Effects of cyclic AMP, glucocorticoids and insulin on the activities of phosphatidate phosphohydrolase, tyrosine aminotransferase and glycerol kinase in isolated rat hepatocytes in relation to the control of triacylglycerol synthesis and gluconeogenesis

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1. Rat hepatocytes were incubated in monolayer culture in modified Leibovitz L-15 medium containing either 10% (v/v) newborn-calf serum or 0.2% (w/v) fatty-acid-poor bovine serum albumin. The addition of 100 nM-dexamethasone increased the activities of both phosphatidate phosphohydrolase and tyrosine aminotransferase by about 3.5-fold after 8 h, and these activities continued to rise until at least 24 h. 2. Incubating the hepatocytes in the albumin-containing medium with 10 μ M- or 100 μ M-8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate increased the activities of the phosphohydrolase and aminotransferase by 2.6- and 3.4-fold respectively after 8 h. These increases were blocked by actinomycin D. 3. The increases in the activities that were produced by the cyclic AMP analogue and dexamethasone were independent and approximately additive. 4. Insulin when added alone did not alter the phosphohydrolase activity, but it increased the aminotransferase activity by 34%. The dexamethasone-induced increase in the phosphohydrolase activity was completely blocked by 7–144 μ M-insulin, whereas that of the aminotransferase was only partly suppressed. Insulin had no significant effects on the increases in the activities of phosphatidate phosphohydrolase and tyrosine aminotransferase that were produced by the cyclic AMP analogue, but this may be because the analogue is fairly resistant to degradation by the phosphodiesterase. 5. The activity of glycerol kinase was not significantly changed by incubating the hepatocytes with insulin, dexamethasone and the cyclic AMP analogue alone or in combinations. 6. It is proposed that high concentrations of cyclic AMP and glucocorticoids increase the total activity of phosphatidate phosphohydrolase in the liver and provide it with an increased capacity for synthesizing triacylglycerols and very-low-density lipoproteins, which is expressed when the availability of fatty acids is high. There appears to be a co-ordinated hormonal control of triacylglycerol synthesis and gluconeogenesis in diabetes and in metabolic stress to enable the liver to supply other organs with energy.

In diabetes and conditions of metabolic stress, the liver secretes glucose, ketones and very-low-density lipoproteins in order to supply other organs with a source of energy. Glucose and ketones are used by the brain, and the triacylglycerols of the

very-low-density lipoproteins are hydrolysed to provide fatty acids for heart and skeletal muscle (Brindley & Lawson, 1983). The increased ability of the liver to synthesize and secrete triacylglycerols appears to be related to the high activities of PAP that occur in diabetes and in stress conditions (Brindley & Sturton, 1982; Brindley & Lawson, 1983). This occurs because glucocorticoids appear to stimulate the synthesis of PAP, since the increases are sensitive to inhibitors of RNA and protein synthesis (Lehtonen *et al.*,

Abbreviations used: GK, glycerol kinase (EC 2.7.1.30); PAP, phosphatidate phosphohydrolase (EC 3.1.3.4); TAT, tyrosine aminotransferase (EC 2.6.1.5); CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate.

1979; Jennings *et al.*, 1981), and the relatively low concentrations of insulin are unable to antagonize the glucocorticoid effect (Lawson *et al.*, 1982a,b). The glucocorticoid-induced stimulation of PAP activity increases the potential of the liver to synthesize triacylglycerols. This potential is expressed by an increased availability of fatty acids that would normally be mobilized from adipose tissue as a result of the relatively high concentrations of glucagon, adrenaline and corticotropin relative to insulin. The high concentrations of fatty acids stimulate the activity of PAP in the liver (Lamb & McCue, 1983; Cascales *et al.*, 1984). They also cause the functional activation of PAP by promoting its translocation from the cytosol of the hepatocytes to the membranes on which the phosphatidate is being synthesized (Cascales *et al.*, 1984; Butterwith *et al.*, 1984; Martin-Sanz *et al.*, 1984). Vasopressin (and probably α -agonists) also produce a rapid stimulation of PAP activity in the liver, and they increase triacylglycerol synthesis (Pollard & Brindley, 1984).

It therefore appears that there may be a co-ordinated control of triacylglycerol synthesis and gluconeogenesis in stress conditions. The present work was designed to investigate this possibility, by using monolayers of rat hepatocytes that were viable for a culture period of at least 24 h. In particular, the long-term effects of cyclic AMP in the presence and absence of insulin and a glucocorticoid (dexamethasone) on the activity of PAP were determined. For comparison, TAT was chosen as an example of an enzyme that is involved in amino acid breakdown and gluconeogenesis, since there is a considerable literature on its hormonal control. In addition GK activity was also measured. This was considered to be important because this enzyme is involved in both gluconeogenesis and triacylglycerol synthesis. GK participates in the uptake by the liver of the glycerol that is released from adipose tissue in stress and diabetes. The glycerol phosphate that is formed can be converted into glucose and triacylglycerol, or it can be used for energy production. GK activity in the liver has been reported to be increased by insulin and glucagon and to parallel the rate of triacylglycerol synthesis (Lamb *et al.*, 1982). Fatty acids (Kida *et al.*, 1973), oestradiol and ethanol (Schneider, 1975; Lamb *et al.*, 1977) have also been shown to increase GK activity.

Experimental

Animals and materials

The sources of the rats and of most of the materials have been described (Jennings *et al.*, 1981; Pollard & Brindley, 1984; Cascales *et al.*, 1984). Dexamethasone, CPT-cAMP and actino-

mycin D were from Sigma (London) Chemical Co. Falcon Primaria Tissue Culture Dishes (60 mm $\times$ 15 mm) were from Becton Dickinson U.K. Ltd., Cowley, Oxford, U.K. Fatty-acid-poor bovine serum albumin was from Miles Laboratories, Stoke Poges, Slough, U.K., and [1,3- 3 H]-glycerol was from Amersham International, Amersham, Bucks., U.K. The bovine insulin was a gift that was specially purified by Boots Co., Nottingham, U.K. (Lawson *et al.*, 1982a).

Preparation and incubation of hepatocytes

Hepatocytes were prepared and attached to tissue-culture dishes in a modified Leibovitz L-15 medium containing 10% (v/v) newborn-calf serum as described by Cascales *et al.* (1984). In initial experiments normal tissue-culture dishes that had been coated with collagen were used. However, these were replaced with Primaria Tissue Culture Dishes, which also provide a positively charged substratum for attachment of the hepatocytes. In our experience these dishes gave equivalent results to the collagen-coated dishes, but have the advantage of saving time and expense. After incubation for 1–2 h at 37°C in humidified air, the unattached and non-viable cells were removed by replacing the medium. The incubation was continued for a further 7–8 h, and the medium was then replaced by modified Leibovitz medium containing 0.2% (w/v) fatty-acid-poor bovine serum albumin, and the cells were incubated for 12–15 h. The medium was then replaced and hormone additions were made so that the total volume of the medium changed by less than 2% (Jennings *et al.*, 1981). At the times indicated, the cells were washed once with Leibovitz medium that did not contain serum or albumin. The cells were then scraped from the plates in 1 ml of ice-cold 0.25 M-sucrose adjusted to pH 7.4 with KHCO_3 and containing 0.5 mM-dithiothreitol and 0.2 mM-pyridoxal phosphate. Homogenates were prepared by sonication at 22 kHz with an amplitude of 8 μ m peak-to-peak, and they were stored at –20°C until required.

Measurement of enzyme activities

The activities of lactate dehydrogenase (Saggerson & Greenbaum, 1969), PAP (Pollard & Brindley, 1984) and TAT (Marston & Pogson, 1977) were determined essentially as described previously. The incubation system for GK was similar to that described by Lamb *et al.* (1977). It contained, in a final volume of 0.35 ml: 10 mM-ATP, 18 mM-NaF, 1.4 mM-dithiothreitol, 3 mM-MgCl₂, 50 mM-Tris adjusted to pH 8.3 with HCl, 1 mM-EDTA, 0.6 mM-[1,3- 3 H]glycerol (1 Ci/mol) and 50–100 μ g of homogenate protein. After incubation for 20 min at 37°C, the reactions were stopped by adding 0.1 ml of ethanol and placing the tubes in a

boiling-water bath for 5 min. A 300 μ l sample of the resulting protein-free solution was then applied to a column (approx. 0.3 ml; about 5.5 mm diameter $\times$ 10–15 mm) of Dowex-1 (1 X8; 200 mesh; formate form). More than 99.7% of the unchanged glycerol was eluted by washing with about 5 ml of water in three portions. Glycerol phosphate was then eluted with 3 $\times$ 1 ml of 4 M-formic acid containing 0.2% (w/v) ammonium formate. Fiso-fluor scintillator (15 ml) was added and radioactivity was determined by scintillation counting. The recovery of glycerol phosphate by this procedure was greater than 97%.

Results

Choice of the incubation system for the hepatocytes

Previous work from this laboratory has studied the glucocorticoid-induction of PAP and TAT activities by using hepatocytes in suspension culture (Jennings *et al.*, 1981; Lawson *et al.*, 1982a,b; Pollard & Brindley, 1984). However, the viability of hepatocytes in this system is limited to

about 10 h. We therefore decided to adopt a monolayer culture technique in which the cells are metabolically stable for several days (Evans & Mayer, 1982, 1983). This is indicated by the high percentage retention of lactate dehydrogenase activity and stable rates of protein turnover. The time course of the effects of dexamethasone on the activities of PAP and TAT in cells that were cultured in the presence of 10% newborn-calf serum shows that PAP activity increased steadily over the 24 h incubation, by which time it was about 7-fold higher in the cells that were incubated with rather than without 100 nM-dexamethasone (Fig. 1a). The activity of PAP also increased by about 50% in the cells that were incubated in the absence of dexamethasone, whereas TAT activity fell by about 50%. A similar decrease in TAT activity was also seen during a 6 h incubation of hepatocytes in suspension culture when no glucocorticoid was added (Jennings *et al.*, 1981). The presence of 100 nM-dexamethasone increased the TAT activity by about 7.5-fold at the 24 h time point (Fig. 1b).

Fig. 1. Effects of dexamethasone on the activities of (a) PAP and (b) TAT in rat hepatocytes. Hepatocytes were incubated with modified Leibovitz L-15 medium containing either 10% (v/v) newborn-calf serum ($\blacktriangle$, $\triangle$), or 0.2% (w/v) fatty-acid-poor bovine serum albumin ($\bullet$, $\circ$) in the presence of 100 nM-dexamethasone ($\bullet$, $\blacktriangle$), or in its absence ($\circ$, $\triangle$), as described in the Experimental section. The results are expressed as means $\pm$ s.d. for three dishes from a single experiment, and similar results were obtained in a further experiment.

It was hoped that the serum used in the cultures could be replaced by fatty-acid-poor albumin, which would provide a more chemically defined basal medium. This had not been feasible in the suspension cultures, since the ATP content of the hepatocytes fell dramatically and dexamethasone failed to induce PAP activity (Jennings *et al.*, 1981). In the present experiments with collagen-coated dishes, the replacement of the serum with 0.2% (w/v) albumin during the initial application of the cells to the culture dishes led to a progressive loss of PAP and TAT activity over 24h and a leakage of lactate dehydrogenase into the medium. The attachment of the hepatocytes to the dishes and their spreading was also much slower in the albumin-containing medium. This suggests that some components of the serum are needed for these processes, and these could include hormones, binding proteins such as transferrin, attachment factors such as cold-insoluble globulins and fibronectin, or serum spreading factor (Barnes & Sato, 1980).

The hepatocytes were therefore allowed to bed down, spread and stabilize in medium containing 10% newborn-calf serum for about 8h. This medium was then replaced with medium containing 0.2% fatty-acid-poor bovine serum for 12–15h before beginning the experiments. This washed the cells and allowed some time for the degradation of hormones and other compounds derived from the serum. The medium was then replaced by more albumin-containing medium. These cells retained relatively constant ratios of lactate dehydrogenase, PAP and TAT to protein for the next 48h, although there was a loss of about 30% of lactate dehydrogenase and protein. This indicated the detachment of some non-viable cells from the monolayer. The activities of PAP and TAT were increased in parallel by dexamethasone at 1nM–1 μ M with an optimum stimulation for both activities at 100nM. The responses for this last concentration are shown in Fig. 1. These results demonstrate that the cultures containing 0.2% albumin provide a system that can be used to investigate the induction of PAP and TAT activities. The system will be particularly useful in other work for studying the effects of compounds that would be difficult to remove from serum. The magnitude of the changes in the activities of PAP and TAT that are shown in Fig. 1 are much higher than the increases of approx. 2-fold that were obtained with the suspension cultures (Jennings *et al.*, 1981; Lawson *et al.*, 1982a,b; Pollard & Brindley, 1984).

Interaction of cyclic AMP with dexamethasone and insulin on the activities of PAP, TAT and GK

In these experiments CPT-cAMP was used as a

potent and relatively stable analogue of cyclic AMP (Miller *et al.*, 1975). When the hepatocytes were incubated with 100 μ M-CPT-cAMP there were increases of 2.6- and 3.4-fold in the activities of PAP and TAT respectively, but no change in GK activity (Table 1). The increase for TAT was statistically greater than for PAP at 10 μ M- and 100 μ M-CPT-cAMP ($P < 0.005$ and $P < 0.05$ respectively). The stimulations in both of these activities were almost identical between 10 μ M- and 100 μ M-CPT-cAMP (Fig. 2), and similar results were obtained with 6-*N*,2'-*O*-dibutyryl cyclic AMP (results not shown). The maximum stimulations of both PAP and TAT activities occurred after 8h (Fig. 3), and most of the studies were performed at this time (Table 1).

Incubation of the hepatocytes with both 100nM-dexamethasone and 100 μ M-CPT-cAMP produced a greater stimulation of both PAP and TAT activities than was obtained with either compound alone (Table 1). There was again no significant change in the activity of GK. The increases in PAP and TAT activities in the presence of dexamethasone or CPT-cAMP were abolished by adding 1 μ g of actinomycin D/ml.

Fig. 2. Effects of the concentration of CPT-cAMP on the activities of PAP and TAT in rat hepatocytes

Hepatocytes were incubated in modified Leibovitz L-15 medium containing 0.2% (w/v) fatty-acid-poor bovine serum albumin for 8h (see the Experimental section). The activities of PAP (●) and TAT (▲) are given as means $\pm$ S.D. for eight independent experiments at 100 μ M, or for three experiments at 10 μ M, and as the means $\pm$ ranges for two experiments at 0.1 μ M- and 1 μ M-CPT-cAMP.

Table 1. *Effects of cyclic AMP, dexamethasone and insulin on the activity of PAP, TAT and GK in rat hepatocytes*
Hepatocytes were incubated for 8 h in modified Leibowitz medium containing 0.2% (w/v) fatty-acid-poor bovine serum albumin (see the Experimental section). The enzyme activities are given as means $\pm$ S.D. for the numbers of independent experiments shown in parentheses, and they are expressed relative to the activity with the basic incubation medium. Where there are only two experiments the means $\pm$ range are presented. The absolute activities for the basic incubation system were 0.43 ± 0.30 , 0.63 ± 0.50 and 9.9 ± 6.1 nmol of product formed/min per unit of lactate dehydrogenase activity for PAP, TAT and GK respectively. The significance of the difference between groups for the same enzyme is indicated by: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$; † $P < 0.005$; †† $P < 0.001$.

		Relative activity (%)		
Additions		PAP	TAT	GK
I	None	100 (9)	100 (9)	100 (6)
II	Dexamethasone (100 nM)	345 $\pm$ 99 (6) I versus II††	351 $\pm$ 105 (6) I versus II†	111 $\pm$ 18 (5)
III	CPT-cAMP (100 μ M)	257 $\pm$ 122 (8) I versus III***	338 $\pm$ 87 (8) I versus III††	115 $\pm$ 12 (5)
IV	Dexamethasone (100 nM) + CPT-cAMP (100 μ M)	554 $\pm$ 249 (3) I versus IV†	515 $\pm$ 82 (3) I versus IV†	94 $\pm$ 14 (2)
V	Actinomycin D (1 μ g/ml)	83 $\pm$ 4 (3)	110 $\pm$ 24 (3)	Not measured
VI	Dexamethasone (100 nM) + actinomycin D (1 μ g/ml)	78 $\pm$ 2 (2)	121 $\pm$ 14 (2)	Not measured
VII	CPT-cAMP (100 μ M) + actinomycin D (1 μ g/ml)	93 $\pm$ 4 (3) III versus VII***	123 $\pm$ 23 (3) III versus VII*	Not measured
VIII	Insulin (36 μ M)	101 $\pm$ 16 (5)	134 $\pm$ 21 (5) I versus VIII*	101 $\pm$ 27 (4)
IX	Dexamethasone (100 nM) + insulin (36 μ M)	133 $\pm$ 38 (5) II versus IX††	215 $\pm$ 72 (5) I versus IX* II versus IX*	88 $\pm$ 29 (5)
X	CPT-cAMP (100 μ M) + insulin (36 μ M)	212 $\pm$ 89 (4) I versus X**	437 $\pm$ 33 (4) I versus X**	88 $\pm$ 19 (4)
XI	Dexamethasone (100 nM) + CPT-cAMP (100 μ M) + insulin (36 μ M)	240 $\pm$ 39 (4) IV versus XI*	499 $\pm$ 146 (4)	Not measured

Fig. 3. Time course for the effect of CPT-cAMP on the activities of PAP and TAT in rat hepatocytes

Hepatocytes were incubated with 100 μ M-CPT-cAMP (●, ▲) or in its absence (○, △) as described in Fig. 2. The activities of PAP (●, ○) and TAT (▲, △) are the means $\pm$ S.D. for three separate dishes from an individual experiment. Similar results were obtained in an additional independent experiment.

Insulin (36 μ M) did not significantly stimulate the activity of PAP and GK, but it did produce a slight but significant increase in the activity of TAT (Table 1). Insulin decreased the effect of dexamethasone in stimulating the increase in PAP and TAT activities, but it had no significant effect on the increases produced by CPT-cAMP. The effects of insulin were approximately constant between 7 and 144 μ M (results not shown). When insulin (36 μ M) was added in combination with both dexamethasone and CPT-cAMP there was some suppression of the increase in PAP activity, but no significant effect on the activity of TAT (Table 1).

In the present experiments almost all of the PAP was calculated to be in the cytosolic fraction of the hepatocytes when they were lysed with digitonin (Cascales *et al.*, 1984). This probably resulted from the lack of fatty acid in the 0.2% albumin that was added to the incubation medium rather than the 10% newborn-calf serum that we normally employ (Cascales *et al.*, 1984; Butterwith *et al.*, 1984).

Discussion

The results in Fig. 1 establish that the monolayer-culture system can be used for investigating the control of PAP and TAT activities, and in particular that the serum in the incubations can be replaced by albumin provided that the cells are allowed first to bed down in serum. This provides well-defined culture conditions for investigating the interactions of various hormones. The stimulatory effects of dexamethasone are compatible with results that have been reported previously for TAT (Haung & Ebner, 1969; Wicks *et al.*, 1969; Gelehrter *et al.*, 1972; Bonney *et al.*, 1974; Michalopoulos & Pitot, 1975; Michalopoulos *et al.*, 1978; Marston & Pogson, 1977; Savage & Bonney, 1978) and PAP (Lehtonen *et al.*, 1979; Jennings *et al.*, 1981; Lawson *et al.*, 1982*a,b*).

Also, as expected from previous work (Wicks *et al.*, 1969; Lin & Snodgrass, 1975; Michalopoulos *et al.*, 1978; Ernest & Feigelson, 1978; Evans, 1981), there was an increase in the activity of TAT when the hepatocytes were incubated with cyclic AMP analogues. One mechanism that can account for this increase is the higher production of mRNA for TAT (Noguchi *et al.*, 1978; Ernest & Feigelson, 1978). The effects of actinomycin D in suppressing the effects of CPT-cAMP on TAT activity (Table 1) are compatible with this explanation. We are now able to report that the synthesis of PAP is probably controlled in a similar manner by cyclic AMP, although the increases in activity of PAP are less ($P < 0.05$) than for TAT (Table 1; Figs. 2 and 3).

Incubating the hepatocytes with 100 μM -CPT-cAMP and 100 nm-dexamethasone in combination gave increases in the activities of PAP and TAT that were approximately equivalent to the sum of their stimulations when added alone (Table 1), even though these concentrations were approximately optimum. These results suggest an independence of the effects of CPT-cAMP and dexamethasone, even though both may be stimulating transcription. Furthermore, these compounds may have other effects when used alone or in combination. For example, dexamethasone and CPT-cAMP can decrease the rate of degradation of TAT (Levitan & Webb, 1970; Boctor & Grossman, 1970; McNamara & Webb, 1974; Voigt *et al.*, 1978) and cyclic AMP may increase the rate of translation (Roper & Wicks, 1978). Apparently additive or synergistic effects of cyclic AMP and glucocorticoids in increasing the activity of TAT have also been reported by other investigators (Wicks *et al.*, 1969; McNamara & Webb, 1973; Gurr & Potter, 1980; Auberger *et al.*, 1983).

When insulin was added to the hepatocytes at concentrations of 7–144 μM there was no change in

the total activity of PAP after 8 h (Table 1) and after 24 h (results not shown). These results agree with those of Lawson *et al.* (1982*a,b*). There were, however, significant increases in the TAT activity (Table 1), in agreement with work of Gelehrter *et al.* (1972) and Das *et al.* (1984) in hepatocytes and of Sorimachi & Yasumura (1981) in hepatoma cells. The mechanism of insulin action is unclear, but it may cause the interconversion of the isoenzymes of TAT (Iwasaki *et al.*, 1973), or it may decrease the rate of degradation of TAT (Spencer *et al.*, 1978).

Insulin antagonized the increase of PAP activity by dexamethasone (Table 1), as expected from the work of Lawson *et al.* (1982*a,b*), and it was equally active between 7 and 144 μM (results not shown). Only partial antagonism was seen with TAT (Table 1), as compared with a total antagonism in foetal hepatocytes (Ho *et al.*, 1981), additive effects in Reuber H35 cells (Barnett & Wicks, 1971; Wicks *et al.*, 1974) and the effect of insulin in increasing the effect of dexamethasone in enhancing TAT activity in hepatocytes (Gelehrter *et al.*, 1972). The ability of insulin to decrease the dexamethasone-induced increases in PAP and TAT activity is probably caused by a decrease in the production of mRNA, which has been reported for TAT (Spencer *et al.*, 1978) and phosphoenolpyruvate carboxykinase (Granner *et al.*, 1983).

Insulin did not significantly alter the effect of CPT-cAMP in increasing the activities of PAP and TAT (Table 1). Normally insulin can antagonize cyclic AMP action by stimulating the activity of the phosphodiesterase that degrades cyclic AMP. However, since the CPT-cAMP used in the present experiments is fairly resistant to the phosphodiesterase, this mechanism of insulin action should be largely inoperative.

The results from the present experiments demonstrate further similarities between the control of the activities of PAP and TAT, particularly by cyclic AMP analogues (see also Pittner *et al.*, 1985). Although the responses of the two enzymes were not always identical, it is evident that the total PAP activity should increase in the liver in parallel with an expected increased capacity for gluconeogenesis. However, there was no evidence that the activity of GK was altered by cyclic AMP, dexamethasone or insulin when these were added to the cultures for 8 h alone or in combination (Table 1). The stimulation of GK that was reported to be produced by insulin and glucagon were seen after at least 24 h (Lamb *et al.*, 1982). If these changes are important in regulating triacylglycerol synthesis, they are relatively slow by comparison with the alterations in PAP activity.

The present results demonstrate that the relatively high concentrations of cyclic AMP that occur in the liver in stress and diabetes could augment the

action of glucocorticoids in increasing the total PAP activity. This increases the potential of the liver to synthesize triacylglycerols. However, cyclic AMP rapidly translocated PAP from the membrane-associated compartment into the cytosol (Butterwith *et al.*, 1984). This cytosolic reservoir of potential activity can be expressed at high fatty acid concentrations, which counteract the effect of cyclic AMP in displacing PAP from the membrane-associated compartment (Butterwith *et al.*, 1984) and in inhibiting triacylglycerol synthesis (Pelech *et al.*, 1983). Thus the increased PAP activity can be activated by translocation to match the increased supply of fatty acids that is mobilized from adipose tissue in stress and diabetes. The high rate of triacylglycerol synthesis protects the liver against the toxic effects of the fatty acids. The increased secretion of triacylglycerol in very-low-density lipoproteins is co-ordinated with the secretion of ketones and glucose, and the liver is able to supply other organs with sources of energy.

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