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The Effect of Glucose and of Potassium Ions on the Interconversion of the Two Forms of Glycogen Phosphorylase and of Glycogen Synthetase in Isolated Rat Liver Preparations

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1. In the isolated perfused rat liver, increasing glucose concentration from 5.5 to 55mM in the perfusion medium caused a sequential inactivation of glycogen phosphorylase and activation of glycogen synthetase. The latter change was preceded by a lag period which corresponded to the time required to inactivate the major part of the phosphorylase. 2. The same sequence of events was observed in isolated rat hepatocytes incubated at 37°C. In this preparation, the rate of phosphorylase inactivation was greatly increased by increasing the concentration of glucose and/or of K^+ ions in the external medium. The same agents also caused the activation of glycogen synthetase, but this effect was secondary to the inactivation of phosphorylase. 3. In both types of preparations, the rate of synthetase activation was modulated by the residual amount of phosphorylase *a* that remained after the initial phase of rapid inactivation and was independent of glucose concentration. 4. In isolated hepatocytes, the rate of conversion of glucose into glycogen was proportional to the activity of synthetase *a* in the preparation. This conversion was preceded by a lag period which could be shortened by increasing either glucose or K^+ concentration in the medium. The incorporation of labelled glucose into glycogen was simultaneous with a glycogenolytic process which could not be attributed to the activity of phosphorylase *a*.

De Wulf & Hers (1967) showed that the administration of glucose to mice greatly increased the rate of conversion of glucose into liver glycogen through a parallel activation of glycogen synthetase. These changes were preceded by a short lag (1–2min). Further studies in fed mice and rats have shown that this lag is the time required for the inactivation of glycogen phosphorylase (Stalmans *et al.*, 1974*a*). The primary effect of glucose is to bind to phosphorylase *a* (Stalmans *et al.*, 1974*b*) and to favour the conversion of this enzyme into phosphorylase *b* by phosphorylase phosphatase (Stalmans *et al.*, 1970). Since phosphorylase *a* is a strong inhibitor of synthetase phosphatase (Stalmans *et al.*, 1971), its disappearance is a prerequisite for the activation of glycogen synthetase, which occurs in a second step.

The purpose of the present work was to investigate the effect of glucose concentration on the interconversion of the two forms of glycogen phosphorylase and of glycogen synthetase in isolated liver preparations, either perfused rat liver or isolated rat hepatocytes. Since the latter preparation has many advantages over the former, it was used in most of our experiments.

Early work by others, performed with rat liver slices, had shown that the synthesis of glycogen in this preparation is greatly dependent on glucose concentration (Cahill *et al.*, 1958) and on the presence of K^+ as the predominant cation in the incubation medium (Hastings & Buchanan, 1942). The latter effect was explained by a faster and more complete inactivation of phosphorylase in the presence of K^+ (Cahill *et al.*, 1957). More recently it had also been observed that a high glucose concentration caused the inactivation of glycogen phosphorylase and the activation of glycogen synthetase in the perfused rat liver (Buschiazzo *et al.*, 1970; Glinsmann *et al.*, 1970), but the temporal sequence at which these conversions occurred was not established, nor was their obligatory connexion.

In the present paper we show that a high concentration of glucose and of K^+ in the incubation medium greatly increased the rate of inactivation of phosphorylase in isolated hepatocytes. The same effectors caused the activation of glycogen synthetase and, in a parallel fashion, the incorporation of labelled glucose into glycogen. In all conditions, the activation of glycogen synthetase was secondary to the

inactivation of the major part of phosphorylase, and the rate of this activation was controlled by the concentration of residual phosphorylase *a* in the cell. Part of this work has been reported in a preliminary form (Hue *et al.*, 1974).

Materials and Methods

Reagents

Glucose, shellfish glycogen (type II), UDP-glucose, bovine albumin (fraction V), Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid], glucose oxidase, peroxidase and collagenase (type I) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Glucose 1-phosphate was from Merck, Darmstadt, Germany. Amylo-(α-1,4–α-1,6)-glucosidase was purchased from Boehringer und Soehne G.m.b.H., Mannheim, Germany. [U-^{14}C]Glucose and UDP-[U-^{14}C]glucose were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Liver perfusion

Livers of male Wistar rats (200–250g) were perfused *in situ* as described by Hems *et al.* (1966). The perfusion medium was prepared as described by Hems *et al.* (1966) and consisted of 130ml of Krebs–Henseleit medium (118mM-NaCl, 4.75mM-KCl, 2.5mM-$CaCl_2$, 1.18mM-KH_2PO_4, 1.18mM-$MgSO_4$, 25mM-$NaHCO_3$) containing 2.5% (w/v) bovine albumin and washed aged human erythrocytes (15ml of packed erythrocytes/100ml of perfusion fluid) and 5.5mM-glucose. The perfusion medium was in equilibrium with O_2+CO_2 (95:5). The first 20ml of medium was discarded and then the liver was connected to the perfusion apparatus. Livers that were not uniformly perfused were rejected. After an equilibration period of 20min, a biopsy was taken from the papilliform lobule and 5ml of 1.1M-glucose was added to the main reservoir. During the 20min equilibration period, the concentration of glucose in the perfusate of fed-rat livers rose from 5.5mM to 8.03±0.22 (S.E.M., $n=9$)mM.

At different times after the addition of glucose, biopsies were taken successively from the caudate, right main, right accessory and finally the left lobe of the liver. Liver specimens were quick-frozen by the method of Wollenberger *et al.* (1960) and kept in liquid N_2 until homogenization. To avoid excessive bleeding, ligatures, placed around the bases of the hepatic lobes during the equilibration period, were tied soon after cutting the liver specimens. The protein concentration was 215.5±6.4 (S.E.M., $n=9$) mg/g wet wt. of liver measured by the procedure of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Preparation and incubation of liver cells

Hepatocytes were isolated from livers of 200–250g male Wistar rats by the method of Berry & Friend (1969) as modified by Cornell *et al.* (1973) and Krebs *et al.* (1974), except that in the present work hyaluronidase was omitted and the cell suspension was filtered through cheesecloth, centrifuged for 2min at 50*g* and washed in about 10vol. of Krebs–Henseleit medium in which bicarbonate buffer was replaced by 40mM-Hepes at pH7.4. After centrifugation cells were suspended in the same medium to give a concentration of about 100mg of liver cells/ml of suspension. Centrifugation and washing of the cells were performed at room temperature (20°C). The viability of the cells was evaluated by the Trypan Blue test; after 30min of incubation, 80–90% of the cells excluded the stain. Usually 2ml of the cell suspension was incubated at 37°C in 20ml vials with rapid shaking (170 strokes/min) in the presence of air. Except where otherwise stated, the various additions were present in the vials before starting the experiment. When the effects of K^+ were studied, the cells were isolated and washed as described above. They were resuspended either in a Na^+ or in a K^+ medium. The Na^+ medium consisted of 120mM-NaCl, 4.75mM-KCl, 1.25mM-$CaCl_2$, 1.18mM-KH_2PO_4, 1.18mM-$MgSO_4$, 20mM-NaOH, 40mM-Hepes, pH7.4. The K^+ medium contained 120mM-KCl, 4.75mM-NaCl, 1.25mM-$CaCl_2$, 1.18mM-KH_2PO_4, 1.18mM-$MgSO_4$, 20mM-KOH, 40mM-Hepes, pH7.4. The viabilities of cells incubated in Na^+ or K^+ media did not differ from each other. To measure enzyme activities, 0.2ml portions of cell suspension were pipetted and immediately frozen in Potter–Elvehjem tubes kept in a cooling mixture (solid CO_2 in acetone). Each experiment was performed with a separate preparation of cells; 1g of packed cells corresponds to 221.2±7.8 (S.E.M., $n=25$) mg of protein.

Measurements of enzyme activities

Hepatocytes or liver biopsies were homogenized in 4 or 10vol. respectively of an ice-cold medium containing 100mM-NaF, 20mM-EDTA, 0.5% (w/v) glycogen and 50mM-glycylglycine, pH7.4. The activity of phosphorylase *a* was determined in the direction of glycogen synthesis and in the presence of 0.5mM-caffeine. The importance of caffeine as an inhibitor of phosphorylase *b* was first reported by Stalmans *et al.* (1971) and extensively studied by the same group of authors (Stalmans & Hers, 1975). A portion (0.1ml) of homogenate was incubated for 30–60min with an equal volume of a solution containing 100mM-glucose 1-phosphate, 2% (w/v) glycogen, 0.30M-NaF and 1mM-caffeine adjusted to pH 6.1 with HCl. The reaction was stopped by the addition of 0.5ml of ice-cold 1.2M-trichloroacetic acid followed by 3.6ml of water. The mixture was

centrifuged and 0.5ml of molybdate reagent and 0.2ml of aminonaphtholsulphonic acid were added to the supernatant for the determination of phosphate as described by Fiske & SubbaRow (1925). Total phosphorylase was measured after complete activation of the enzyme by purified phosphorylase kinase as described by Stalmans *et al.* (1974*a*). The total phosphorylase of hepatocytes from fed rats was equal to 59.1 ± 3.5 (S.E.M., $n = 9$) units/g of protein and was not significantly different from total phosphorylase of cells from rats that had been deprived of food (55.9 ± 4.5, $n = 6$). These values correspond to 12.6 units/g wet wt. of liver.

The activity of synthetase *a* was measured by the incorporation of the glucosyl moiety of UDP-glucose into glycogen. A portion (0.02ml) of homogenate was incubated at 20°C for 15min in a final volume of 0.12ml in the presence of 0.25mM-UDP-[U-^{14}C]glucose, 1% (w/v) glycogen, 60mM-glycylglycine buffer, pH7.4, 10mM-Na_2SO_4 and 1mM-EDTA. The dual role of Na_2SO_4 as stimulator of synthetase *a* and inhibitor of synthetase *b* was reported by De Wulf *et al.* (1968). At the end of incubation, 75μl of the assay mixture was spotted on a filter paper which was further processed as recommended by Thomas *et al.* (1968).

One unit of phosphorylase *a* or of synthetase *a* is the amount of enzyme that converts 1μmol of substrate/min under the conditions of the assay.

Measurement of conversion of glucose into glycogen

For the determination of radioactive glycogen, samples (0.5ml) of liver cells were pipetted into tubes containing 0.5ml of 30% (w/v) KOH and 5mg of carrier glycogen. After digestion at 100°C during 15min, glycogen was precipitated in ethanol; the glycogen pellet was drained and resuspended in 1ml of 0.6M-trichloroacetic acid and any insoluble material was eliminated by centrifugation. Glycogen was further purified by three ethanolic precipitations and finally dissolved in water, and its radioactivity was measured in a liquid-scintillation spectrometer by using the scintillation mixture described by Patterson & Greene (1965). The incorporation of [^{14}C]glucose into glycogen was calculated from the specific radioactivity of glucose in the medium. Glucose concentration was measured by the glucose oxidase method (Huggett & Nixon, 1957) in deproteinized extracts obtained by mixing portions of cell suspension with equimolar amounts of $ZnSO_4$ and $Ba(OH)_2$ as described by Weichselbaum & Somogyi (1941). The same extract was used for the measurement of the radioactivity of glucose.

For the determination of the glycogen content of the cells from fed rats, the polysaccharide was isolated as indicated above, except that no carrier was added and that the ethanolic precipitations were performed in the presence of 1.5% (w/v) Na_2SO_4. The glycogen was finally dissolved in 1ml of 20mM-sodium acetate buffer at pH5 and incubated for 60min at 37°C in the presence of 0.1mg of amylo-(α-1,4–α-1,6)-glucosidase. Glucose was determined as described above. The glycogen content of cells from rats that had been deprived of food was measured by the glucose content of neutralized $HClO_4$ extracts before and after enzymic hydrolysis of glycogen performed as described above.

Results

Effect of glucose

Experiments with perfused livers. The effect of glucose on the interconversion of the two forms of glycogen phosphorylase and glycogen synthetase was studied in perfused livers from both fed and food-deprived rats. The activity of phosphorylase *a* found after the equilibration period was close to 3 units/g wet wt. of liver (14 units/g of protein), i.e. about 25% of total (*a*+*b*) phosphorylase. The activity of synthetase *a* was close to zero in the liver of fed rats but markedly higher (about 20% of total synthetase) in the livers of rats that had been deprived of food. In control experiments, these values were maintained with no significant variation over the experimental period of 10min (not shown); in contrast, if glucose was added to the perfusion medium at high (55mM) concentration, phosphorylase *a* was converted into *b* and subsequently synthetase became activated (Fig. 1). The characteristic lag that precedes synthetase activation, previously observed in mice (De Wulf & Hers, 1967) and rats (Stalmans *et al.*, 1974*a*) *in vivo*, was also apparent in these experiments.

Experiments with isolated hepatocytes. Hepatocytes isolated from fed rats contained approx. 70% of their phosphorylase in the active form. Fig. 2 shows that, on incubation of these cells at 37°C, the enzyme was progressively inactivated and this inactivation was markedly stimulated by glucose. Glycogen synthetase was initially mostly (about 90%) in the *b* form and became activated only if and when a considerable proportion of the phosphorylase had been inactivated.

Fig. 3 shows that hepatocytes isolated from rats that had been deprived of food had a lower initial content of phosphorylase *a* and that on incubation of the cells at 37°C this phosphorylase *a* was converted into *b* at a rate not markedly different from that observed in cells from fed rats. However, since the initial amount of phosphorylase *a* was low, the reaction was more rapidly terminated, reaching a plateau at which the value was inversely proportional to glucose concentration. At all glucose concentrations this plateau was two to three times lower than in cells from fed rats. Glycogen synthetase was initially more active than in cells from fed rats. It became activated after a short lag that corresponded to the time required for inactivation of phosphorylase.

At all glucose concentrations, this activation was larger than in cells from fed rats. This behaviour was observed in the hepatocytes obtained from rats that had been deprived of food for only 12h, if the glycogen concentration in their livers was lower than 100–120 μmol of glucose equivalent/g of protein.

Effect of K^+

All the experiments with isolated liver cells described in the preceding section were performed in the presence of an incubation medium in which Na^+ was the predominant cation. Figs. 4 and 5 show that when Na^+ ions were replaced by K^+ the rate of conversion of glucose into glycogen was greatly increased. This effect was particularly striking at low concentrations (up to 14mM), since under these conditions almost no synthesis occurred in the Na^+ medium. Fig. 4 shows that the effect of K^+, like that of glucose, was both to shorten the lag that precedes the synthesis of labelled glycogen and to increase the rate of this synthesis. Comparison of Figs. 5(*a*)

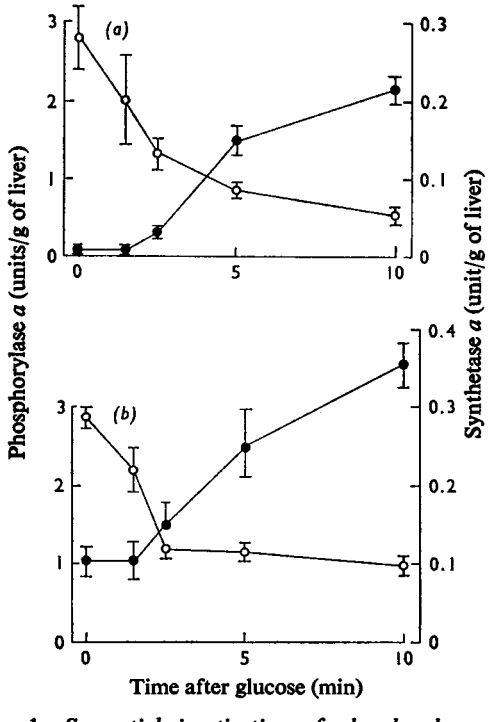

Fig. 1. *Sequential inactivation of phosphorylase and activation of synthetase in perfused liver from fed rats (a) and rats deprived of food for 30h (b)*

At zero time, glucose concentration was raised from 5.5 to 55mM. Values shown are means±S.E.M. of three (*a*) and four (*b*) experiments. Activity of phosphorylase *a* (○); activity of synthetase *a* (●).

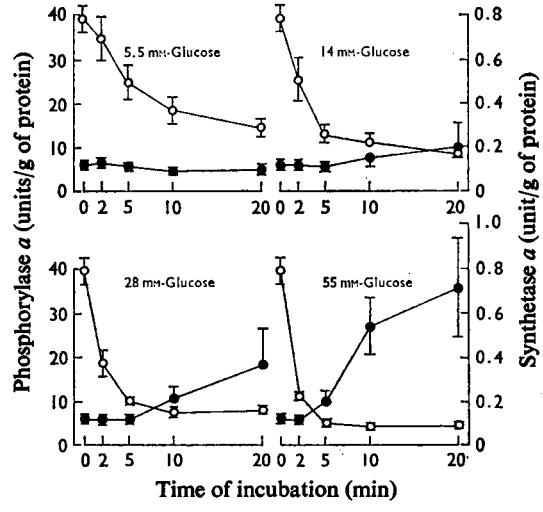

Fig. 2. *Sequential inactivation of phosphorylase and activation of synthetase in hepatocytes isolated from fed rats*

Liver cells were incubated at 37°C in the presence of glucose as indicated. Values shown are means±S.E.M. of six experiments. Symbols are as in Fig. 1.

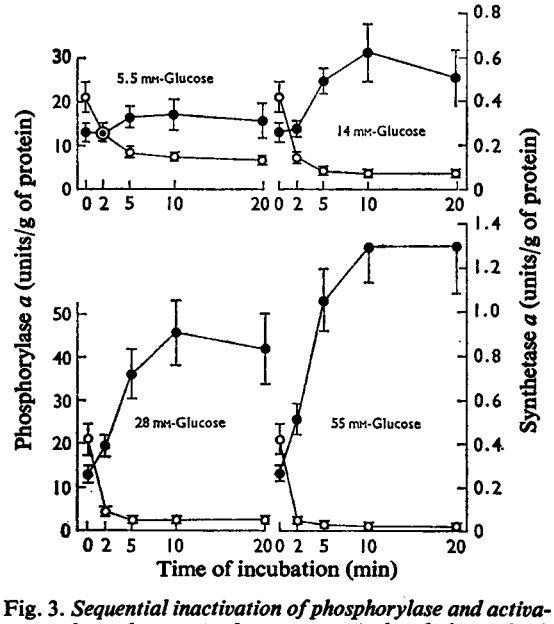

Fig. 3. *Sequential inactivation of phosphorylase and activation of synthetase in hepatocytes isolated from food-deprived rats*

Rats were deprived of food for 12–30h. The glycogen content of their livers was below 100–120 μmol of glucose equivalent/g of protein. Values shown are means±S.E.M. of eight experiments. Symbols are as in Fig. 1.

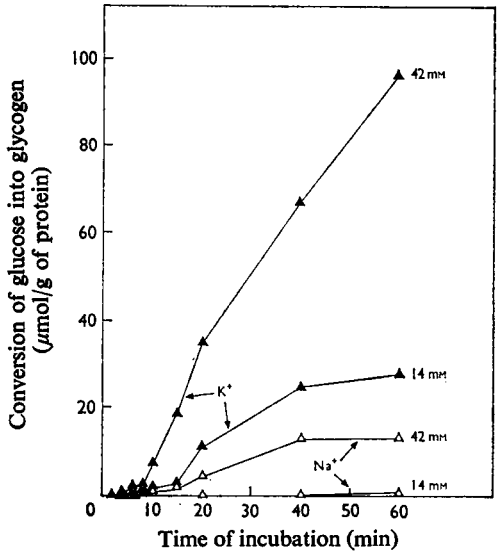

Fig. 4. *Effect of K^+ on the time-course of conversion of glucose into glycogen in hepatocytes isolated from fed rats*

Cells were incubated in the Na^+ (△) or in the K^+ (▲) medium containing either 14mM- or 42mM-[U-^{14}C]glucose as indicated.

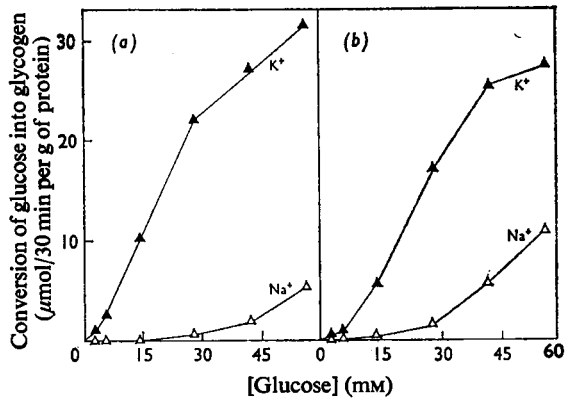

Fig. 5. *Effect of glucose concentration on conversion of glucose into glycogen in cells incubated in the Na^+ or in the K^+ media*

Cells were incubated for 30min at 37°C. Symbols are as in Fig. 4. (*a*) Cells from fed rats; (*b*) cells from rats deprived of food.

and 5(*b*) confirms that cells from rats that had been deprived of food were more sensitive to glucose than were cells from fed rats in the Na^+ medium; this difference was not apparent in the K^+ medium.

Figs. 6(*a*) and 6(*b*) show that the effect of K^+ on conversion of glucose into glycogen was secondary to a more rapid and therefore more complete inactivation of phosphorylase. Indeed at 14mM-glucose the initial rate of inactivation of phosphorylase increased continuously with increasing K^+ concentration in the medium, reaching an optimum at 140mM-K^+. In contrast, the activation of glycogen synthetase (Fig. 6*b*) and the incorporation of labelled glucose into glycogen (Fig. 6*a*) occurred only above 80mM-K^+. At lower K^+ values the concentration of phosphorylase *a* remained, within the 20min of the experiment, sufficiently elevated to maintain synthetase phosphatase completely inactive and consequently no activation of synthetase occurred. The same general mechanism explains the lag that precedes the incorporation of glucose into glycogen (see Fig. 4).

Further analysis of the glucose effect

Similar causal links in the control of glycogen metabolism by glucose are shown in Fig. 7. Advantage was taken of the fact that the sensitivity of the system to glucose is modulated by K^+ concentration. At 80mM-K^+ radioactive glycogen was formed and the initial rate of phosphorylase inactivation could be measured with accuracy, over a wide range of glucose concentration. It appears that increasing glucose concentration caused an almost proportional increase in the rate of phosphorylase inactivation up to a concentration of 20mM. Accordingly, the residual amount of phosphorylase *a* at 20min decreased continuously and glycogen synthesis occurred only above a threshold glucose value which can itself be related to this residual amount of phosphorylase *a*. At very high glucose concentration the effect of glucose on the initial rate of phosphorylase inactivation was presumably underestimated, because this inactivation was too fast and not linear with time. The very low residual phosphorylase activity at 20min indicates, however, that the large amount of labelled glycogen formed at 45mM-glucose was secondary to a higher rate of phosphorylase inactivation.

The results shown in Figs. 4–7 are illustrative of a series of experiments which gave consistently the same type of results with, however, some quantitative variation with regard to the magnitude of glycogen synthesis (compare, for instance, Fig. 4 with Fig. 5).

Influence of the concentration of phosphorylase a on the rate of activation of glycogen synthetase

The inhibition of synthetase phosphatase by phosphorylase *a in vitro* (Stalmans *et al.*, 1971) and the absence of synthetase activation *in vivo*, in livers in which phosphorylase *a* is present at a concentration above threshold (Stalmans *et al.*,

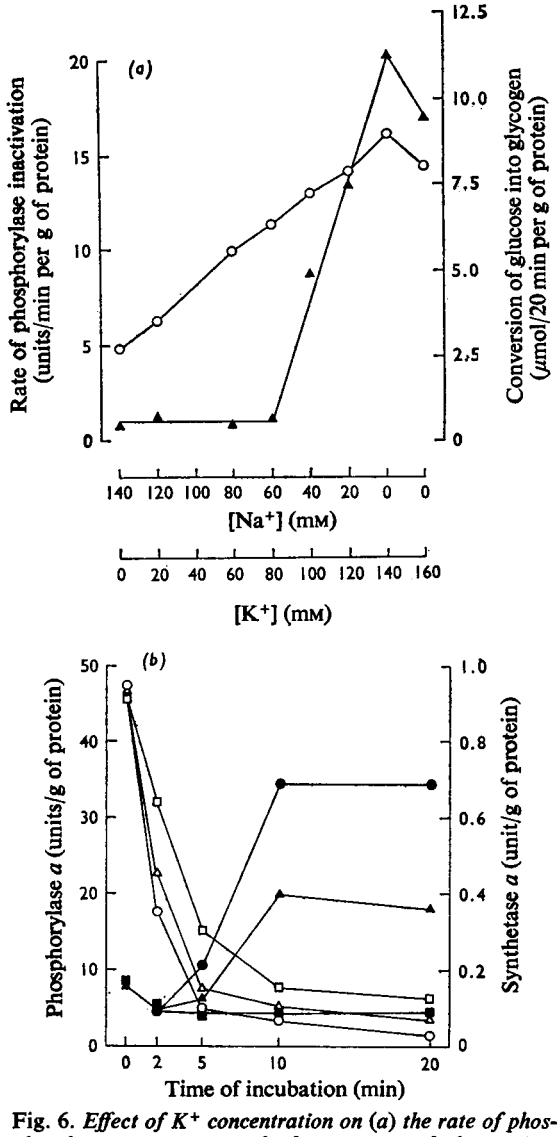

Fig. 6. *Effect of K^+ concentration on (a) the rate of phosphorylase inactivation and of conversion of glucose into glycogen and (b) the sequential changes in enzyme activities*

Hepatocytes from fed rats were suspended in either the Na^+ or the K^+ medium at room temperature and mixed in different proportions to obtain the concentrations of Na^+ and K^+ indicated. They were then incubated at 37°C in the presence of 14mM-[U-^{14}C]glucose. (*a*) The rate of inactivation of phosphorylase *a* (○) was measured by the amount of enzyme inactivated during the first minute of incubation at 37°C; radioactive glycogen (▲) was measured at 20min. (*b*) The sequential changes of activities of phosphorylase *a* (open symbols) and of synthetase *a* (filled symbols) were measured in three ionic conditions: (○, ●), $K^+ = 140$mM; (△, ▲), $K^+ = 100$mM and $Na^+ = 40$mM; (□, ■), $K^+ = 60$mM and $Na^+ = 80$mM). Results shown in (*a*) and (*b*) were obtained within the same experiment.

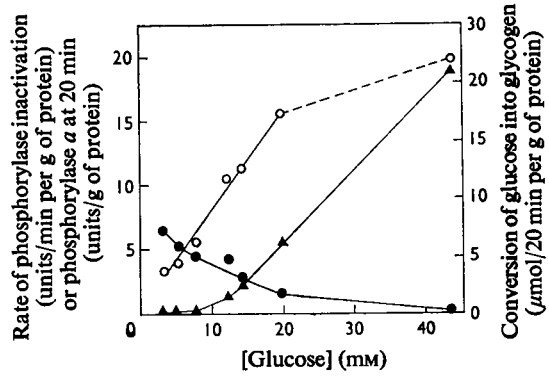

Fig. 7. *Effect of glucose concentration on the rate of phosphorylase a inactivation and of conversion of glucose into glycogen*

Hepatocytes from fed rats were incubated for 20min at 37°C in a medium containing 60mM-Na^+, 80mM-K^+ and glucose at the concentration indicated. The rates of inactivation of phosphorylase *a* (○) and of conversion of glucose into glycogen (▲) were measured as in Fig. 6. The activity of phosphorylase *a* at the end of the 20min incubation period (●) was also recorded.

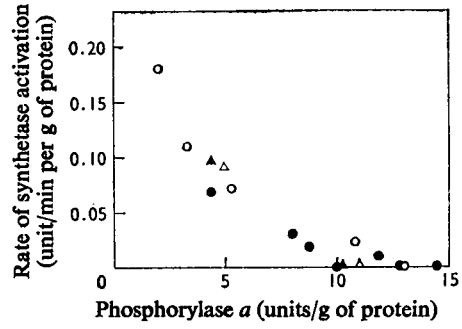

Fig. 8. *Correlation between the rate of activation of glycogen synthetase and the activity of phosphorylase a*

Rates of synthetase activation were calculated as indicated in the text from data of Figs. 1(*a*) (▲), 1(*b*) (△), 2 (●) and 3 (○).

1974*a*, Curnow *et al.*, 1975) is well documented. Figs. 1, 2, 3 and 6(*b*) show that three periods can be recognized in the activation of glycogen synthetase in isolated liver preparations. The first is the lag period, during which the concentration of phosphorylase *a* is so high that no activation is possible. The second starts when the concentration of phosphorylase *a* has passed below the threshold value at which

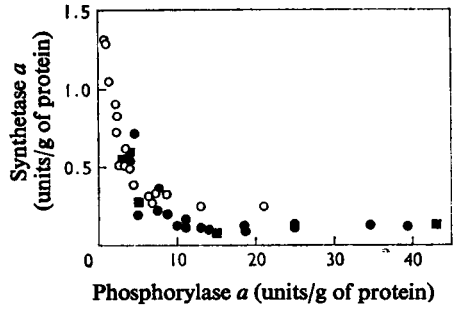

Fig. 9. *Correlation between the activity of synthetase a and of phosphorylase a in isolated hepatocytes*

Values of synthetase *a* shown in Figs. 2 (●) and 3 (○) have been plotted versus the corresponding values of phosphorylase *a*. Results of experiments similar to those shown in Fig. 2 but performed in the K^+ medium are also included (■).

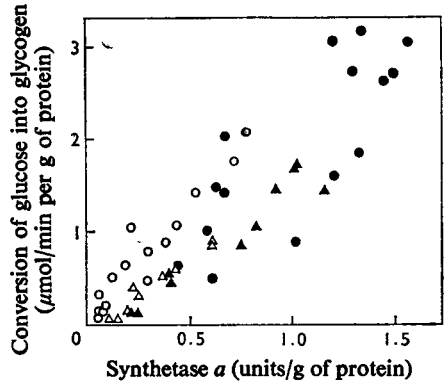

Fig. 10. *Correlation between the rate of conversion of glucose into glycogen and the activity of synthetase a*

Cells isolated from fed (○,●) or food-deprived (△, ▲) rats were incubated in the presence of various concentration of glucose in either a Na^+ (○, △) or K^+ (●, ▲) medium. After 18min of incubation at 37°C, a sample was taken for the assay of synthetase *a* and a trace amount (1 μCi) of [U-^{14}C]glucose was added to the medium; the cells were further incubated for 5min and the radioactive glycogen was isolated and counted.

the inhibition ceases to be complete; the activation starts slowly and becomes faster if phosphorylase is further inactivated. In the third period, the activation of the synthetase becomes slower and finally reaches a plateau, presumably because synthetase phosphatase is balanced by synthetase kinase. Fig. 8 shows a plot of the rate of activation of glycogen synthetase in periods 1 and 2 versus the activity of phosphorylase *a*. It appears that little or no activation occurred when the activity of phosphorylase *a* was higher than 10 units/g of protein and that, below this threshold value, the rate of activation was inversely proportional to the activity of phosphorylase *a*. This phenomenon is apparent in experiments with perfused livers and in those with isolated hepatocytes.

Fig. 9 shows the inverse correlation that exists between the activities of phosphorylase *a* and synthetase *a* in isolated hepatocytes. With regard to the preparations from fed rats, the relationship is essentially similar to that observed by Stalmans *et al.* (1974*a*) for the liver of fed mice *in vivo*. In contrast, the preparations from animals that had been deprived of food displayed a relatively high activity of glycogen synthetase before phosphorylase *a* started to be inactivated.

Glycogen synthesis and glycogen breakdown

Since the rate of conversion of glucose into glycogen in the liver *in vivo* is known to be proportional to the concentration of synthetase *a* in that tissue (De Wulf & Hers, 1968), we have tried to establish a similar correlation in isolated hepatocytes. For this, the conversion of glucose into glycogen was measured during short periods, by adding a trace amount of radioactive glucose to the incubation medium for the last 5min of experiments similar to those shown in Figs. 2, 3 and 6(*b*). It was expected that the changes in synthetase *a* activity would be minimal during this period. Fig. 10 shows that the rate of conversion of glucose into glycogen correlated satisfactorily with the activity of synthetase *a*; 1 unit of enzyme (measured at 20°C) allowed the incorporation of about 2 μmol of glucose into glycogen by the cells incubated at 37°C, a value which is easily explained by the temperature difference (Devos & Hers, 1974).

Cells obtained from food-deprived rats contained very little glycogen at the beginning of the incubation period [30±7.7 (S.E.M., $n=11$) μmol of glucose equivalent/g of protein], and the conversion of labelled glucose into glycogen in these cells corresponded to an equivalent increase in glycogen content. This was not the case for cells obtained from fed rats; their glycogen content was equal to 698±109 (S.E.M., $n=10$) μmol of glucose equivalent/g of protein and decreased at a rate close to 4.25±0.66 (S.E.M., $n=5$) μmol of glucose equivalent/min per g of protein in the presence of 5.5mM-glucose, so that incorporation of labelled glucose into the polysaccharide was in most cases simultaneous with a net decrease in glycogen content. Preliminary ex-

periments indicated that this degradation is not correlated with the concentration of phosphorylase *a* in the cells (not shown).

Discussion

Control of glycogen metabolism by glucose

The similarities between the enzymic changes induced by a glucose load in isolated liver preparations (the present paper) and in the liver of rats *in vivo* (Stalmans *et al.*, 1974*a*) indicate that isolated preparations may be used with confidence in investigation of glycogen metabolism. Isolated hepatocytes have the great advantage over the perfused liver in giving a much greater amount of information from a single experiment.

The fundamental significance of the sequential inactivation of glycogen phosphorylase and activation of glycogen synthetase initiated by glucose has been extensively discussed in previous publications from this laboratory (Stalmans *et al.*, 1974*a*,*b*; see the introduction). It is enough to recall that this sequence of events is the biochemical expression of the concept of hepatic threshold to glucose, initially proposed by Soskin (1940). Since this sequence has now been observed in isolated cell preparations, free of hormonal interactions, one can conclude, once more, that the glucose effect is not mediated by insulin. The threshold for glucose is particularly apparent in Fig. 7, where it is shown that increasing glucose concentration caused a progressive increase in the rate of phosphorylase inactivation, even at low glucose concentrations, whereas labelled glycogen was formed only above a threshold glucose concentration; this threshold is the glucose concentration at which phosphorylase inactivation was fast enough to stabilize the concentration of phosphorylase *a* below the point where it completely inhibits synthetase phosphatase. Further, Fig. 8 shows clearly that the rate of synthetase activation is modulated by the residual amount of phosphorylase *a* which remains in the cell after the initial phase of rapid inactivation. In contrast, the great differences in the rate of synthetase activation that were observed at different times of the same experiment could not be related to the concentration of glucose, which remains about the same over the whole experimental period. The glucose effect therefore cannot be explained by a hypothetical release by the hexose of the inhibition of synthetase phosphatase by ATP, as was proposed by Gilboe & Nuttall (1973).

The higher sensitivity of cells from rats deprived of food to glucose is also of interest and is in agreement with the rapid rates of glycogen synthesis known to occur *in vivo* in the livers of food-deprived rats on re-feeding (Cori, 1926; Friedmann *et al.*, 1963). Again, the rapid activation of glycogen synthetase appears secondary to an extreme inactivation of phosphorylase, which stabilized at a plateau two to three times lower than in cells from fed rats. The reason for this difference between cells from fed and food-deprived rats is not clear. It seems to be related to a low glycogen content, since it was already observed after a few hours of starvation, provided that the glycogen concentration of the cells was below 0.5%. In the livers of rats deprived of food, there was also a higher initial content of synthetase *a*, in agreement with the report by Curnow & Nuttall (1973). This suggests that, in these livers, synthesis of glycogen could operate at least at a low rate, simultaneously with degradation. Such a recycling of glucose may be useful for the cell in preventing a complete disappearance of glycogen molecules during starvation.

Effect of K^+

Our observation that increasing the concentration of K^+ in the incubation medium greatly increased the rate of glucose incorporation into glycogen and the rate of inactivation of phosphorylase, and also decreased the activity at which phosphorylase *a* tends to stabilize, confirms previous work by Cahill *et al.* (1957) with liver slices. Our work establishes a link between the concentration of phosphorylase *a* in the cells and the rate of glycogen synthesis. This link is the control of synthetase activation by phosphorylase *a*. Indeed, similarly to what was discussed for the glucose effect, the activation of glycogen synthetase in the presence of K^+ is secondary to the inactivation of phosphorylase. Here also, the initial rate of phosphorylase inactivation increased proportionally with increasing K^+ concentration, and this effect could be noticed even at low K^+ concentrations, at which no labelled glycogen was formed; in contrast, glycogen was formed from glucose only above the threshold concentration of K^+ that allowed inactivation of phosphorylase to the point where it no longer inhibited synthetase phosphatase (Fig. 6).

It is remarkable that the sensitivity of the hepatocytes to glucose can be modulated by the external K^+ concentration (Figs. 5 and 6) and vice versa. In this respect, increasing K^+ concentration mimics the effect of the endocrine balance which, according to Soskin (1940), controls the threshold for glucose. The K^+ effect is actually the one that would be expected from insulin. It is therefore worth while to recall that glucagon and adrenaline cause an extrusion of K^+ from the liver (see Friedmann, 1972), whereas insulin has the reverse effect (Mortimore, 1961).

The primary effect of a high K^+ concentration on liver cells is not clear. Hastings & Buchanan (1942) incubated liver slices in a K^+-rich medium to compensate for the loss of K^+ from this preparation. Iso-

lated liver cells are also known to have lost about 60% of their K^+ (Berry & Friend, 1969). It is remarkable therefore that an increase in the rate of phosphorylase inactivation was already observed at 20mM-K^+ (Fig. 6). Since this concentration is lower than that existing in the cells, one cannot make the simple assumption that K^+ diffuses freely into the cell. The situation is further complicated by the report by Frimmer & Kroker (1973) that K^+ is excreted from the perfused liver at high external K^+ concentration. Dambach & Friedmann (1974) have observed that perfusing rat livers with a medium containing 100mM-K^+ decreased the glucose production by 50% and slightly increased the concentration of K^+ in the tissue.

Assuming that increasing external K^+ causes an increase in intracellular K^+ with a concomitant decrease in Na^+, it would be of primary importance to establish what reaction is regulated by these ions. A faster inactivation of phosphorylase could be due to stimulation of phosphorylase phosphatase or to an inhibition of phosphorylase kinase or of phosphorylase kinase kinase.

Glycogen synthesis and glycogen breakdown in isolated liver cells

As shown in Fig. 10, there is a clear correlation between the amount of synthetase *a* in the cells and the rate of incorporation of radioactive glucose into glycogen as measured during a 5min incubation period. The rates of incorporation that we observed are similar to those reported by Cahill *et al.* (1957) with liver slices. The general agreement between our work and that of the latter authors indicates that the low concentration of ATP known to exist in liver slices (Krebs, 1970) does not result in a major impairment of glycogen synthesis.

In a few preliminary experiments we have observed that cells often produce glucose from glycogen even at very low concentrations of phosphorylase *a*. This observation suggests that an unknown mechanism of glycogenolysis, possibly involving α-amylase, α-glucosidase or phosphorylase *b*, might operate in liver cells incubated *in vitro* or, at least, in some damaged cells. This predominance of glycogenolysis in isolated liver preparations was previously reported by others (Berthet *et al.*, 1956; Cahill *et al.*, 1957; Wagle *et al.*, 1973; Seglen, 1974). An extensive investigation of this problem is outside the scope of the present work.

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