase (pyruvate dehydrogenase *b* phosphatase). Wieland et al. [7] showed in isolated perfused rat heart and Patzelt et al. [17] in isolated perfused rat liver, that an increased supply of fatty acids resulted in a conversion of pyruvate dehydrogenase *a* to pyruvate dehydrogenase *b*. The feed-back inhibition of pyruvate dehydrogenase complex by acetyl-CoA and/or NADH, described by Garland et al. [8,18], provides an additional mechanism for the control of pyruvate decarboxylation. During fatty acid oxidation both acetyl-CoA and NADH concentrations are increased [6]. In an earlier study from this laboratory [19] it was shown, that octanoate and oleate both inhibited glucose oxidation in vascularly perfused intestine. Under the conditions employed, glycolysis was not inhibited, suggesting a lowered energy charge [20]. No conclusion could be drawn in those studies about the mechanism involved in the inhibition of pyruvate dehydrogenase complex in rat small intestine. It is the purpose of the present study to shed some light on the mechanism(s), involved in the inhibition of pyruvate dehydrogenase complex during fatty acid oxidation in rat small intestinal epithelial cells.

Materials and Methods

Reagents

All chemicals were of analytical purity. Enzymes and cofactors were obtained from Boehringer and Co., Mannheim, Germany. Sodium [1-^{14}C] pyruvate was supplied by The Radiochemical Centre, Amersham (specific activity 13.1 Ci/mole). In every experiment a part of solid [1-^{14}C] pyruvate was dissolved in a freshly prepared non-radioactive sodium pyruvate solution.

Preparation and extraction of epithelial cell suspensions

Normal fed male Wistar rats, weighing approx. 200 g and maintained on a normal laboratory diet and water ad libitum were used. The rats were killed by cervical fracture. Villous cells, originating from the whole length of the small intestine, were harvested according to the high frequency vibration technique of Harrison and Webster [21,22], in a medium containing 0.01 M Tris—HCl buffer, 0.13 M NaCl and 5 mM EDTA (final pH 7.4). Cells and cell sheets were collected by centrifugation for 15 s at 800 × g_{max}. The cell pellet was resuspended by passage through a plastic pipette and once washed with incubation buffer. As indicated in the legends in some experiments homogenates of villous cells were prepared by rapid homogenization of the unwashed cell pellet in 20 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 2 mM dithiothreitol for 40 s with a small Polytron homogenizer (type PT_{10}, Kinematica, Luzern, Switzerland), set at position 4.5 (8000 rev./min), followed by freezing and thawing once. Samples of 100 μl were taken for the pyruvate dehydrogenase complex enzyme assays.

Extraction of rapidly frozen small intestine, after preloading with glucose or octanoate, or liver

In intraluminal loading, 4 ml 5 mM glucose or 5 mM octanoate in 0.9% NaCl was delivered through a canula in the small intestine. The operation technique has been described elsewhere [23]. In experiments with liver, the rats were not intraluminally fed. After 10 min a part of the intestine was freeze-clamped [20]. The frozen tissue was pulverized in a mortar, while liquid N_2 was continuously added. 1 g of the powder was then mixed with 4 ml buffer, containing 40 mM Tris–HCl, pH 7.4, 2 mM dithiotreitol, 30 mM glucose and 1.4 units/ml hexokinase (EC 2.7.1.1.) for rat small intestine or 40 mM Tris–HCl, pH 7.4, 5 mM EDTA and 2 mM dithiotreitol for rat liver. The powder was thawed and homogenized with a Polytron homogenizer as described in the previous section. Extraction of pyruvate dehydrogenase complex was completed by three times freezing and thawing the homogenate. Samples of 100 μl were taken for the pyruvate dehydrogenase complex enzyme assays.

Incubation of intact epithelial cells

Pyruvate metabolism was measured in 1 ml of a Krebs–Henseleit–bicarbonate buffer [24] (pH 7.4), containing 1.4 mM $CaCl_2$ and 0.6 mM $MgSO_4$ and fortified with 2 mM sodium [1-^{14}C]pyruvate (the specific activity varied from 5 to 30 mCi/mole). Villous cell suspensions of the rat were incubated in polyethylene vials for 30 min at 30°C. The measurements were started after saturating the vials with O_2–CO_2 (95:5, v/v), by injecting labelled pyruvate. $^{14}CO_2$ was trapped in hyamine, which was introduced at the end of the incubation in a plastic cup connected to the rubber cap closing the reaction vessel, prior to the addition of 1 ml 5% $HClO_4$ to terminate the reaction. The plastic cup was cleaned of condensed water and transferred to a counting vial. Counting was performed in a liquid scintillation counter with automatic standardization. The counting vials contained 10 ml Dimilume (Packard), a counting liquid inhibiting chemiluminescence. Lactate [25] and pyruvate [26] were determined after neutralizing the $HClO_4$-deproteinized samples with KOH. $^{14}CO_2$ production rates were linear between 0 and 30 min incubation.

Assay of the pyruvate dehydrogenase complex

Pyruvate dehydrogenase complex activity was measured in crude epithelial cell homogenates and extracts from freeze-clamped tissue, prepared as described in the previous sections by an assay system, measuring the rate of formation of $^{14}CO_2$ from [1-^{14}C]pyruvate. The reaction mixture contained 100 mM Tris–HCl, pH 8.0, 2.8 mM $MgCl_2$, 10 mM mercaptoethanol, 3 mM NAD^+, 0.02 mM thiamine pyrophosphate, 1.2 units/ml lactate dehydrogenase

(EC 1.1.1.27), 1.2 units/ml carnitine acetyltransferase (EC 2.3.1.7), 20 mM D,L-carnitine. The reaction mixture was freshly prepared for each series of experiments. Carnitine acetyltransferase and carnitine were added to prevent accumulation of acetyl-CoA [18], and lactate dehydrogenase to ascertain the removal of inhibitory NADH [8,18]. The incubations were carried out in polyethylene vials for 5 min at 30°C, as described in the previous section. Into each vial were pipetted, 100 μl 10 mM CoASH solution (pH 6.0), 500 μl of the reaction mixture (in the blanks was added 1 mM arsenite and CoASH was replaced by water), 50 μl 0.01 M sodium [1-^{14}C]pyruvate (50—200 mCi/mole) and water to the final volume of 900 μl. The reaction was started by the addition of 100 μl homogenate and stopped with 1 ml 5% $HClO_4$. The blank values were in the range of 500—1000 dpm, depending on the age of the [1-^{14}C]pyruvate. Experimental values were 3 to 15 times the blank values. The reactions were proportional to the enzyme concentration used to an amount of 2.5 mg/ml. Protein was determined by the biuret method [27] after solubilization of membrane-bound protein by treatment with deoxycholate and by sonication. Statistics refer to ± S.E.

Results and Discussion

Pyruvate metabolism in intestinal villous cells

In earlier experiments from our laboratory [28,29] it was observed that in isolated intestinal villous cells the rate of lactate production from glucose exceeded by far the rate of CO_2 production. The explanation of the high flow through the glycolytic pathway appeared to be a low energy charge [20], which is responsible for the stimulation of phosphofructokinase. The rate at which pyruvate is oxidized may also be measured directly with this substrate and these data are given in Table I. Under these conditions, at 2 mM pyruvate, the rate of oxidative decarboxylation is of the same order of magnitude, as found with pyruvate derived from glucose by glycolysis [28,29]. This is in contrast to the striking difference in the rate of lactate production. The latter may be a consequence of supply of extramitochondrial NADH, when glucose is used as the substrate.

In the assay employed in Table I, the evolution of $^{14}CO_2$ from [1-^{14}C]pyruvate was followed as a measure for the conversion of pyruvate to acetyl-CoA. It is possible that exchange reactions contribute to the evolution of $^{14}CO_2$ to some extent. Kneer and Ball [30] have suggested that an exchange reaction between pyruvate and CO_2 can occur in adipose tissue. This exchange reaction presumably involves the reversible reactions, catalysed by the enzymes pyruvate carboxylase (EC 6.4.1.1), malate dehydrogenase (EC 1.1.1.37) and fumarase (EC 4.2.1.2), which are also present in intestinal mucosa [29,31,32]. This then introduces the possibility of overestimating the rate of conversion of pyruvate to acetyl-CoA, when $^{14}CO_2$ formation was followed. For these reasons we used 1 mM arsenite in the blank. Arsenite, as a dithiol reagent, reacts with the reduced lipoamide in pyruvate dehydrogenase complex. The blank reaction rates were subtracted and amounted to about 8% of the uninhibited $^{14}CO_2$ production rate. Side reactions, as mentioned above, are not likely to contribute to the release of $^{14}CO_2$ from [1-^{14}C]pyruvate to a large extent,

TABLE I

EFFECT OF FATTY ACIDS ON PYRUVATE METABOLISM IN ISOLATED INTESTINAL EPITHELIAL CELLS

Villous cells were incubated with [1-^{14}C]pyruvate in the concentration indicated. The incubation medium and the method used for the determinations of $^{14}CO_2$, lactate and pyruvate are mentioned under Materials and Methods.

Additions to the incubation medium	$^{14}CO_2$ production (percent of control*)	*n*	Δ $^{14}CO_2$ (nmoles/min per mg protein)	Δ lactate (nmoles/min per mg protein)	$-\Delta$ pyruvate (nmoles/min per mg protein)
2 mM pyruvate	100	6	2.09 ± 0.27	1.78 ± 0.18	4.17 ± 0.12
2 mM pyruvate + 5 mM octanoate	41.9 ± 2.2	5	0.89 ± 0.18	1.69 ± 0.21	2.69 ± 0.32
2 mM pyruvate + 1 mM octanoate	72.1	2	1.70	—	—
2 mM pyruvate + 5 mM D, L-3-hydroxybutyrate	63.2 ± 4.8	5	1.32 ± 0.21	1.83 ± 0.13	3.68 ± 0.18
2 mM pyruvate + 1 mM D, L-3-hydroxybutyrate	71.8	2	1.66	—	—
2 mM pyruvate + 5 mM acetate	82.9 ± 8.9	4	1.65 ± 0.44	1.71 ± 0.10	3.82 ± 0.31
2 mM pyruvate + 1 mM acetate	77.3	2	1.43	—	—
2 mM pyruvate + 5 mM propionate	99.7 ± 0.7	4	1.95 ± 0.40	1.56 ± 0.12	3.75 ± 0.10
5 mM pyruvate	—	2	3.97	—	—
5 mM pyruvate + 5 mM octanoate	—	2	1.98	—	—

* The values of this column are derived from individual experiments, in which no additions to the incubation medium with 2 mM pyruvate were made 100%.

since the pyruvate dehydrogenase complex is a very active enzyme complex, when compared with e.g. pyruvate carboxylase.

Effects of fatty acids on pyruvate metabolism

Table I also demonstrates, that the rate of $^{14}CO_2$ production from [1-^{14}C]pyruvate was decreased by 5 mM octanoate (58%). This is in accordance with the results of Hulsmann [19] who observed that during vascular perfusion of intestine, octanoate inhibited glucose oxidation. A less pronounced effect was obtained with a lower concentration of octanoate or by replacing 5 mM octanoate by either 5 mM D,L-3-hydroxybutyrate or acetate. In mitochondria of rat small intestine D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) and acetoacetyl-CoA-thiolase (EC 2.3.1.9) are present [32], as well as 3-ketoacid CoA transferase [32] (EC 2.8.3.5). The combined action of these enzymes leads to acetyl-CoA formation from D,L-3-hydroxybutyrate. Another part of added D,L-3-hydroxybutyrate may be converted to acetyl-CoA after activation of the substrate by medium-chain fatty acyl-CoA synthetase (EC 7.2.1.2), which is also active in rat small intestine [32]. This explains the finding (Table I) that D,L-3-hydroxybutyrate inhibits pyruvate oxidation in epithelial cells to some extent. Acetyl-CoA synthetase (EC 6.2.1.1) is present in rat small intestinal epithelium (Scholte, H.R., unpublished observations). In the present results (Table I) 17% inhibition of pyruvate oxidation by 5 mM acetate also indicates acetate activation. Garland et al. [8] concluded from their studies on pyruvate metabolism in heart and in diaphragm, that inhibition of pyruvate dehydrogenase complex may be caused by high [acetyl-CoA]/[CoASH] and [NADH]/[NAD$^+$] ratios. That propionate has no effect on pyruvate oxidation (Table I), compared with the effects of octanoate, D,L-3-hydroxybutyrate and acetate is in agreement with the proposal of Garland et al. [8]. Propionate may be activated by acetyl-CoA synthetase and possibly also by a separate propionate activating enzyme [33]. Propionyl-SCoA is likely to give rise to the synthesis of dicarboxylic acids, rather than to acetyl-CoA generated by the other compounds mentioned.

The fatty acids mentioned have virtually no effect on the rate of lactate production, with the possible exception of propionate, indicating that reduction of oxidative decarboxylation is not compensated by increased rates of lactate production. This is unexpected, since in other tissues but intestine at least octanoate and 3-hydroxybutyrate increase the cellular [NADH]/[NAD$^+$] ratio and since lactate dehydrogenase (EC 1.1.1.27) is very active, an increase of rate of lactate production may be expected if pyruvate could enter the cell freely. It therefore seems possible, that fatty acids interfere with pyruvate transport in the epithelial cells more directly than through their influence on cellular metabolism, since 1 or 5 mM octanoate, D,L-3-hydroxybutyrate or acetate are all expected to increase ATP, acetyl-CoA and NADH generation and therefore should have a similar effect. This possible influence of fatty acids on pyruvate transport is a matter of further investigation.

Effects of Mg^{2+} and ATP on the activity of pyruvate dehydrogenase complex in homogenates of intestinal epithelial cells

Recent studies on pyruvate dehydrogenase complex in crude homogenates

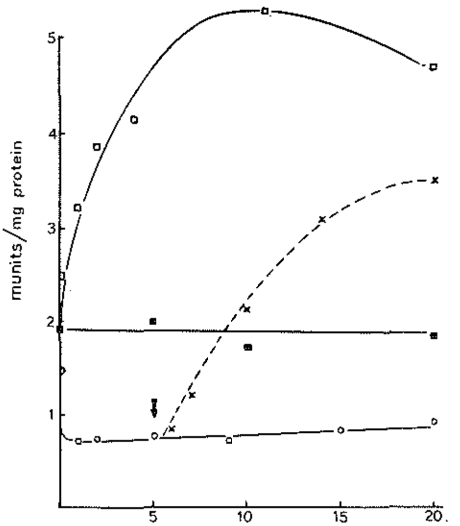

Fig. 1. Time course of the effect of addition of Mg^{2+} or Mg^{2+}—ATP on the pyruvate dehydrogenase complex activity in homogenates of small intestinal cells. Cells were isolated according to Materials and Methods. The unwashed pellet (125 mg protein) was homogenized in 5 ml potassium phosphate buffer (pH 7.4), 1 mM EDTA and 2 mM dithiothreitol. The homogenate was incubated at 30°C with 10 mM MgCl$_2$, 10 mM glucose, 7 units/ml hexokinase (□—□); 1.5 mM MgCl$_2$, 0.1 mM ATP, 2 mM creatinephosphate, 1.8 units/ml creatinekinase (EC 2.7.3.2) (○—○); no additions ■—■. At the time indicated, samples of 100 μl were taken for the pyruvate dehydrogenase complex enzyme assay, as described in Materials and Methods. At the time indicated by the arrow, 10 mM glucose, 10 mM MgCl$_2$ and 7 units/ml hexokinase were added to the Mg^{2+}—ATP preincubated homogenate (x--x). Additional MgCl$_2$ was included in the enzyme assays to obtain uniformly a free Mg^{2+} concentration of 1.3 mM.

from kidney [11], heart muscle [12—14], liver [13], brain [15] and adipose tissue [16,34] indicated that pyruvate dehydrogenase complex from these organs is regulated by a phosphorylation—dephosphorylation reaction sequence. Linn et al. [13] concluded from their experiments on purified subunits of pyruvate dehydrogenase complex from beef kidney mitochondria, that the site of this reaction sequence was on the pyruvate dehydrogenase component of pyruvate dehydrogenase complex. A phosphatase (pyruvate dehydrogenase *b* phosphatase), requiring a high free Mg^{2+} concentration, removes a phosphate residue from pyruvate dehydrogenase *b*, thereby converting it to the active form (pyruvate dehydrogenase *a*). Phosphorylation, dependent on Mg^{2+}—ATP on the other hand, may regenerate the inactive enzyme (pyruvate dehydrogenase *b*). Homogenates of intestinal epithelial cells were prepared from normal fed rats. When incubated for 20 min at 30°C, little change in pyruvate dehydrogenase complex occurred (Fig. 1). When 10 mM Mg^{2+} and an ATP-removing system were added, an increase in activity occurred. The addition of Mg^{2+}—ATP and an ATP-generating system rapidly decreased pyruvate dehydrogenase complex activity. After inactivation of the enzyme with Mg^{2+}—ATP, the enzyme was reactivated in a time-dependent fashion by the addition of 10 mM Mg^{2+} and an ATP-removing system. In a few experiments, however, the activation of the enzyme by incubation with Mg^{2+} was incomplete due to a more pronounced lability of the enzyme at 30°C. The maximal activity shown in Fig. 1 is probably also underestimated. The results described in Fig. 1 then provide evidence that a similar phosphorylation—dephosphorylation cycle [11—16] occurs with the pyruvate dehydrogenase complex from rat small intestinal cells.

Assay of pyruvate dehydrogenase complex activity in extracts from small intestinal epithelial cells, preincubated with 1 mM pyruvate in the presence or absence of 5 mM octanoate

It has been demonstrated by several authors [16,23,36—38], that the dynamic equilibrium between pyruvate dehydrogenase *a* and pyruvate dehydrogenase *b* in adipose tissue in vitro is under metabolic and hormonal control. Metabolic control of pyruvate dehydrogenase complex is also shown in vivo in rat liver [9], heart [39] and kidney [39], as well as in vitro with perfusion studies in heart [7] and liver [17,40]. The significance of this mechanism for the regulation of pyruvate metabolism in these organs, relative to the feedback control of pyruvate dehydrogenase complex, is not yet clear. Therefore, we were interested in pyruvate dehydrogenase complex activity under conditions, when pyruvate oxidation is inhibited in epithelial cells. In experiments, shown in Table II, the activity of pyruvate dehydrogenase complex was decreased by preincubation of the cells with 5 mM octanoate or 5 mM D,L-3-hydroxybutyrate (averaging to 13 and 15%, respectively). The inhibition of pyruvate oxidation found in intact cell incubations (Table I) is large compared with these small inhibitions of pyruvate dehydrogenase complex. The latter may suggest that other mechanisms contribute to the action of fatty acids in intact cells, as also was concluded from results discussed in the previous section. Because mitochondrial processes probably also operate during isolation of the cells with the vibration procedure at 7°C, substrate was added at this step of the isolation. This is shown in Table III, which also demonstrates that 2 mM octanoate had some inhibitory effect on pyruvate dehydrogenase complex activity. Clearcut differences in pyruvate dehydrogenase complex activity were found in isolated cells from different rats, suggesting an influence of the metabolic state

TABLE II

ACTIVITY OF PYRUVATE DEHYDROGENASE IN EXTRACTS FROM EPITHELIAL CELLS, PREINCUBATED WITH PYRUVATE IN THE PRESENCE OR ABSENCE OF FATTY ACIDS

A suspension (3 ml, approx. 30 mg protein) of villous cells in Krebs—Henseleit—bicarbonate buffer was incubated for 10 min at 30°C. At the end of the incubation period, the suspension of cells was rapidly centrifuged at 0°C and the cell pellet homogenized in 4 ml 20 mM potassium phosphate buffer (pH 7.4). 1 mM EDTA, 2 mM dithiolthrietol and once frozen and thawed. Samples of 100 μl were taken for the pyruvate dehydrogenase complex enzyme assays, as described in Materials and Methods. Each assay was carried out in triplicate.

Additions to the incubation medium	n	Specific activity (units/g protein)	Percent of control*
1 mM pyruvate	6	4.67 ± 0.26	—
1 mM pyruvate + 5 mM octanoate	6	$4.02 \pm 0.29^{**}$	86.8 ± 4.9
1 mM pyruvate + 5 mM D,L-3-hydroxybutyrate	6	$3.98 \pm 0.18^{**}$	84.1 ± 4.8

* The values of this column are derived from individual experiments, in which incubation with 1 mM pyruvate alone was made 100%.

** $0.025 < P < 0.05$.

TABLE III

ACTIVITY OF PYRUVATE DEHYDROGENASE IN EXTRACTS FROM EPITHELIAL CELLS, ISOLATED IN THE PRESENCE OF GLUCOSE OR OCTANOATE

Cells were isolated according to Materials and Methods, except that a substrate was present during the vibration procedure. The unwashed pellet (60 mg protein) was homogenized in 3 ml 20 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 2 mM dithiolthreitol and once frozen and thawed. Samples of 100 μl were taken for the pyruvate dehydrogenase complex enzyme assays, as described in Materials and Methods. Each assay was carried out in triplicate.

Substrate added to the isolation buffer	Specific activity (units/g protein)				
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5
2 mM glucose	3.13	4.09	2.79	4.35	5.30
2 mM octanoate	2.94	4.06	2.10	3.42	5.20
Decrease by octanoate (%)*	6.1	0.7	24.7	21.4	1.9

* The mean percentage decrease in these five experiments amounted to 10.9 ± 5.0%.

of the small intestine on the pyruvate dehydrogenase complex activity measured.

Assay of pyruvate dehydrogenase complex activity in Tris buffer extracts from freeze-clamped rat small intestine and in liver in situ

Whereas in the previous section the enzyme was extracted by homogenization in potassium phosphate buffer (pH 7.4), 1 mM EDTA, 2 mM dithiothreitol and once freezing and thawing, in earlier experiments with freeze-clamped samples, extraction was carried out with Tris buffer (pH 7.4), containing 4 mM EDTA. This type of extraction, now with 5 mM EDTA, of livers from fed rats, freeze-clamped in situ, led to the measurement of an average (three experiments) pyruvate dehydrogenase complex activity of 0.77 units/g protein. On subsequent incubation of these liver extracts in 15 mM $MgCl_2$ for 40 min at 30° C, the activity was maximally activated to 3.63 units/g protein. These values correspond very well with the values, given for perfused rat livers by Patzelt et al. [17]. In small intestine, however, freeze-clamping followed by Tris—EDTA extraction of pyruvate dehydrogenase complex, led to very low

TABLE IV

ACTIVITY OF PYRUVATE DEHYDROGENASE IN EXTRACTS FROM RAT SMALL INTESTINE, FREEZE-CLAMPED AFTER INTRALUMINAL GLUCOSE OR OCTANOATE LOADING IN SITU

10 min after intraluminal loading of 4 ml 5 mM glucose or octanoate in 0.9 % NaCl, freeze-clamping was performed (see Materials and Methods), followed by an extraction of pyruvate dehydrogenase complex, by homogenizing the pulverized tissue in a 4-fold volume of Tris buffer. For details see Materials and Methods.

Substrate used	*n*	Specific activity (units/g protein)
5 mM glucose	6	2.03 ± 0.18
5 mM octanoate	8	1.67 ± 0.21*

* $P \leqslant 0.1$.

activities (results not shown). Under these conditions Mg^{2+} addition resulted in a large stimulation, which is not necessarily due to pyruvate dehydrogenase *b* phosphatase activity, but may be due to the requirement of Mg^{2+} in the assay for binding thiamine pyrophosphate to the pyruvate dehydrogenase complex [41,42]. Freeze-clamping of the small intestine after intraluminal administration of 2 mM glucose or octanoate and extracting the pyruvate dehydrogenase complex enzyme with Tris buffer (no EDTA), containing glucose and hexokinase to remove traces of ATP from the extracts, as shown in Table IV, resulted in a significant lowering of pyruvate dehydrogenase complex by prior intraluminal administration of fatty acid. Why the activities in total tissue extracts (Table IV) are lower than in isolated cells (Table III) is not known. It could be that pyruvate dehydrogenase complex is enriched in epithelial cells (no muscular or connective tissue elements present) or that homogenization in potassium phosphate buffer may result in a better extraction of the enzyme. The data in Table IV then suggest, that also in situ the inhibition of pyruvate dehydrogenase complex during fatty acid oxidation in rat small intestine may contribute to the inhibition of pyruvate oxidation, which was observed in in vitro perfusion experiments earlier [19].

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BBA Report

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ABSENCE OF SIGNIFICANT RATES OF GLUCONEOGENESIS IN INTESTINAL MUCOSA OF STARVED GUINEA PIGS

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Summary

Homogenates of jejunal mucosa from 72 h fasted guinea pigs showed glucose production rates of 3—4 μmoles$\cdot h^{-1} \cdot g^{-1}$ wet weight under the conditions recommended in the literature (IDP present). However, we found no incorporation of the gluconeogenic precursors lactate, pyruvate or alanine in glucose. Further results led to the conclusion that glucose was formed from nucleotides, added to the homogenates.

Isolated intact epithelial cells showed a very low rate of glucose production, suggesting only a low contribution of intestinal mucosa to whole body gluconeogenesis in starved guinea pig.

It has recently been shown by Anderson and Rosendall [1] that homogenates of jejunal mucosa from 72 h starved guinea pigs catalyze gluconeogenesis from lactate, malate or pyruvate at rates between 3 and 5 μmoles$\cdot$ $h^{-1} \cdot g^{-1}$ tissue weight. Although this rate is one order of magnitude lower than the rate of gluconeogenesis in isolated parenchymal liver cells [2, 3] or in the in vitro perfused liver [4, 5], we considered the findings of considerable importance since tissue homogenates even from liver or kidney generally fail to show significant rates of gluconeogenesis. It can be seen from Table I that homogenates of mucosal scrapings of 72 h fasted guinea pigs, when tested under the conditions described by Anderson and Rosendall [1], indeed catalyze net glucose synthesis. The strong stimulatory effect of IDP, as described by these authors, was confirmed. However, IDP addition stimulated similarly when the gluconeogenic precursors lactate or pyruvate were omitted,

TABLE I

GLUCOSE AND GLUCOSE-6-*P* PRODUCTION AND INCORPORATION OF [1-^{14}C]PYRUVATE, [U-^{14}C]ALANINE AND [1-^{14}C]LACTATE IN GLUCOSE IN MUCOSAL HOMOGENATES

Experiments were done essentially according to the method described by Anderson and Rosendall [1]. Homogenization of intestinal scrapings was carried out in a PT_{10} Polytron homogenizer (60 s at 8000 rev./min) in a buffer containing 113 mM NaCl, 4.7 mM KCl, 1.7 mM $CaCl_2$, 0.8 mM $MgCl_2$, 1.2 mM $MgSO_4$, 2.5 mM sodium acetate, 25 mM $NaHCO_3$ saturated with CO_2 and 38 mM imidazole buffer (pH 7.0). After incubation, glucose and glucose-6-*P* were measured in deproteinized samples in a combined assay [14] using dual wavelength spectrophotometry (wavelength pair 350 and 375 nm). The specific activities of the substrates [1-^{14}C]pyruvate, [U-^{14}C]alanine and [1-^{14}C]lactate were 47, 5 and 40 mCi/mole, respectively. Glucose was separated from labeled ionic species by passage through Amberlite-MB-3, as described by Hawkins et al. [15].

Additions to the incubation medium		µmoles·g^{-1} wet weight·h^{-1} at 37°C		µmoles of labeled precursor converted to glucose·g^{-1} wet weight·h^{-1}
	n	ΔGlucose	ΔGlucose-6-*P*	
None	3	1.31	0	—
2 mM IDP	3	3.11	2.85	—
10 mM lactate	1	1.02	0	—
10 mM pyruvate	1	1.10	0	—
10 mM malate, 2 mM IDP	3	4.07	1.71	—
10 mM [1-^{14}C]lactate, 2 mM IDP	3	2.93	2.23	0.026
10 mM [1-^{14}C]pyruvate, 2 mM IDP	3	3.54	2.28	0.012
10 mM [U-^{14}C]alanine, 2 mM IDP	3	3.86	2.00	0.027

TABLE II

GLUCOSE, GLUCOSE-6-*P* AND FRUCTOSE-6-*P* PRODUCTION IN HOMOGENATES OF ISOLATED EPITHELIAL CELLS FROM 72 h FASTED GUINEA PIG SMALL INTESTINE

Epithelial cells were isolated according to Harrison and Webster [6] and homogenized by Polytron treatment (see Table I). Incubations and determinations were carried out as described in Table I. Protein was determined by the biuret method [16] after solubilization of the membrane-bound protein by treatment with deoxycholate and by sonication. (Statistics refer to ± S.E.).

Additions to the incubation medium	*n*	µmoles·g^{-1} protein·h^{-1} at 37°C		
		ΔGlucose	ΔGlucose-6-*P*	ΔFructose-6-*P*
None	4	10.4±1.4	0.3±0.2	1.2±0.2
5 mM pyruvate	5	8.6±1.0	1.9±1.3	0.6±0.2
2 mM IDP	4	29.3±6.1	15.2±4.3	6.4±1.5
5 mM pyruvate, 2 mM IDP	6	28.5±3.4	13.3±3.5	6.2±1.9
5 mM pyruvate, 2 mM IMP	2	37.8; 22.4	6.1; 10.1	0.0; 0.0
5 mM pyruvate, 2 mM ITP	1	29.6	7.1	2.7
5 mM pyruvate, 2 mM AMP	3	26.3±5.8	11.9±0.9	5.9±0.3
5 mM pyruvate, 2 mM ADP	3	24.5±6.8	9.2±0.6	5.1±0.4
5 mM pyruvate, 2 mM IDP, 1 mM MIA*	3	22.6±6.8	11.3±0.7	5.0±0.9
5 mM malate, 2 mM IDP	3	22.6±5.0	13.8±2.8	5.7±1.0
5 mM malate, 2 mM IDP, 1 mM MIA*	3	23.8±5.5	11.6±1.8	5.8±1.0
5 mM pyruvate, 2 mM inosine, 2 mM P_i	6	56.2±5.4	13.0±2.1	9.8±0.9
5 mM pyruvate, 2 mM P_i	3	11.9±2.1	1.3±0.7	1.7±0.9
5 mM pyruvate, 2 mM inosine	3	21.7±1.4	5.4±3.0	5.2±2.4
5 mM pyruvate, 2 mM ribose-5-*P*	4	36.9±2.7	1.3±0.8	4.5±0.4

*Monoiodoacetate.

which casts serious doubt on the interpretation that IDP stimulates gluconeogenesis from pyruvate. Indeed, when [1-^{14}C]pyruvate, [1-^{14}C]lactate or [U-^{14}C]alanine were included in the assay system, the amount of labeled glucose formed amounted to only 1% of the glucose formed (Table I).

It can be seen from Table II that when guinea pig small intestinal epithelial cells were isolated by the vibration method of Harrison and Webster [6] prior to the homogenization (in order to reduce the heterogeneity of the cellular elements isolated), IDP also stimulated glucose formation and that IDP could be replaced by IMP, ITP, ADP or AMP. Inhibition of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) by the addition of 1 mM monoiodoacetate hardly affected the stimulatory effect of IDP on glucose formation in the presence of pyruvate or malate. No conclusive evidence was obtained for glycogenolysis as a significant contribution to the AMP- or IMP-dependent glucose formation, although we found that 5 mM IMP or AMP stimulated phosphorylase (EC 2.4.1.1) two-fold in guinea pig intestine (results not shown, compare also results on muscle phosphorylase *b* [7]). Moreover, experiments with intact isolated epithelial cells from 72 h starved guinea pigs (Fig. 1) showed that the glycogen concentration, expressed in glucose equivalents, is considerably less than the glucose formed in homogenates (Table II). These results were interpreted such that probably the IMP or AMP degradation may be involved in glucose formation. Indeed, Table II shows that inosine plus P_i or ribose-5-*P* stimulated hexose- and hexose-*P* production equally well. In older studies done by Horecker et al. [8], and also more recently [9], it was shown that on incubation of ribose-5-*P* with

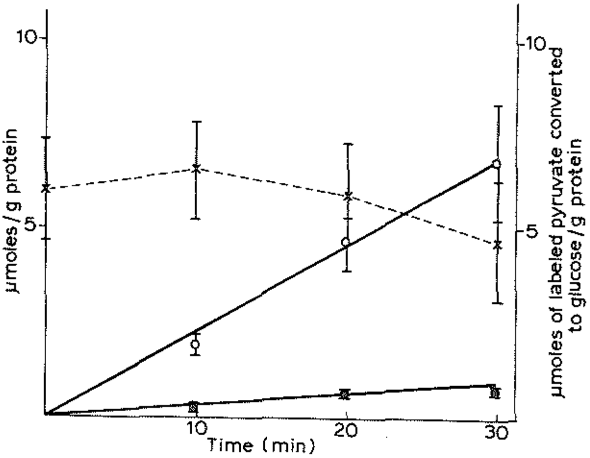

Fig. 1. Glucose formation and rate of incorporation of [1-^{14}C]pyruvate in glucose in isolated epithelial cells from 72 h fasted guinea pig small intestine. Epithelial cells, harvested according to the vibration method of Harrison and Webster [6], were incubated in 1 ml of a Krebs—Henseleit—bicarbonate buffer [17] (pH 7.4) containing 1.4 mM $CaCl_2$ and 0.6 mM $MgSO_4$ and fortified with 5 mM sodium [1-^{14}C]-pyruvate (specific activity 47 mCi/mole). Incubations were carried out in polyethylene vials at 37°C in an atmosphere of O_2—CO_2 (95:5, v/v). At the end of the incubation period the reaction was stopped by the addition of 0.1 ml of 30% $HClO_4$, centrifuged, neutralized with 2 M $KHCO_3$ and again centrifuged. Glucose and specific activity of glucose were determined as described in Table I. ○——○, glucose formed; ●——●, incorporation of [1-^{14}C]pyruvate into glucose. Glycogen (X- - -X), expressed as glucose equivalents, was determined after enzymic hydrolysis by $\alpha(1\rightarrow4)$, $\alpha(1\rightarrow6)$ amyloglucosidase (EC 3.2.1.-). The cell suspensions used (1 ml) were rapidly cooled in ice, sonicated for 30 s with a Branson sonifier (model S 75) at 1.5 A and 0.6 ml. 0.2 M acetate buffer (pH 4.8) was added. This mixture was incubated for 60 min at 37°C in the presence of 1.6 units/ml amyloglucosidase. After incubation the samples were deproteinized and assayed for glucose. In some determinations a glycogen standard was included to check recovery. Each point represents the mean of four experiments (±S.E.).

liver supernatants triose-*P* and hexose-*P* accumulated, which correlates very well with data on guinea pig intestine. Hence it may be concluded that the glucose production appeared to be due to a breakdown of nucleotides to nucleosides and finally to ribose-5-*P*, because the enzymes that catalyze the initial steps: 5-nucleotidase [10] (EC 3.1.3.5) and adenosine deaminase [10, 11] (EC 3.5.4.4), are very active in jejunal mucosa. That nucleoside phosphorylase (EC 2.4.2.1) may be involved in further degradation of nucleotides, is strengthened by the finding (Table II) that inosine plus P_i more effectively stimulated net hexose- and hexose-*P* formation than IMP or IDP. Pentose-*P* can be converted in the non-oxidative part of the pentose cycle to triose-*P* and hexose-*P* [8, 9]. These phosphorylated intermediates can be converted to glucose by combined action of fructose-1,6-diphosphatase (EC 3.1.3.11) and glucose-6-phosphatase (EC 3.1.3.9). That this pathway is also operative in guinea pig intestine can also be concluded from results of Ginsburg and Hers [12], who showed in vitro a rapid conversion of fructose to glucose via triose-*P*. The contribution of pentose cycle intermediates during in vitro conversion of fructose to glucose in human intestinal strips was shown by White and Landau [13].

Finally we studied gluconeogenesis in intact intestinal cells isolated from small intestine of 72 h fasted guinea pig (Fig. 1). The incorporation of [1-^{14}C]-pyruvate in glucose amounts to only 10% of the already low rate of glucose production, as compared with liver cells [2, 3]. This suggests that intestinal mucosa in starved guinea pig may not contribute to overall gluconeogenesis in the intact animal significantly. Recent work from our laboratory supports this conclusion, since pyruvate kinase (EC 2.7.1.40) from guinea pig intestine has been found to be of the muscle type (Van Berkel, Th.J.C., unpublished).

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INHIBITION OF PYRUVATE TRANSPORT BY FATTY ACIDS IN ISOLATED CELLS FROM RAT SMALL INTESTINE

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SUMMARY

1. A technique is described for the rapid separation of intestinal epithelial cells from the incubation medium by passage through a silicon-oil layer and collection in acid, in which their soluble constituents are released.

2. The inhibition by fatty acids of pyruvate oxidation is further studied. Measurement of pyruvate transport in epithelial cells at 0 °C showed that short- and medium-chain fatty acids as well as ricinoleate inhibit this transport. Propionate inhibits pyruvate transport by another mechanism than octanoate.

3. Differences between pyruvate, propionate and octanoate transport across the epithelial cell membrane were obtained in efflux studies. These studies revealed that acetate, propionate, butyrate and high concentrations of bicarbonate readily stimulate the efflux of pyruvate, probably by anionic counter-transport. No effects were seen with octanoate and hexanoate. The data obtained in these efflux studies suggest that lipophilicity and the pK_a values of the monocarboxylic acids determine the contribution of non-ionic diffusion to overall transport.

4. Saturation kinetics, competitive inhibition by short-chain fatty acids and counter-transport suggest a carrier-mediated transport of pyruvate.

INTRODUCTION

Earlier experiments with rat heart [1, 2], diaphragm [1], liver [3] or isolated kidney tubules [4] showed that during fatty acid oxidation, oxidative decarboxylation of pyruvate is inhibited. Determination of the state of the pyruvate dehydrogenase complex [5, 6] during fatty acid oxidation has established that enzyme phosphorylation plays an important role in the decrease of pyruvate oxidation in these tissues. Studies in rat intestine [7, 8] on the inhibition of fatty acids of pyruvate oxidation suggested that a small part of this decrease can be ascribed to phosphorylation of the enzyme complex too. The decrease in pyruvate dehydrogenase activity measured under the same conditions, however, was much lower than the depression of $^{14}CO_2$ production from [1-^{14}C]pyruvate. To a minor extent this discrepancy was also found by Wieland et al. [9] in studies with perfused rat heart. Inhibition of the pyruvate dehydrogenase complex by increased levels of acetyl-CoA and NADH have been mentioned as additional metabolic mediators of fatty acid action [1, 8, 9]. Randle et al. [10] concluded from the effects of fatty acids on pyruvate oxidation in rat heart that there may be an effect of fatty acids on the permeability of cellular membranes for pyruvate. Moreover, we concluded from earlier results that fatty acids not only inhibit the oxidative decarboxylation of pyruvate in rat small intestine, but also its conversion to lactate, possibly by inhibition of pyruvate transport.

It has generally been assumed [11–13] that pyruvate freely permeates the cell membrane by passive diffusion so that changes of the intracellular level will be reflected by similar changes in the blood. There are only a few reports [12, 14, 15] of studies on the mechanism of pyruvate translocation across cellular membranes. Some evidence has been obtained for the involvement of a carrier system in the transport of pyruvate in heart [16] and more recently in erythrocytes [17]. In the latter case this is clearly demonstrated by the specific inhibition of the transport by α-cyano-4-hydroxycinnamate.

The access of pyruvate to its dehydrogenase in mitochondria is also of great interest in view of the central role of pyruvate oxidation in carbohydrate metabolism. Evidence has now become available [17–21] for the occurrence of a transport carrier for pyruvate in the mitochondrial membrane, although it has been argued by Klingenberg [22] that sufficient undissociated monocarboxylic acid exists intracellularly to render such a transport carrier redundant. Recently Bakker et al. [23] described the mechanism by which monocarboxylic acids, such as pyruvate and acetate move across artificial membrane vesicles (liposomes). Their results suggest that pyruvate crosses the membrane in the undissociated form and readily exchanges with other weak monocarboxylic acids. It is the purpose of the present study to investigate the mechanism of translocation of pyruvate across the epithelial membrane, to compare it with that of other monocarboxylic acids and to study their mutual influence.

MATERIALS AND METHODS

Reagents

All chemicals were of analytical purity. Sodium [1-^{14}C]pyruvate (13.1 Ci/mol), sodium [2-^{14}C]pyruvate (9.89 Ci/mol), [^{3}H]inulin (860 Ci/mol), [U-^{14}C]sorbitol (8.7 Ci/mol), [1-^{14}C]propionic acid (57.5 Ci/mol), [1-^{14}C]octanoic acid (31.8 Ci/mol). $^{3}H_2O$ (90.9 Ci/mol) were supplied by The Radiochemical Centre (Amersham, England). Because of instability of aqueous solutions of pyruvate, in every experiment a part of solid [^{14}C]pyruvate was dissolved in a freshly prepared non-radioactive sodium pyruvate solution. Ricinoleic acid was purchased from Sigma (St. Louis, U.S.A.). Prostaglandin E_1 was a generous gift of the Unilever Research Laboratories (Vlaardingen, the Netherlands).

Preparation and extraction of epithelial cell suspensions

Normal fed male Wistar rats, weighing approx. 200 g, were maintained on a normal laboratory diet and H_2O ad libitum. The rats were narcotized with ether and killed by removal of the heart. The small intestine was removed and the lumen flushed with 100 ml ice-cold saline. Villous cells, originating from the jejenum, were harvested according to the high frequency vibration technique of Harrison and Webster [24, 25], as described earlier [8]. The cell pellet (20–30 mg protein) was washed two times with incubation buffer, Krebs/Henseleit/bicarbonate [26] or Krebs/phosphate [27], both containing 1.4 mM $CaCl_2$, 0.6 mM $MgSO_4$ and 0.1 % fatty acid-poor Pentex serum albumin from Fluka (Buchs, Switzerland). The cells were resuspended, by passage through a plastic pipette, in 2–3 ml buffer and directly used for transport measurements. Care was taken that the cell suspension was standing not longer than 5 min before the start of the transport studies, since strong aggregation may occur after 5 min and this was found to interfere with the reproducibility of the transport rates. This aggregation was greatly reduced when the cells were standing as dilute suspensions (1 mg protein/ml). Metabolism of [1-^{14}C]pyruvate was measured as described in the previous report [8].

Silicon oil filtration technique

Eppendorf plastic microtest tubes (No. 3810, 1.5 ml volume) were filled with 0.1 ml 0.5 M $HClO_4$ and 0.4 ml silicon oil (TEGILOXAN® A. V. 100, Goldschmidt Ltd., Essen, G.F.R.) and 0.5 ml incubation buffer, with labelled substrate present and [U-^{14}C]sorbitol or [^{3}H]inulin as marker for extracellular space (1 mM and 0.4 mM final concentrations, respectively). After layering the tubes, they were briefly centrifuged in an Eppendorf 3200 centrifuge to separate the silicon oil from both aqueous layers. They were stood for a minimum of 15 min in an ice-bath to insure that the incubation layer was at 0 °C. When uptake of substrate was to be determined, 50 μl of a concentrated, disaggregated, cell suspension was added to the incubation layer and during incubation the tubes were gently shaken by hand (about twice/min). At the end of the incubation the cells were sedimented at $10\,000 \times g$ for 4 min in the Eppendorf centrifuge. This centrifuge reaches maximal g values after 5 s and the cells were separated from the medium by the silicon oil layer within 15 s. The cellular content was released when the cells came into contact with the bottom perchloric acid layer. Parallel tests were done with incubation buffer (sometimes with the inhibitors present) containing ^3H_2O and [U-^{14}C]sorbitol to find out the intracellular space of the cells and the total water space that was mixed with the acid underphase. After samples had been taken from the cell-free upperphase for radioactivity measurement, it was washed away with the help of acetone/water (50 : 50, v/v). A sample of 70 μl of the clear acid underphase was then taken with a Hamilton syringe for the analysis of radioactivity. When substrates or inhibitors (e.g. fatty acids) had to be added in a higher concentration than 1 mM they were added as 123 mM sodium salt solutions in NaCl-free Krebs/phosphate or Krebs/Henseleit/bicarbonate, for maintenance of isotonicity. The description of the method of studying efflux of substrates from cells is given in the legend to Fig. 6.

Calculation of results

Cellular ^{14}C-labelled substrate content was calculated by correcting the ^{14}C-labelled substrate content of the pellet for substrate in the extracellular space, as determined from the distribution of [^{3}H]inulin. Calculation of the intracellular water in a typical experiment (example 0.77 mg cell protein) proceeds as follows. Specific activity of ^3H_2O in the upperphase was 1149 cpm/μl and ^3H_2O measured in underphase (70 μl) was 7780 cmp; the total water space then is

$$\frac{100}{70-\frac{7780}{1149}} \times \frac{7780}{1149} = 10.71\ \mu l.$$

Furthermore the specific activity of [U-^{14}C]sorbitol in upperphase was 285 cpm/μl and [U-^{14}C]sorbitol measured in underphase (70 μl) yielded 1372 cpm. The total adherent space then equals

$$\frac{100+10.71}{70} \times \frac{1372}{285} \text{ or } 7.61\ \mu l.$$

The intracellular water in this experiment then amounted to (10.71—7.61) = 3.10 μl.

Counting of radioactivity

The counting vials contained 10 ml Instagel (Packard) and ^{3}H and ^{14}C activities were measured in a liquid scintillation counter (Packard, model 3380) with automatic standardization.

RESULTS AND DISCUSSION

Effects of fatty acids and the fatty acid derivatives ricinoleic acid and prostaglandin E_1 on oxidative decarboxylation of pyruvate at 30 °C

In earlier experiments from our laboratory [8] it was observed that short-chain and medium-chain fatty acids decrease the rate of $^{14}CO_2$ production from [1-^{14}C]pyruvate in intestinal epithelial cells. Interconversion of the pyruvate dehydrogenase complex determines to a minor extent the inhibition of pyruvate oxidation by fatty acids. Therefore we proposed two possible mechanisms for this discrepancy. Firstly the inhibition of the pyruvate dehydrogenase complex may be caused by high acetyl CoA/CoASH- and NADH/NAD$^+$ ratios as the result of fatty acid oxidation. Indeed in later experiments we found a clearcut increase in acetyl-CoA/CoASH ratio after incubation of cells with octanoate or albumin-bound oleate (results not shown). Secondly, fatty acids may interfere with pyruvate transport across the membrane of the epithelial cells.

The stronger inhibition of pyruvate oxidation by 5 mM octanoate than by 5 mM hexanoate (Table I) might suggest chainlength dependency. Therefore the long-chain fatty acids palmitate and myristate were also tested. However, they were not more effective than octanoate when tested at 80 μM.

Recently Ammon and Phillips described the influence of fatty acids on the absorption of water and electrolytes in canine Thiry-Vella fistulas of ileum [28] and human jejunum [29]. Long-chain fatty acids, particularly the hydroxy fatty acids ricinoleate (the active principle of castor oil) and 10 (9)-hydroxystearic acid (major fatty acid of fatty stools [30]), strongly impaired electrolyte- and water absorptions in vivo; some inhibition was seen with octanoate and hexanoate [28]. Ricinoleate at a lower concentration (0.12 mM; see Table I) was as effective as 80 μM octanoate, but at 0.5 mM it inhibited pyruvate oxidation almost completely. Perfusion of human jejunum in vivo with ricinoleic acid or oleic acid evokes water secretion [31], the latter has been described also for prostaglandin E_1 in vivo [32, 33]. Prostaglandin E_1 at 0.1 mM (Table I) resulted in a 30 % decrease of pyruvate oxidation, but no effect

TABLE I

EFFECT OF FATTY ACIDS, RICINOLEIC ACID AND PROSTAGLANDIN E, ON OXIDATIVE DECARBOXYLATION OF PYRUVATE IN RAT SMALL INTESTINE

Pyruvate oxidation ([1-^{14}C]pyruvate → $^{14}CO_2$) was measured in Krebs/Henseleit/bicarbonate buffer (pH 7.4) fortified with 1 mM sodium [1-^{14}C]pyruvate (spec. act. varied from 5 to 30 μCi/mol) at 30 °C, as described in detail in the previous report [8]. The values are derived from individual experiments, in which in each case the rate of CO_2 production was set at 100 % when no additions were made.

Addition to the incubation medium	Concentration (mM)	*n*	$^{14}CO_2$ Production (percentage of control)
—	—	8	100
Hexanoate	5	3	67.2 ± 2.1
Octanoate	0.08	3	81.8 ± 3.1
Octanoate	5	4	44.4 ± 2.0
Myristate	0.08	3	82.3 ± 4.0
Palmitate	0.08	4	84.3 ± 5.5
Ricinoleate	0.12	5	81.7 ± 7.5
Ricinoleate	0.5	5	14.0 ± 2.1
Prostaglandin E_1	0.1	4	69.5 ± 5.6
Prostaglandin E_1	0.02	1	98.8

was observed at 0.02 mM, the concentration needed for maximal activation of adenylcyclase [32]. The latter result might suggest a fatty acid-like effect of prostaglandin E_1 on pyruvate utilization. Moreover, octanoate and ricinoleate are not able to increase cyclic AMP levels in intestinal villous cells under the same conditions as described here (H. R. de Jonge, unpublished).

Effect of octanoate on pyruvate transport in epithelial cells at 0 °C

In studies on the uptake of substrates that readily cross the membrane it is important to separate the cells from the incubation medium rapidly; secondly, it is essential that the solute composition of the cells should not change during the separation. Techniques for the separation of intestinal cells have been described, in particular in studies on aminoacid- and glucose uptake [35–37]. Reiser and Christiansen [35] followed millipore filtration of intestinal cells microscopically and observed extensive morphological damage, probably attributed to the presence of mucus. Also, collection of the cells by centrifugation reduced the viability of the cells, although in this case amino acid uptake against a concentration gradient could be measured [35]. Therefore our attention was directed to the centrifugal filtration method, successfully used in many mitochondrial uptake studies [38, 39]. Recently this method has also been applied to cell separations (thymocytes [40], kidney tubules [4] and fat cells [41]). The viability of the cells after passage through the oil appeared to be unchanged [40, 41], as judged by their hormone sensitivity, oxygen consumption and sodium content.

The rate of utilization of pyruvate in intestinal epithelial cells at physiological temperature has been found to be very rapid [8], as judged by CO_2– and lactate production rates. For that reason we decided to study transport at 0 °C, since at that temperature its metabolism is relatively sluggish. Experiments not shown indicated

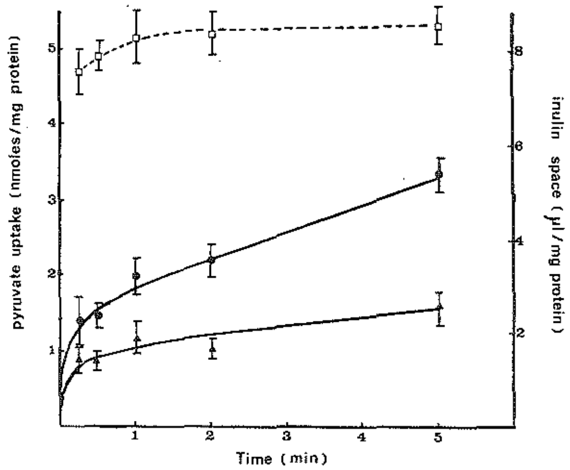

Fig. 1. Effect of 5 mM octanoate on transport of 1 mM pyruvate in epithelial cells at 0 °C. The incubation was carried out at 0 °C in Krebs/Henseleit bicarbonate buffer (pH 7.4), containing 1 mM [2-^{14}C]pyruvate, 0.4 mM [^{3}H]inulin and 0.1 % albumin. After the time indicated, the cells were rapidly centrifuged (10 000 × *g*) through a silicon oil layer, as described under Methods: ● — ●, control; ▲ — ▲, 5 mM octanoate present; □- - -□, adherent water, calculated from [^{3}H]inulin transported with the cells. Intracellular water volumes, calculated from the distribution of ^3H_2O and [U-^{14}C]sorbitol were 4.18±0.21 (*n* = 16) µl/mg protein, determined after 5 min incubation. Each point represents the mean of six experiments (±S.E.).

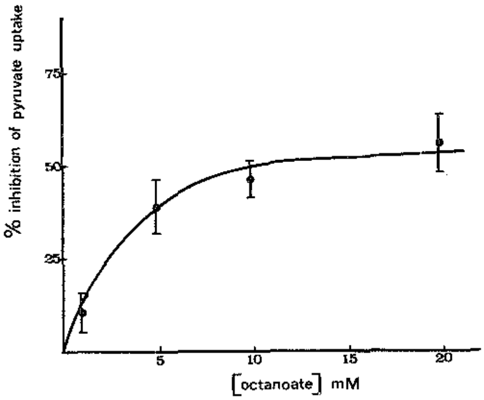

Fig. 2. Inhibition of pyruvate transport at 0 °C as a function of the concentration of octanoate. The incubations are carried out at 0 °C (5 min) in Krebs/phosphate buffer (pH 7.4), containing 1 mM labeled substrate, 0.4 mM [^{3}H]inulin and 0.1 % albumin. For details of the transport assay see Methods. Each point represents the mean of four experiments (±S.E.).

that at 0 °C the rate of conversion of [1-^{14}C]pyruvate to $^{14}CO_2$ and lactate amounted only to 0.1 nmol/min/mg protein, whereas the data of Fig. 1 indicate that the initial rate of pyruvate transport exceeds 5 nmol/min/mg protein. In agreement with the discrepancy between the rates of metabolism and transport is the observation that the radioactivity within the cells is largely due to the presence of pyruvate as measured by double-beam spectrophotometry (data not shown). Fig. 1 demonstrates furthermore that 5 mM octanoate clearly inhibited the translocation of pyruvate across the membrane. The study of the effects of different concentrations of octanoate on pyruvate transport (5 min) revealed that at 10 mM octanoate the inhibition of pyruvate transport was nearly maximal (Fig. 2). In Fig. 1 it is also demonstrated that the adherent space, calculated from [^{3}H]inulin transported with the cells, appeared not to be constant during the first min of incubation. This might be due to the relatively long time required for the equilibration of inulin with the adherent space of the cells, as pointed out in another report [42]. Therefore, the uptake values for [2-^{14}C]pyruvate at times shorter than 30 s could be somewhat overestimated. It seems more probable to us that at short incubation times there is an increase of adherent water with time.

Comparison of the effects of fatty acids on the rate of pyruvate transport

In order to compare the effects of fatty acids other than octanoate on pyruvate transport we decided to measure uptake rates at 5 min. In the first experiments we used Krebs/Henseleit/bicarbonate buffer (Table II). From the relative inhibitions of octanoate, hexanoate and D,L-3-hydroxybutyrate we concluded that there was the same chainlength dependency as observed in the effect of fatty acids on pyruvate oxidation at 30 °C (compare ref. 8 and the previous section). In later studies we measured transport in Krebs/phosphate buffer owing to its stronger buffering capacity. Surprisingly, in this buffer a significantly higher rate of pyruvate uptake was measured, but the effect of octanoate was relatively the same. Ricinoleate and prostaglandin E_1 were also inhibitory to pyruvate transport.

TABLE II

INFLUENCE OF FATTY ACIDS ON PYRUVATE TRANSPORT IN INTESTINAL EPITHELIAL CELLS AT 0 °C

The incubations were carried out for 5 min at 0 °C. The conditions are described in the legend to Fig. 1 and under Materials and Methods. The number of experiments is indicated in parentheses.

Addition to the influx medium	Concentration (mM)	Buffer used for influx measurements	
		Krebs/Henseleit/ bicarbonate	Krebs/phosphate
		Influx rates of pyruvate (nmol/mg protein/5 min)	
—	—	3.25±0.35 (5)	4.69±0.13 (9)
Acetate	10	—	3.34±0.08 (4)
Propionate	10	—	3.52±0.12 (4)
Butyrate	10	—	3.14±0.12 (4)
Octanoate	5	2.00±0.20 (5)	2.82±0.15 (5)
Hexanoate	5	2.55±0.24 (5)	—
D,L-3-hydroxybutyrate	5	2.97±0.21 (4)	—
Prostaglandin E_1	0.1	2.82±0.27 (4)	3.85±0.15 (3)
Ricinoleate	0.5	1.99±0.22 (4)	2.78±0.19 (5)

The short-chain fatty acids, acetate, propionate and butyrate at 10 mM concentration were found to inhibit pyruvate transport (Table II). To elucidate the character of the inhibition of the fatty acids tested, we studied the effect of fatty acids on the rate of transport at different pyruvate concentrations (Fig. 3). In the first experiments we tried to investigate the kinetics during very short incubation periods (30 s, results

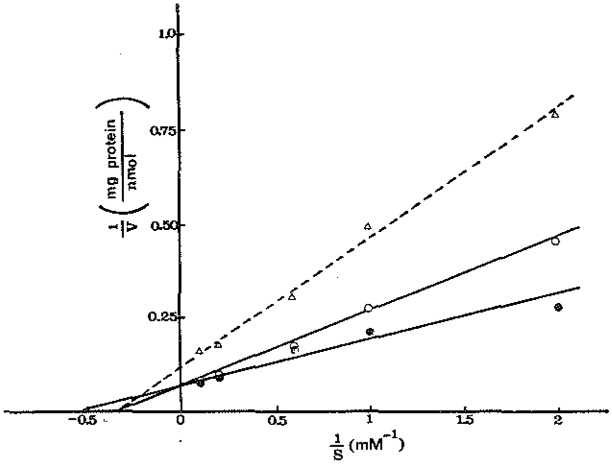

Fig. 3. Lineweaver-Burk plots of the rates of pyruvate transport as a function of the concentration of pyruvate. Effects of 10 mM octanoate and 10 mM propionate. For details of transport assay see Methods. The incubations were carried out at 0 °C (5 min) in Krebs/phosphate buffer (pH 7.4), containing 0.4 mM [^{3}H]inulin and 0.1 % albumin. ● — ●, pyruvate alone; ○ — ○, pyruvate in the presence of 10 mM propionate; △- - -△, pyruvate in the presence of 10 mM octanoate. Each point represents the mean of four experiments.

not shown). It was found that the amount of adherent substrate accounted for a large percentage of the total substrate found in the sample. This introduced a large error in the uptake rate measurements at 0 °C and made the results unreliable. Therefore, in the experiments shown (Fig. 3) uptake was measured over 5 min, after it had been shown in Fig. 1 that at 5 min the uptake of pyruvate was not maximal. Lineweaver-Burk plots of the results suggested that uptake of pyruvate at 0 °C was a saturable process. This argues for the presence of a carrier for pyruvate translocation in epithelial cells, as already described for the cell membranes of heart [16] and erythrocytes [17]. This proposal was further supported by the competitive nature of the propionate inhibition (Fig. 3), suggesting the presence of a translocator for monocarboxylic acids in intestinal epithelial cell membranes. The same experiments were done in the presence of octanoate, the inhibition of which appeared to be of non-competitive nature. This led us to study the nature of diffusion of pyruvate, propionate and octanoate across the epithelial cell membrane.

Effect of pH on the influx of pyruvate, propionate and octanoate at 0 °C

The mechanism involved in the transport of weak electrolytes by small intestine has been the subject of several previous investigations [42–48]. The transport of short-chain fatty acids may be a combination of carrier-facilitated or active transport [43–45] and non-ionic diffusion [42, 46–48]. In these studies net mucosal to serosal fluxes were measured. Our studies, however, involve isolated epithelial cell sheets in which both brush borders and basolateral plasma membranes are exposed to the incubation fluid simultaneously, not allowing definitive conclusions to be made about the localization of the translocation sites. The contribution of diffusion of the non-ionized form of short-chain fatty acids to overall transport should be influenced by varying the pH of the incubation medium. While changing the pH of the Krebs/

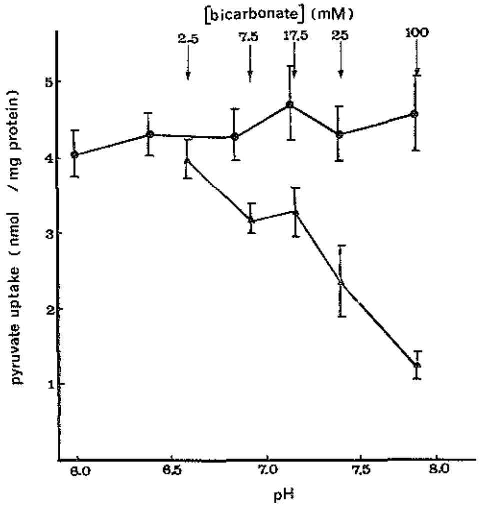

Fig. 4. Influence of pH on transport of 1 mM pyruvate into epithelial cells at 0 °C, measured in two isotonic buffers. The incubations were carried out at 0 °C (5 min) in buffer containing 1 mM [2-^{14}C]-pyruvate, 0.4 mM [^{3}H]inulin and 0.1 % albumin. The pH of the Krebs/Henseleit/bicarbonate buffer was varied by adjustment of the concentrations of HCO_3^- and Cl^-, while maintaining the other ion concentrations constant. For details of the transport assay see Methods. ● — ●, pH varied in Krebs-phosphate buffer; ▲ — ▲, pH varied in Krebs/bicarbonate buffer. Each point represents the mean of four experiments (±S.E.).

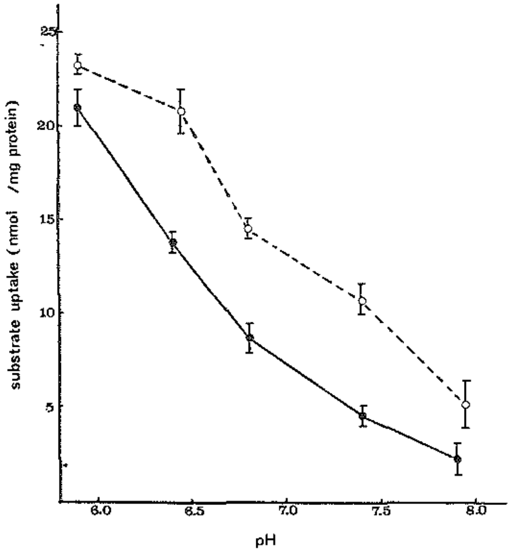

Fig. 5. Influence of pH on the transport of 1 mM propionate and 1 mM octanoate in epithelial cells at 0 °C, measured in Krebs/phosphate buffer. For details of transport assay see Methods. ● — ●, 1 mM [1-^{14}C]propionate; ○- - -○, 1 mM [1-^{14}C]octanoate. The measurement of transport of octanoate by silicon oil filtration was complicated by the precipitation of the fatty acid in the acid underphase, and the strong influence of the pH on the distribution of octanoate between adherent water and silicon oil during passage of the cells. Therefore in these experiments the silicon oil layer, the acid extract and the pellet were all tested for radioactivity. Each point represents the mean of three experiments (±S.E.). The incubation time was 5 min.

Henseleit bicarbonate buffer (by varying the bicarbonate concentration from 0–100 mM), as used by Barry and Smyth [44] in studies on propionate transport in everted sacs, we found a dependence of pyruvate transport on the pH (Fig. 4). But repeating the same experiments in Krebs/phosphate, we found no significant variation between pH 5.9 to pH 7.9 (Fig. 4). The effect found in Krebs/bicarbonate can be ascribed to inhibition of pyruvate transport by high concentrations of bicarbonate (compare also Table II). By contrast, propionate or octanoate influx, measured in Krebs/phosphate, were both strongly dependent on pH (Fig. 5). Assuming a homogeneous partition of carboxylic acid in the cell water (intracellular water of cells amounted to 4.18 ± 0.21 ($n = 16$) μl/mg protein), the calculated concentrations of pyruvate, propionate and octanoate at pH 7.4 were 1.03, 1.10 and 2.50 mM respectively. This leads to the conclusion that a concentration gradient of octanoate across the membrane is probably present at 0 °C at physiological pH. Octanoate transport in intestine has been studied by Gallagher and Playoust [49] and by Bloch et al. [45]. The latter authors showed an accumulation of octanoate against a concentration gradient in vitro at physiological pH. Taking into account our results obtained at 0 °C (Fig. 5), a part of the fatty acid concentration gradient may be explained by non-ionic diffusion [46]. The fatty acids used in the present study have pK_a values close to 5. Pyruvate on the other hand has a pK_a value of 2.5, so that the absence of an effect of pH lowering on the influx rate tends to support the conclusion that the ionic form is the only species transported. From the data of Fig. 5 it can be concluded that at pH 7.4 the amount of propionate taken up after 5 min amounts to 4.6 nmol/mg protein. When it is considered that the initial rate is many times larger (as concluded from unpublished experiments carried out for 30 s when a rate of 8 nmol/min/mg protein was found) and assuming a Q_{10} of 3.5 (as was suggested by experiments on propionate efflux carried out at 0 °C and at 10 °C; not shown) propionate transport is very fast when compared with propionate metabolism [43]. Since the presence of substrates at 5 min incubation is not solely due to influx, but a result of influx accompanied by efflux, efflux studies were undertaken.

Efflux of pyruvate, propionate and octanoate at 0 °C from epithelial cells and the effects of monocarboxylic acids and pH on these processes

Efflux studies approximate unidirectional fluxes better than the 5 min uptake studies as presented above. They were performed after preloading the cells for 15 min with labeled pyruvate or fatty acid at 0 °C. A small aliquot of these suspensions was added to a medium with or without exchangeable substrate. The concentration of labeled extracellular substrate then rapidly decreased to approx. 6 % of its original value in the preloading period. Fig. 6 clearly shows that pyruvate moves slowly out of the cells. This process was not found to be pH-dependent (compare Fig. 7). The initial phase (1 min) of pyruvate efflux could be stimulated by propionate, probably by countertransport. However, this propionate-pyruvate exchange was greatly inhibited when the pH was lowered to pH 5.9 (Fig. 7), probably due to very rapid equilibration of propionate between the various compartments at that pH. Fig. 5 indeed shows a strong stimulation of propionate influx by lowering the pH.

Propionate moves out much faster than pyruvate (Fig. 6). This rate was clearly depressed at pH 5.9 (Fig. 7). This, together with the results on influx, indicates that the pH gradient across the membrane influences the rate of propionate movement.

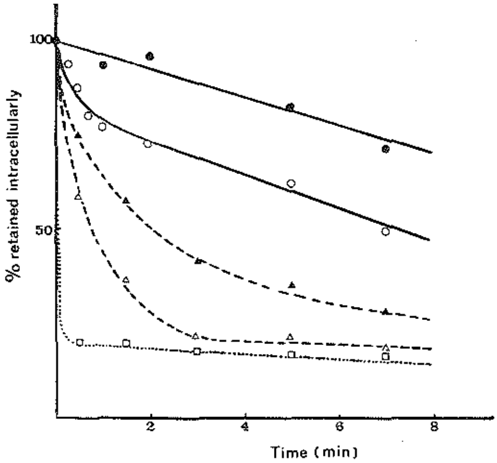

Fig. 6. Efflux of [2-^{14}C]pyruvate, [1-^{14}C]propionate and [1-^{14}C]octanoate from epithelial cells in Krebs/phosphate buffer (pH 7.4); effect of counter ions. Epithelial cells were preincubated at 0 °C with 1 mM labeled substrate. After 15 min 50 μl of these preincubated cell suspensions were added to 0.7 ml buffer layered on silicon oil. The buffer contained counter-substrate and [^{3}H]inulin. After the time indicated the cells were rapidly centrifuged (10 000 × g) through the oil layer as described in Methods. The substrate content at "zero time" was determined by filtration of 0.5 ml of undiluted cell suspension, to which [^{3}H]inulin was added, through a silicon oil layer. This initial ^{14}C-labelled substrate content of the cells amounted for pyruvate, propionate and octanoate 5.16, 5.05 and 12.50 nmol/mg protein respectively. ● — ●, [2-^{14}C]pyruvate efflux; ○ — ○, [2-^{14}C]pyruvate efflux in the presence of 2 mM propionate; ▲ — ▲, [1-^{14}C]propionate efflux; △- - -△, [1-^{14}C]propionate efflux in the presence of 2 mM pyruvate; □. . . .□, [1-^{14}C]octanoate efflux.

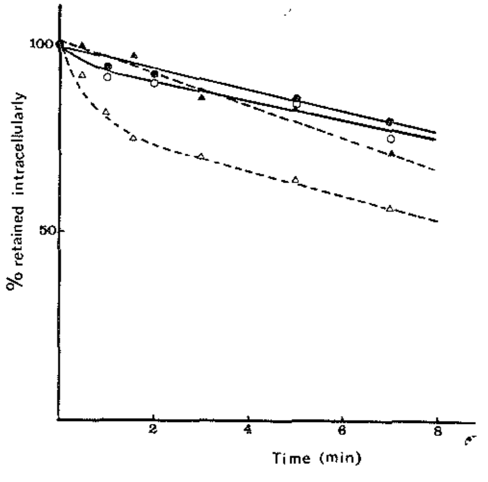

Fig. 7. Efflux of [2-^{14}C]pyruvate and [1-^{14}C]propionate from epithelial cells in Krebs/phosphate buffer (pH 5.9). Effect of counter-ions. Details are described in the legend to Fig. 6. ● — ●, [2-^{14}C]-pyruvate efflux; ○ — ○, [2-^{14}C]pyruvate efflux in the presence of 2 mM propionate; ▲- - -▲, [1-^{14}C]propionate efflux; △- - -△, [1-^{14}C]propionate efflux in the presence of 2 mM pyruvate.

TABLE III

INFLUENCE OF FATTY ACIDS AND BICARBONATE ON THE EFFLUX OF PYRUVATE OR PROPIONATE FROM INTESTINAL EPITHELIAL CELLS AT 0 °C

Epithelial cells were preincubated for 15 min at 0 °C in Krebs/phosphate buffer (pH 7.4) with 1 mM labelled substrate. Then 50 μl cell suspension were added to 0.7 ml buffer layered on a silicon-oil layer and containing counter-substrate or other compounds, as indicated as well as [^{3}H]-inulin. After 2 or 5 min, as indicated, the cells were rapidly centrifuged (10 000 × g) through the oil layer, as described under Methods.

Addition to the efflux medium	Concentration (mM)	*n*	[2-^{14}C]pyruvate-loaded cells		*n*	[1-^{14}C]propionate loaded cells	
			Efflux in nmol/mg protein/5 min	% Control*		Efflux in nmol/mg protein/2 min	% Control*
—	—	5	0.716	—	4	2.012	—
Acetate	2	3	0.924	166±11	4	2.258	111±5
Pyruvate	2	—	—	—	4	3.011	148±6
Propionate	2	5	1.744	256±30	4	—	—
Butyrate	2	5	1.683	253±26	4	2.699	134±4
Hexanoate	2	3	0.614	117±32	4	2.681	132±7
Octanoate	2	5	0.758	104±20	4	2.820	139±7
Propionate + Octanoate	2	3	1.898	230±24	—	—	—
Butyrate + Octanoate	2	3	1.950	230±30	—	—	—
Pyruvate	1	3	1.500	199±18	—	—	—
Octanoate	5	5	0.755	99±18	—	—	—
Ricinoleate	0.5	5	0.718	89±18	—	—	—
Bicarbonate	25	4	1.280	176±20	—	—	—
Bicarbonate	100	4	1.628	227±22	—	—	—

* In each experiment the efflux rate was set at 100% when no addition to efflux medium was made. The mean (±S.E.) values are given in this column.

Furthermore, propionate efflux could be stimulated by pyruvate (Fig. 7). Lowering the pH to 5.9 did not influence this exchange process, which is in agreement with the finding (Fig. 4) that pyruvate translocation across the membrane was not pH-dependent. The efflux of octanoate at pH 7.4 was too rapid to follow (Fig. 6). Comparison then of these data may indicate that lipophilicity of the undissociated form as well as the pK_a value of the carboxylic acid determines the rate of non-ionic diffusion. That for pyruvate or propionate the efflux rate is lower than the influx rate, reflects either the absence of a counterion or a low affinity of these substrates for the carrier at the inside of the membrane.

Efflux of pyruvate and propionate from epithelial cells at 0 °C; effects of the presence of fatty acids in the efflux medium

In a previous section it was shown that fatty acid could inhibit the net influx of pyruvate. It was also suggested that the nature of the inhibitory effect of e.g. propionate compared with octanoate on pyruvate influx was different (Fig. 3). Table III demonstrates the strong stimulations of 2 mM acetate, propionate or butyrate and 25 mM bicarbonate on pyruvate efflux, measured over 5 min. This suggests the presence of a monocarboxylate carrier with a high substrate affinity compared with that for bicarbonate. Hexanoate and octanoate in 2 mM concentrations, however, had no effect on the exit of pyruvate. This might be due to an inhibitory effect of these fatty acids on both in- and outward directed pyruvate fluxes, for instance by perturbation of the membrane lipoproteins or non-competitive binding to the monocarboxylate carrier. These possibilities were excluded by the observation that 2 mM hexanoate or 2 mM octanoate had no effect on the pyruvate efflux stimulated by 2 mM propionate or butyrate (Table III). This supports the hypothesis that octanoate and hexanoate are diffusing exclusively in the undissociated or perhaps in the micellar form across the membrane. Even higher concentrations (5 mM) of octanoate and 0.5 mM ricinoleate were not found to be inhibitory to the efflux of pyruvate. This was unexpected since 5 mM octanoate or 0.5 mM ricinoleate strongly inhibited the influx of pyruvate (Table II). This again would suggest an asymmetrical behaviour of the monocarboxylate carrier.

Propionate efflux may be stimulated by fatty acids and pyruvate in 2 mM concentrations. Comparable stimulations of pyruvate, hexanoate and octanoate on propionate efflux were obtained (Table III), which contrasts their dissimilar effects on pyruvate efflux. From the previous section it was concluded that propionate was moving out in the undissociated form at pH 7.4. Therefore stimulation of propionate exit by octanoate or hexanoate may be due to fatty acid exchange. The possible mechanism of this exchange might depend on the provision of protons at the inside of the membrane by influx of undissociated fatty acid, as described by Bakker and Van Dam [23] for liposomes.

CONCLUDING REMARKS

The transport of pyruvate in intestinal epithelial cells at 0 °C is characterized by saturation kinetics, competitive inhibition by short-chain fatty acids and countertransport. These results could be explained by carrier-facilitated diffusion of monocarboxylic anions across the cell membrane. Since the outward flux rates of the substrates are lower than the inward flux rates, a localization of the carrier within the brush border part of the cellular envelope, would explain the net mucosa to serosa fluxes of these monocarboxylic acids.

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SOME CHARACTERISTICS OF MONOCARBOXYLIC ACID TRANSFER ACROSS THE CELL MEMBRANE OF EPITHELIAL CELLS FROM RAT SMALL INTESTINE

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SUMMARY

1. The translocation of monovalent organic anions (pyruvate, propionate, acetate and butyrate) across the cell membrane of isolated epithelial cells from rat small intestine was studied by measuring competitive inhibition kinetics, exchange diffusion and temperature dependency of the efflux rate. A possible function of a monocarboxylate carrier in intestine will be discussed.

2. Earlier studies on the inhibition of pyruvate transport by fatty acids were extended to propionate and were found to show the same characteristics. The kinetics, however, appeared to be more complex by the contribution of several diffusion pathways for propionate.

3. The mechanism of countertransport was most compatible with an "accelerated exchange diffusion" and could be studied at both sides of the membrane. This exchange diffusion exhibited saturation kinetics. It is proposed that different monocarboxylate anions may have different affinities for a common carrier.

4. Temperature dependency of the efflux of pyruvate and propionate was studied. Arrhenius plots obtained were not found to be linear between 0 and 5°C. Between 5 and

15^{o}C activation energies for pyruvate and propionate efflux rates were found to be 19.6 and 12.6 kcal/mole respectively.

INTRODUCTION

In a previous report [1] evidence was presented showing the existence of a carrier-mediated diffusion of pyruvate anions across the cell membrane of isolated epithelial cells. Experiments on the properties of the transport process, demonstrating inhibition by fatty acids, countertransport and influences of pH alterations, were presented in that study. The competitive inhibition by short-chain fatty acids found, indicated that this translocation system might have a function in transepithelial transport of short-chain fatty acids in rat small intestine. A specific transfer mechanism for the transport of volatile fatty acids in rat small intestine was first proposed by Smyth and Taylor [2]. This mechanism was dependent on metabolic activity. Later, several investigations [3-8] presented similar processes. Generally, these short-chain fatty acids are quantitatively not important as metabolic fuel for the rat. In ruminants, however, they are a major product of cellulose fermentation in the rumen [9,10]. In these species an apparent correlation between fatty acid uptake and the appearance of bicarbonate in the lumen occurs [10].It may be related to our finding of exchange of pyruvate and bicarbonate in epithelial cells [1]. Jackson [6] proposed an additional mechanism for mucosal to serosal flux of weak electrolytes as a three compartment model, including an intermediate compartment of high pH (intercellular space). Inhibition of transport of a weak acid by another weak acid was explained by alteration of the pH by nonionic diffusion of the inhibitor. The model suggested that the two barriers separating the three compartments had different permeabilities to the ionic form of the acid. Using isolated epithelial cell preparations [1], we concluded from studies on pyruvate transfer that the anionic form was the only species transported. Therefore, we extended our investigations with the study of the kinetics of the anionic transport of monocarboxylate acids to get more insight in the contribution of this process to the overall transfer.

Recently it was discovered that pyruvate and lactate transport across the erythrocyte membrane was specifically inhibited by α-cyanohydroxycinnamate [11]. No effect of this inhibitor was seen on acetate and butyrate influx [12], suggesting that pyruvate and short-chain fatty acids do not share a common transport system in this cell type. This then would be at variance with our findings in rat small intestinal epithelium. Deuticke [13,14] studied the transport of organic anions, such as pyruvate, acetate and propionate, in bovine red cells. A number of environmental parameters, which influence anion permeability in the red cell, were varied, including the concentration, the temperature and the pH. Clearcut differences were found [14] between acetate and pyruvate permeation. Therefor a second purpose of the present study was to compare more thoroughly the transfer characteristics of short-chain fatty acids with those of pyruvate with respect to the result obtained earlier [1]. The kinetics of organic anion transfer are solely based on measurements at 0°C, because of the extremely rapid rate of transmembrane flux in small intestinal epithelial cells, which is also reported for the red blood cell [15]. Moreover, at 0°C the possible interference of metabolism of the anions studied, will be minimized.

MATERIALS AND METHODS

Reagents

All chemicals were of analytical purity. Sodium [1-^{14}C]-pyruvate (13.1 Ci/mol), [1-^{14}C]-propionic acid (57.5 Ci/mol), [^{3}H]-inulin (860 Ci/mol), [^{14}C]-polyethyleneglycol (average mol. weight 6000; 75 Ci/mol), ^3H_2O (90.9 Ci/mol) were supplied by the Radiochemical Centre (Amersham, England). Because of instability of aqueous solutions of pyruvate, in every experiment a part of solid $[^{14}C]$-pyruvate was dissolved in a freshly prepared non-radioactive sodium pyruvate solution. Ricinoleic acid was purchased from Sigma (St. Louis, U.S.A.).

Measurement of uptake of monocarboxylate acids

The procedures of isolation of epithelial cells from rat jejunum by the high frequency vibration technique of Harrison and Webster [16,17] as well as the centrifugal filtration of the cells through silicon oil were described in detail previously [1]. The minor modifications of this procedure employed will be indicated in the legends to the figures and tables.

Measurement of efflux

Epithelial cells (4-8 mg protein/ml) were first preincubated at 0°C with 1 mM labelled substrate. After 20 min, 50 µl of this preincubated cell suspension was added to 700 µl Krebs-phosphate buffer (pH 7.4) (in some experiments 300 µl, as indicated) which was layered on silicon oil. The buffer sometimes contained countersubstrate. After the time indicated, the cells were rapidly centrifuged (10,000 g) through the oil layer in an Eppendorf 3200 centrifuge. The substrate content at "zero time" was determined by the filtration of 200 µl undiluted cell suspension, to which $[^3H]$-inulin had been added. In the studies on temperature dependency of efflux rates, $[1\text{-}^{14}C]$-pyruvate or $[1\text{-}^{14}C]$-propionate efflux was followed for a short time (30 sec). At this stage of the investigations it was found that the initial ^{14}C-labelled substrate content at 0°C was sometimes lower than the values after 30 sec efflux at 0°C. It was supposed that the ^{14}C-labelled substrate content estimated in efflux studies was more reliable since the adherent ^{14}C-substrate transported with the cells through the silicon oil amounted to only 5-10% from the labelled cellular content. In order to explore further this curious finding, another marker for the extracellular space, ^{14}C-labelled polyethyleneglycol was tested. A comparison of the values in these experiments showed that $^{3}H_2O$, $[^{3}H]$-inulin and $[^{14}C]$-polyethyleneglycol yielded 11.35 ± 0.58, 6.83 ± 0.37 and 5.85 ± 0.45 µl/mg protein (n=5) respectively. This overestimation of the volume of adherent fluid when $[^{3}H]$-inulin was used as a marker, became considerably important when the efflux was small. Sallee et al. [18] also noted a slightly larger adherent mucosal fluid in everted sacs of intestine, when measured with $[^{3}H]$-inulin. It was ascribed to the rapid exchange of labelled tritium with tissue protons. Calculations of results on temperature dependency were corrected by using the $[^{14}C]$-polyethyleneglycol space instead of $[^{3}H]$-inulin in parallel incubations. The adherent spaces were found to vary between different cell suspensions, which makes corrections in earlier experiments impossible.

RESULTS AND DISCUSSION

Inhibition of propionate transport by monocarboxylic acids

TABLE I shows the inhibition of propionate (1 mM) uptake at 30°C by pyruvate, octanoate or ricinoleate in epithelial cells from rat jejunum. It can be seen that pyruvate (10 mM) inhibition at 0°C and 30°C was largely abolished at higher propionate concentrations (4-5 mM), suggesting a competitive nature of the inhibition. On the other hand, the increase of the propionate concentration was without effect on the relative inhibition by octanoate or ricinoleate. These data agree with our earlier studies on pyruvate transport [1] and probably reflect the presence of a common translocation system for pyruvate and propionate. The differences between pyruvate and propionate transport across epithelial cell membrane obtained in

TABLE I

EFFECT OF MONOCARBOXYLIC ACIDS ON THE UPTAKE OF PROPIONATE IN RAT SMALL INTESTINAL EPITHELIUM

The incubations were carried out at 0°C and 30°C in Krebs-phosphate buffer (pH 7.4) containing different concentrations of [1-^{14}C]-propionate, 0.4 mM [^{3}H]-inulin and 0.1% albumin. After 5 min the cells were rapidly centrifuged (10.000 g, 2 min) through a silicon oil layer in the perchloric acid phase. Further treatment of the samples is described under METHODS. Each value represents the mean of four separate experiments (± S.E.).

Propionate concentration (mM)	Temperature (°C)	Control	+ pyruvate (10 mM)	+ octanoate (10 mM)	+ ricinoleate (0.5 mM)
		in nMoles/mg protein/5 min			
0.5	30	2.91±0.21	2.32±0.13	1.69±0.18	1.96±0.17
1.0	30	5.33±0.50	4.15±0.04	2.79±0.35	3.04±0.16
2.0	30	9.62±0.69	9.63±0.14	5.34±0.67	6.95±0.92
5.0	30	21.9±1.6	19.9±1.3	12.5±1.2	12.2
10.0	30	37.5±3.0	35.6±1.6	22.6±0.9	26.5±0.8
0.2	0	1.06±0.13	0.63±0.08	-	-
0.4	0	1.72±0.26	1.06±0.22	-	-
1.0	0	4.18±0.35	2.73±0.50	-	-
2.0	0	7.16±1.11	5.80±0.82	-	-
4.0	0	11.9±1.7	9.87±0.48	-	-

earlier efflux studies [1], were also confirmed with respect to the kinetics of the uptake at different concentrations of propionate (TABLE I). An important contribution of nonionic diffusion to the overall transfer of propionate made it an ill-suited model for a kinetic study of anionic translocation. The more so as only an approximation of the initial rate could be determined. Lineweaver-Burk plots (not shown) of the values given in TABLE I showed non-linear relationships. The rate of propionate transport was obviously not proportional with the concentration used. The data of TABLE I, suggesting affinity of both anions for a common (hypothetical) carrier were used to approximate the (apparent) affinity values of both anions for this carrier.

Mutual competitive inhibition between pyruvate and propionate

The K_i of propionate inhibition on pyruvate uptake was calculated from double reciprocal plots of the rate of pyruvate transport versus the pyruvate concentration used [1]. The K_i (21 mM) differed markedly from the apparent K_m (2 mM) of pyruvate. These affinities might be overestimated by the interference of other anionic diffusion pathways for pyruvate. For that reason we attempted to measure the affinity or half saturation constant K_m by varying the concentration of the inhibitor at a fixed pyruvate concentration. If a carrier mediated transport is involved in monocarboxylate anion transport, this process might be described by the equation:

$$\frac{v_i}{v-v_i} = \frac{K_i}{[I]}\left(\frac{[S]}{K_m} + 1\right)$$ which is derived from

Michaelis-Menten equations:

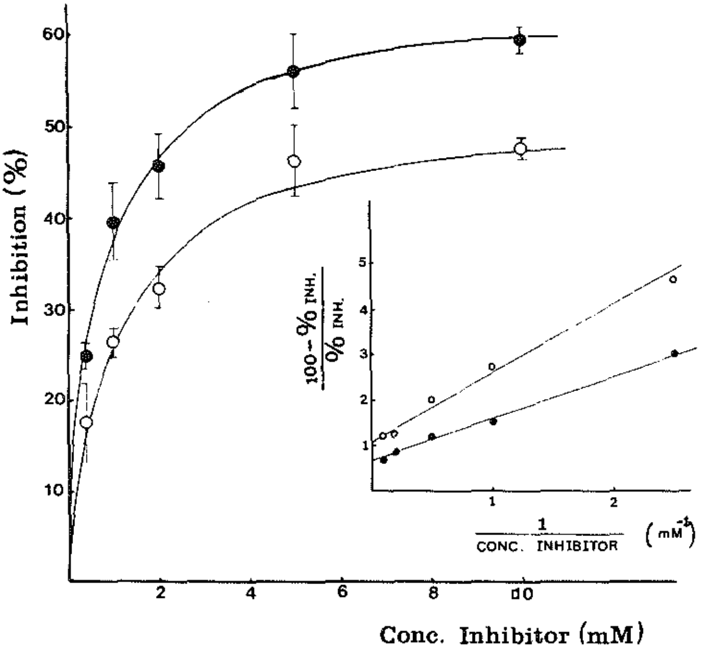

Fig. 1. Mutual competitive inhibition between pyruvate and propionate. The incubations were carried out at 0^{0}C (5 min) in Krebs-phosphate buffer (pH 7.4), containing 0.4 mM [^{3}H]-inulin and 0.1% albumin. ●-●, 0.4 mM [1-^{14}C]-pyruvate in the presence of different concentrations of propionate; O-O, 0.4 mM [1-^{14}C]-propionate in the presence of different concentrations of pyruvate. The theoretical description of the $\frac{100-\%\ \text{inh}}{\%\ \text{inh}}$ versus $\frac{1}{[I]}$ plots was given in the text. Each point represents the mean of four experiments.

$$v = \frac{V_{max}}{1 + \frac{K_m}{[S]}} \quad \text{and} \quad v_i = \frac{V_{max}}{1 + \frac{K_m}{[S]}\left(1 + \frac{[I]}{K_i}\right)}$$ in which v is the

rate with substrate alone and v_i the rate in the presence of an inhibitor of concentration [I]. K_i and K_m are the proposed affinity constants of inhibitor and substrate respectively. Experiments on the effects of different concentrations of propionate on the influx of pyruvate (0.4 mM) and the reversed process are summarized in Fig. 1.

The inset clearly shows a straight line in a plot of the ratio uninhibited influx to inhibited influx $\left(\frac{v_i}{v-v_i} \text{ or } \frac{100-\% \text{ inh}}{\% \text{ inh}}\right)$ versus the inverse inhibitor concentration $\left(\frac{1}{[I]}\right)$. Affinity constants of 1.7 and 1.2 mM could be calculated from the slopes of the two plots for pyruvate and propionate respectively. Likewise it was concluded from Fig. 1 that the maximal inhibition differed from 100%, indicating that the monocarboxylate entry process was composed of several contributions. Consequently an attempt was made to find a method that could distinguish between the carrier-mediated anionic diffusion and other processes. Therefore the exchange diffusion between pyruvate and short-chain fatty acids was used to confirm results obtained from the influx studies.

Exchange diffusion of monocarboxylate anions

Earlier studies [1] on the efflux of propionate and pyruvate showed that the movement of the substrate across the membrane could be accelerated by the presence of the substrate or its homologue on the opposite side of the membrane. The non-competitive inhibitor of influx, octanoate, had no effect on this exchange diffusion, indicating an asymmetrical behaviour of the monocarboxylate carrier. On the other hand, if binding of anions to the membrane constituents would occur, efflux stimulation could be explained by inhibition of the reentry of the effluxed anion due to the blockade of binding sites by "counter"-anions. In view of this possibility, we performed experiments measuring the effect of preloading cells for 5 min with unlabelled substrate, on the influx of another labelled substrate. The results are shown in TABLE II. It can be seen that cells preloaded with unlabelled propionate (2 mM) show an increase of the rate of influx of $[1-^{14}C]$-

TABLE II

EFFECT OF PRELOADING OF CELLS WITH UNLABELLED SUBSTRATE ON THE INFLUX RATE OF [1-^{14}C]-PYRUVATE OR [1-^{14}C]-PROPIONATE AT 0°C.

Cells preloaded at 0°C, for 5 min, with 1 or 2 mM unlabelled substrate (50 µl) were added to 700 µl Krebs-phosphate buffer (pH 7.4) containing 1 mM labelled substrate, 0.4 mM [^{3}H]-inulin and 0.1% albumin. After 5 min the cells were rapidly sedimented through silicon oil, as described unter METHODS. The results are the mean of four experiments (± S.E.). The significance of each comparison with the control was determined using Student's "t" test for paired data.

Preincubation	Influx rate of pyruvate (nMol/ mg protein/5 min)	Influx rate of propionate (nMol/ mg protein/5 min)
-	3.18 ± 0.27	4.64 ± 0.19
1 mM propionate	3.82 ± 0.26 ($P<0.05$)	-
2 mM propionate	4.51 ± 0.50 ($P<0.05$)	-
1 mM pyruvate	-	5.25 ± 0.13 ($P<0.05$)
2 mM pyruvate	-	5.63 ± 0.17 ($P<0.01$)

pyruvate of 42%. The degree of stimulation was dependent on the concentration of propionate used. These findings are compatible with a reversible membrane transport model in which the organic anions penetrate the membrane in the form of a complex with some unknown carrier.

Countertransport is a term that has been used for two different processes at the cellular level, which have been designated by Stein [19], as "competitive exchange diffusion" and "accelerative exchange diffusion". In a recent paper Robinson [20] described a method for distinguishing between these two models for transport of amino acids in rat small intestine. The volume of the external medium was changed and it was investigated whether the stimulation of efflux was affected. A change of the external medium in the present efflux experiments (TABLE III) from 0.7 to 0.3 ml might be expected to approximately double the extracellular concentration of [1-^{14}C]-propionate so that the reentry may be stimulated and the shielding of external binding sites for the labelled propionate by the unlabelled pyruvate might become less efficient. In the "accelerative model" the stimulation of efflux should be unaffected. It can be seen from TABLE III that the intracellular concentration of [1-^{14}C]-propionate

TABLE III

THE INFLUENCE OF THE EXTERNAL VOLUME OF THE STIMULATION OF EFFLUX BY A COUNTERANION

Epithelial cells, preincubated for 15 min at 0°C with 1 mM [1-^{14}C]-propionate (50 µl), were added to 0.7 or 0.3 ml buffer, layered on silicon oil. The buffer contained countersubstrate and [^{3}H]-inulin. After 30 sec the cells were rapidly centrifuged through silicon oil as described under METHODS. The results are the mean of four experiments (± S.E.)

Volume efflux medium (ml)	Counteranion	Propionate intracellularly after 30 sec incubation	Efflux stimulation by counteranion
		nMoles/mg protein	
0.70	-	3.70±0.16*	-
0.70	2 mM pyruvate	2.82±0.13	0.88±0.06**
0.30	-	4.15±0.21*	-
0.30	2 mM pyruvate	3.00±0.19	1.15±0.14**

Between * marked values significance of difference was: $P<0.01$. Between ** marked values significance of difference was: $P<0.01$.

was higher after 30 sec when 0.3 ml external medium was used, indicating increased backflux of labelled substrate. The stimulation of efflux however, was significantly increased under this condition. This disagrees with the concept that the presence of external unlabelled pyruvate decreases the backflux of [1-^{14}C]-propionate. Analogous results were obtained with the stimulated efflux of [1-^{14}C]-pyruvate by external propionate (results not shown). Stein [19] clearly formulated the basis for this phenomenon, that some component of the membrane moves through the membrane at a faster rate when combined with substrate than when free: "accelerative exchange diffusion" The possibility remains, however, that the pyruvate anion could induce the exchange for propionate of equivalent electrical charge. Therefore the specificity of the stimulation of efflux was studied by examining the ability of four substrates, pyruvate, acetate, propionate and butyrate, to accelerate exchange. In this substrate sequence the contribution of ionic diffusion to overall transport would be expected to decrease, because of the increase in lipophilicity of the undissociated acid [1]. Previous results [1] clearly showed that pyruvate in contrast to its rapid rate of influx moves slowly out of the cells. The initial phase (1 min) of pyruvate flux could be stimulated by counteranions (Fig. 6 of ref. 1). For convenience of measurement, an approximation of concentration-dependent efflux-stimulation by different counteranions could still be obtained after 5 min incubation, since only the initial phase showed large differences. It can be seen from TABLE IV that indeed butyrate is more efficient than propionate or acetate to remove pyruvate from the cells. The reciprocal efflux-stimulation is plotted versus the reciprocal of counteranion concentration in Fig. 2. These plots reveal that the stimulation evoked by acetate, propionate or butyrate gave the same V_{max}. However, the affinity constants (1.8, 1.0 and 0.4 mM respectively) were con-

TABLE IV

DEPENDENCE OF THE INTRACELLULAR PYRUVATE CONCENTRATION ON THE PRESENCE OF COUNTERSUBSTRATES

Epithelial cells preincubated for 15 min at 0°C with 1 mM [1-^{14}C]-pyruvate (50 µl) were added to 0.7 ml buffer present on top of silicon oil. The buffer contained countersubstrates at the concentration indicated as well as [^{3}H]-inulin. After 5 min incubation the cells were rapidly centrifuged through silicon oil as described under METHODS. The results are the mean of four experiments (± S.E.).

Counter-anion concentration (mM)	Pyruvate intracellularly after 5 min incubation (nMoles/mg prot.)			
	Pyruvate*	Acetate*	Propionate*	Butyrate*
0	4.04±0.13	4.06±0.14	3.76±0.27	4.28±0.30
0.4	3.34±0.25	3.85±0.18	3.43±0.27	3.65±0.32
0.8	2.90±0.12	3.68±0.20	3.26±0.24	3.55±0.30
2.0	2.59±0.15	3.46±0.20	2.98±0.25	3.26±0.27
4.0	2.11±0.06	3.19±0.15	2.78±0.29	3.22±0.30

* This anion was added to the external medium at the indicated concentration.

siderably different. The affinity constant for propionate obtained here correlated with the value calculated from the results of Fig. 1 (1.2 mM) rather well. It can be seen (Fig. 2) that the V_{max} with pyruvate as counteranion is clearly higher than the V_{max} obtained with short-chain fatty acid anions. As a possible explanation for this discrepancy it is suggested that pyruvate induces not only movement of [1-^{14}C]-pyruvate over the cellular membrane, but also the exchange over the mitochondrial membrane. In a recent report [21] evidence was presented, showing the existence in rat liver mitochondria of a translocator,

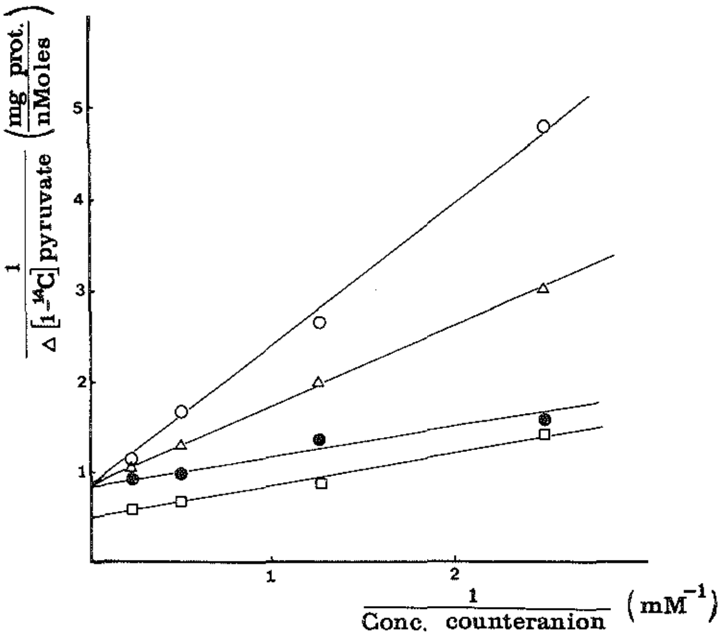

Fig. 2. Lineweaver-Burk plots of the stimulation of efflux as a function of the concentration of counteranion. The values represent the difference in efflux in the presence and absence of counteranion calculated from the data in TABLE IV. Different counteranions were used: ●-●, butyrate; Δ-Δ, propionate; O-O, acetate and □-□, pyruvate.

which mediates an exchange between mitochondrial [^{14}C]-pyruvate and external pyruvate, but no exchange could be demonstrated with external acetate. Indeed, another set of experiments (results not shown) revealed that stimulation of [1-^{14}C]-propionate efflux by pyruvate resulted in a V_{max} close to the one found with propionate as the counteranion.

Temperature dependency of monocarboxylic acid efflux

Temperature dependency of anion transfer has puzzled many investigators. Reported Q_{10} values range from 1.2 to 1.5 [22], as would be expected in the case of a diffusion process. In contrast Q_{10} values for phosphate, sulphate, chloride, pyruvate, lactate and bicarbonate [13,15,23,24] were found to be 5 to 8, corresponding to an activation energy of 30-40 kcal/mol). There appeared to be no simple correlation between the absolute rates of anion permeation and the magnitude of the activation energy. Fig. 3 demonstrates that in the Arrhenius diagrams of initial rates (30 sec) of pyruvate and propionate efflux a deviation from linearity in the temperature interval 0-5°C occurs.

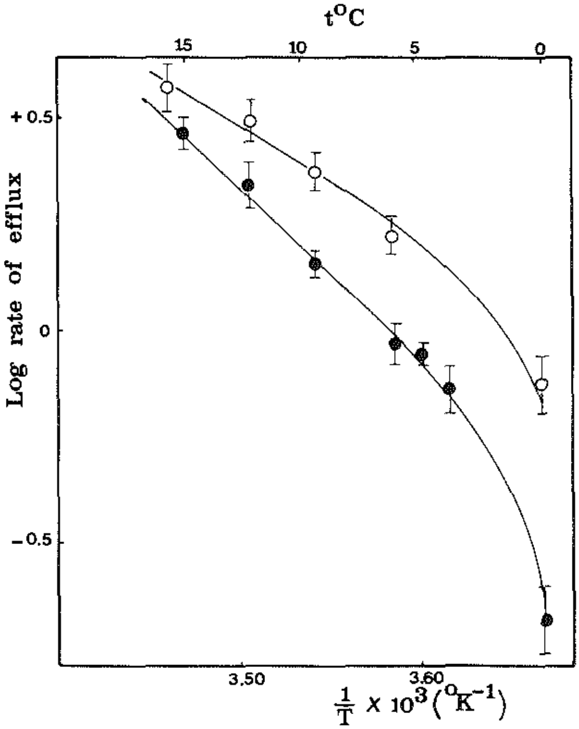

Fig. 3. Temperature dependence of efflux of propionate and pyruvate from isolated epithelial cells. Cells, preincubated for 20 min at 0°C with 1 mM labelled substrate (50 µl), were added to 0.7 ml Krebs-phosphate buffer layered on silicon oil. After 30 sec the cells were rapidly centrifuged through silicon oil. The initial [^{14}C]-substrate content was determined as described under METHODS. The rate of efflux was calculated as nMol/mg protein. ●-●, [1-^{14}C]-pyruvate efflux; O-O, [1-^{14}C]-propionate efflux. Each value represents the mean of four measurements (± S.E.).

Firstly this may confirm some peculiar high temperature dependency in the range 0-5°C, in accordance with the values found between 0-10°C for other anion transfer systems [23,24], but secondly such a change in activation energy may also indicate a transition in the membrane structure at the deflection point of the diagram. The lower activation energy of propionate efflux (12.6 kcal/mol) found between 5 and 15°C, when compared with pyruvate (19.6 kcal/mol) may have its origin in a partial contribution of nonionic diffusion to propionate permeation (compare ref. 1). Unpublished observations showed that the temperature dependency of propionate - [1-^{14}C]-propionate exchange between 6 and 15°C yielded the same activation energy as propionate efflux. Measurements at temperatures higher than 15°C were of limited significance by the inability to estimate linear rates of transport.

CONCLUDING REMARKS

The present experiments strongly point to the involvement of a carrier in the movement of monocarboxylate anions across the cell membrane of the intestinal epithelial cell. Transfer shows competitive phenomena (compare also ref. 1), saturable exchange diffusion and a high temperature coefficient. Most experiments were carried out at 0°C indicative of facilitated translocation, independent of metabolic activity. Furthermore, evidence from literature [11,12,25-28] makes it very likely that monocarboxylate transporters are to be found in the plasma membrane of the cell. Halestrap and Denton [11,29] now obtained conclusive evidence that a carrier for pyruvate is present in erythrocytes, which is sensitive to inhibition by α-cyano-4-hydroxycinnamate, an analogue of the enol form of pyruvate. The failure to demonstrate any inhibition of this compound on acetate transport [11,29] is probably related to the observation of Deuticke [14], that acetate mainly penetrates by nonionic diffusion in erythrocytes. They also failed to show inhibition of pyruvate or lactate transport across the heart cell membrane by α-cyanocinnamate analogues. The latter finding indicates that the proposed pyruvate carrier [25,26] in this organ may have no affinity for these inhibitors. Oldendorf [27] demonstrated a saturable carrier transport system for the uptake of pyruvate, propionate and acetate in brain *in vivo*. In competition experiments it was found that the carrier was half saturated at approximately 1 mM pyruvate and 2.5 mM acetate, affinity constants approaching the values found in the present study on isolated epithelial cells from rat small intestine at 0°C (Fig. 2). However, comparison of the values may not be permitted because of the large temperature differences involved. Because of both rapid metabolism and uptake of monocarboxylic acids, we were unable to measure these parameters at higher temperatures. Monocarboxylic acid transport across the erythrocyte membrane was studied extensively with respect to the ionic contribution to the transfer [13,14]. Temperature-, ion- and pH-dependencies suggested a specific anion transport system in this cell type. Also experimental evidence was provided for the contribution of non-ionic diffusion to the transfer, obscuring the kinetics of ionic transfer. The present paper demonstrates an easy means to discriminate between a specific anion and the non-ionic transport by studying the exchange diffusion. Apparent Michaelis constants determined in the exchange diffusion (Fig. 2) approached the values found in the net influx studies (Fig. 1).

In the earlier report [1] we speculated about the involvement of the monocarboxylate carrier in short-chain fatty acid transport and a possible localization in brush-border membrane. Indeed, several investigations [2-4,7] were indicative of a specific transport system for short-chain fatty acids in rat small intestine. There are, however, contradictory results about the mutual competition between these fatty acids and the existence of saturation kinetics [7,8,30]. Secondly, Jackson [6] demonstrated that the transport of weak acids can be described in terms of a model system of three serial compartments, in which the pH of the intermediate compartment is higher than that of the bulk phases. On this basis he was able to explain the mutual inhibitory interactions between weak acids and the net mucosal to serosal fluxes found in experiments with stripped mucosa. Recently, it was clearly shown by the same group [31], that the development of a compartment of high pH within the intestinal wall may be associated with the serosally directed movement of metabolic anions [32, 33] through lateral intercellular spaces of epithelium. In order to account for the observed mucosal to serosal flux of weak acids, it was necessary to suggest that the brushborder membrane, in contrast to the serosal barrier, was impermeable to the anionic form of the acids. The anionic exchange diffusion described in the present paper may be a property of the basolateral membrane of the epithelial cell, accelerating the anionic transfer at this site with exchangeable anions at the serosal side (for example HCO_3^-). Localization of a monocarboxylate carrier at the blood side of the epithelial cell, is more compatible with the carriers proposed in heart-, brain- or erythrocyte cell membranes [25-29,11,12].

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