Stimulation of Phosphoenolpyruvate Carboxykinase (Guanosine Triphosphate) Activity by Low Concentrations of Circulating Glucose in Perfused Rat Liver

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1. After nicotinic acid treatment, rat liver glycogen is depleted and phosphoenolpyruvate carboxykinase activity increased, to about twice the initial value. 2. The increase in phosphoenolpyruvate carboxykinase activity promoted by nicotinic acid is prevented by cycloheximide or actinomycin D, suggesting that this effect is produced by synthesis of the enzyme *de novo*. 3. Despite the enhancement of phosphoenolpyruvate carboxykinase activity and glycogen depletion, which occurs 5 h after the injection of nicotinic acid, the gluconeogenic capacity of liver is low and considerably less than the values found in rats starved for 48 h. 4. When the livers of well-fed rats are perfused in the presence of low concentrations of glucose, the activity of phosphoenolpyruvate carboxykinase significantly increases compared with the control. 5. This increase is not related to the glycogen content, but seems to be also the result of synthesis of the enzyme *de novo*, since this effect is counteracted by previous treatment with cycloheximide or actinomycin D. 6. Phosphoenolpyruvate carboxykinase activity is not increased in the presence of low concentrations of circulating glucose when 40 mM-imidazole (an activator of phosphodiesterase) is added to the perfusion medium. 7. Addition of dibutyl cyclic AMP to the perfusion medium results in an increase in phosphoenolpyruvate carboxykinase activity, in spite of the presence of normal concentrations of circulating glucose. On the other hand, the concentration of cyclic AMP in the liver increases when that of glucose in the medium is low. 8. These results suggest that, in the absence of hormonal factors, the regulation of phosphoenolpyruvate carboxykinase can be accomplished by glucose itself, inadequate concentrations of it resulting in the induction of the enzyme. The mediator in this regulation, as in hormonal regulation, seems to be cyclic AMP.

The activity of phosphoenolpyruvate carboxykinase [GTP-oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32], a key enzyme of gluconeogenesis, is very sensitive to hormonal and nutritional treatment, in both liver and kidney (Lardy *et al.*, 1964; Weber *et al.*, 1965; Seubert & Huth, 1965; Scrutton & Utter, 1968; Usatenko, 1970).

The activity of the hepatic enzyme is increased by administration of dibutyl cyclic AMP *in utero* or to prematurely delivered rats (Yeung & Oliver, 1968), as well as to intact rats (Wicks *et al.*, 1969), strongly suggesting that cyclic AMP is the intracellular mediator of the action of glucagon and adrenaline on phosphoenolpyruvate carboxykinase synthesis.

The hormonal initiation of liver gluconeogenesis through enhancement of phosphoenolpyruvate carboxykinase activity is well established (Ballard & Hanson, 1967; Yeung & Oliver, 1967; Ashmore &

Weber, 1968; Yeung & Oliver, 1968; Philippidis & Ballard, 1970; Exton *et al.*, 1971). The available data are consistent with a hormone-mediated repression of phosphoenolpyruvate carboxykinase by glucose (Foster *et al.*, 1966; Shrago *et al.*, 1967; Yeung & Oliver, 1968), which is antagonized by dibutyl cyclic AMP (Treadow & Khairallah, 1972). On the other hand, several authors have reported an inverse relationship between glycogen content and phosphoenolpyruvate carboxykinase activity under certain conditions (Foster *et al.*, 1966; Phillips & Berry, 1970a; Lane & Mavrides, 1970).

In a previous paper (Sánchez-Medina *et al.*, 1971), we reported an increase in rat liver phosphoenolpyruvate carboxykinase activity after nicotinic acid administration, which was attributed to the simultaneous depletion of the liver glycogen store. As the primary aim of the present work is to elucidate the relationship between the increase in phosphoenolpyruvate carboxykinase activity and the glycogen depletion produced by nicotinic acid treatment, the time-course of both phenomena and the nature of the

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enhancement in phosphoenolpyruvate carboxykinase activity have been investigated. Moreover, the gluconeogenic capacity of rat liver after nicotinic acid treatment has been investigated.

To examine the role of hormones in the regulation of liver phosphoenolpyruvate carboxykinase activity, we have also studied its variations *in vitro* by using an isolated rat liver system. Although the regulatory role of hormones in physiological conditions is not questioned, our results *in vitro* indicate the existence of a direct non-hormonal increase in phosphoenolpyruvate carboxykinase activity produced by 'hypoglycaemia'.

Experimental

Reagents

Nicotinic acid, imidazole, cycloheximide, actinomycin D, dibutyryl cyclic AMP and glucose oxidase were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The other enzymes used were purchased from Boehringer (Mannheim, Germany). Bovine serum albumin powder (fraction V) was obtained from Armour Pharmaceutical Co. (Eastbourne, Sussex, U.K.). Standard analytical-grade laboratory reagents and coenzymes were obtained from Sigma, Merck (Darmstadt, Germany) or Boehringer. Sodium pentobarbital was a gift from Abbott Laboratories (Madrid, Spain).

Treatment of animals

Fed female rats of the Wistar strain were used. All agents were injected intraperitoneally, the controls being treated with 0.9% NaCl solution. Nicotinic acid was given in neutral solution (500 mg/kg body wt.) 5 h before killing, except in the time-course experiments. Actinomycin D (100 µg/100 g body wt.) was administered ½ h before the nicotinic acid. Cycloheximide (1 mg/rat) was injected along with the nicotinic acid. The injection was repeated 2.5 h later. In all cases, the animals were deprived of food during the experiment, but allowed to take water *ad libitum*. Rats were killed by cervical dislocation, with minimal stress on the animals, and the livers were then removed and used to determine the glycogen content and phosphoenolpyruvate carboxykinase activity, as described below.

Analytical methods

Glycogen was determined as glucose after enzymic hydrolysis, as described by Krebs *et al.* (1963). Blood glucose was assayed by the glucose oxidase method as described by Krebs *et al.* (1963, 1964). Blood samples were collected in a heparinized tube after cutting the jugular vein.

Cyclic AMP was determined by the method of Gilman (1970). Phosphoenolpyruvate carboxykinase activity was measured spectrophotometrically in the direction of oxaloacetate synthesis in the presence of an excess of malate dehydrogenase at 30°C, as described previously (Sánchez-Medina *et al.*, 1971). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Lactate dehydrogenase was measured by the method of Bergmeyer *et al.* (1965). Glucose 6-phosphatase was determined as described by Harper (1965) and fructose diphosphatase as described by Pontremoli *et al.* (1965), by using 0.04 M-glycine buffer, pH 9.4.

Perfusion technique

The perfusion method was as described by Hems *et al.* (1966). The perfusate (150 ml) consisted of Krebs-Henseleit physiological saline (Krebs & Henseleit, 1932), bovine serum albumin powder (fraction V) previously filtered (in Krebs-Henseleit physiological saline) through Millipore filters (1.2 µm pore size), and washed human erythrocytes stored for 30 days at 4°C in citrate-dextrose anticoagulant solution (Don Baxter, Glendale, Calif., U.S.A.).

In the experiments where cyclic AMP was measured, liver samples were taken at 38, 70 and 120 min and immediately frozen in liquid N₂. The powder was then treated as described by Gilman (1970).

Results

Time-course of the effect of nicotinic acid treatment on blood glucose, liver glycogen content and liver phosphoenolpyruvate carboxykinase activity

Nicotinic acid treatment causes a mobilization of glucose from the liver glycogen store (Ammon & Estler, 1967), owing to its antilipolytic effect (Carlson & Nye, 1966). As shown in Fig. 1, injection of nicotinic acid led to a rapid depletion of liver glycogen; it was scarcely detectable after 5 h. Simultaneously, there was an increase in the phosphoenolpyruvate carboxykinase activity, which was already detectable after 1 h, and doubled in value after 5 h. On the other hand, blood glucose fell during the first 2 h, but then remained fairly constant during the last 3 h (Fig. 1).

Effect of actinomycin D and cycloheximide on the increase in phosphoenolpyruvate carboxykinase activity produced by nicotinic acid treatment

To establish whether the changes in phosphoenolpyruvate carboxykinase activity after injection of nicotinic acid were related to the synthesis of the enzyme *de novo*, two inhibitors of protein synthesis

Fig. 1. Time-course of the effect of nicotinic acid treatment on blood glucose (○), liver glycogen (□) and liver phosphoenolpyruvate carboxykinase activity (●)

Nicotinic acid was injected intraperitoneally in neutral solution (500mg/kg body wt). The results are means of four experiments. The S.E.M. values are indicated by error bars.

Table 1. Effect of actinomycin D and cycloheximide on the increase in phosphoenolpyruvate carboxykinase activity produced by nicotinic acid treatment

All agents were intraperitoneally injected, the controls being treated with 0.9% NaCl. Nicotinic acid was given in neutral solution (500mg/kg body wt.) 5h before killing. Actinomycin D (100μg/100g body wt.) was administered 30min before nicotinic acid. Cycloheximide (1mg/rat) was injected at the same time as nicotinic acid and again 2½h later. Enzyme activity is expressed in nmol of oxaloacetate formed at 30°C/min per mg of protein. The results are means ± S.E.M.

Experimental conditions	No. of experiments	Phosphoenolpyruvate carboxykinase activity
Control	12	20.8 ± 0.7
Actinomycin D	4	28.3 ± 1.1
Cycloheximide	5	20.4 ± 0.5
Nicotinic acid	6	43.8 ± 2.7
Actinomycin D + nicotinic acid	4	28.8 ± 1.1
Cycloheximide + nicotinic acid	5	22.2 ± 0.5

(actinomycin D and cycloheximide) were used. Actinomycin D is known to decrease protein synthesis by inhibiting the formation of mRNA (Goldberg *et al.*, 1962), and cycloheximide inhibits by blocking the transfer of amino acids from tRNA (Ennis & Lubin, 1964).

Both actinomycin D and cycloheximide counteracted the stimulatory effect of the nicotinic acid injection on liver phosphoenolpyruvate carboxykinase activity (Table 1), suggesting that, under the experimental conditions, the increase in phosphoenolpyruvate carboxykinase activity is due to the synthesis of the enzyme *de novo*.

Gluconeogenic capacity of the liver from rats previously treated with nicotinic acid

Krebs *et al.* (1966) reported that the gluconeogenic capacity is inversely related to the amount of the glycogen store in mouse liver slices. On the other hand, the close relationship between phosphoenolpyruvate carboxykinase activity and gluconeogenic capacity is well established (see above). Since after 5h of nicotinic acid treatment, the liver glycogen store is almost completely depleted and the phosphoenolpyruvate carboxykinase activity is greatly increased, it was decided to investigate the gluconeogenic ability of liver from rats previously treated with nicotinic acid. Under these conditions, the production of glucose from 10mm-L-lactate in perfused rat liver was rather low (0.39 μmol/min per g wet wt. of liver) when compared with the gluconeogenic capacity of the starved (48h) rats (1.07 μmol/min per g wet wt. of liver).

Liver glycogen content, concentrations of glucose in the perfusate and phosphoenolpyruvate carboxykinase activity in perfused rat liver

As indicated above, the induction of phosphoenolpyruvate carboxykinase in response to hypoglycaemia is mediated by hormones. The rhythmic inverse relationship between glycogen content and phosphoenolpyruvate carboxykinase activity reported in mouse and rat liver (Phillips & Berry, 1970a; Lane & Mavrides, 1970) seems also to be under hormonal control (Phillips & Berry, 1970a). To examine the possible involvement of non-hormonal factors in the regulation of phosphoenolpyruvate carboxykinase, the influence of the liver glycogen and circulating glucose concentrations on the activity of the enzyme in perfused rat liver has been tested.

Livers of fed rats were perfused without any addition to the medium and in the presence of 10 and 17.3mm-glucose. According to Ross *et al.* (1967), a substantial loss of glucose (about 700 μmol) is expected to occur in the first situation, whereas the glycogen depletion should be considerably less in the presence of glucose. As shown in Table 2, the circulating concentrations of glucose were high when livers were perfused with 10 and 17.3mm-glucose, reaching

Table 2. *Liver phosphoenolpyruvate carboxykinase activity after perfusion under several conditions*

Livers of well-fed rats (a) not treated, (b) treated by intraperitoneal injection of cycloheximide (1 mg/rat) 30 min before perfusion, or (c) treated by intraperitoneal injection of actinomycin D (100 µg/100 g body wt.) 3 h before perfusion, were perfused for 130 min. The concentration of glucose oxidase was 50 mg in the total perfusion medium. Phosphoenolpyruvate carboxykinase activity was assayed in liver samples taken at the end of the perfusions. Enzyme activity is expressed in nmol of oxaloacetate formed at 30°C/min per mg of protein. The results are means ± S.E.M.

Experimental conditions	No. of observations	Time (min)	Glucose found (µmol in total perfusion medium)			Final enzyme activity
			0	85	130	
Addition of 17.3 mm-glucose	19		2640 ± 152	2410 ± 129	1940 ± 196	14.9 ± 0.2
Addition of 10 mm-glucose	4		1517 ± 159	1818 ± 59	1797 ± 125	13.8 ± 0.9
No addition	14		47 ± 12	807 ± 41	941 ± 39	14.3 ± 0.5
Addition of glucose oxidase	15		27 ± 5	56 ± 8	55 ± 6	23.6 ± 1.1
No addition: change of medium at 70 min	3		26 ± 5	55 ± 6	244 ± 58	19.8 ± 0.5
No addition: change of medium at 30 and 60 min	6		45 ± 10	175 ± 14	353 ± 28	24.6 ± 1.4
Addition of glucose oxidase: change of medium at 70 min	4		32 ± 6	69 ± 6	70 ± 12	21.5 ± 1.6
No addition: cycloheximide-treated	4		40 ± 1	816 ± 96	947 ± 120	15 ± 0.4
Addition of glucose oxidase: cycloheximide-treated	11		29 ± 3	46 ± 4	41 ± 4	14.6 ± 0.7
No addition: change of medium at 30 and 60 min: cycloheximide-treated	8		28 ± 3	125 ± 7	397 ± 67	12.8 ± 1.2
No addition: actinomycin D-treated	6		42 ± 3	824 ± 43	955 ± 56	11.2 ± 0.7
Addition of glucose oxidase: actinomycin D-treated	6		28 ± 4	55 ± 5	54 ± 6	12.3 ± 0.9
Addition of 40 mm-imidazole	4		29 ± 7	954 ± 119	1215 ± 84	11.6 ± 0.7
Addition of glucose oxidase and 40 mm-imidazole	12		30 ± 2	118 ± 14	49 ± 6	10.7 ± 0.4

a common final concentration of about 12–13 mm. There was a slight increase in perfusate glucose, corresponding to the glucose lost from liver glycogen during perfusion with 10 mm-glucose; glucose in the medium decreased, however, during perfusion with 17.3 mm-glucose, probably indicating formation of glycogen. When no glucose was added to the perfusion medium, there was a continuous loss of glucose (up to about 1 mmol).

The glycogen content in the three conditions (determined at the end of each experiment) was highly variable, ranging from negligible values to near 200 µmol (measured as glucose equivalents)/g wet wt. of liver. These results could be expected, as it is well known that the liver glycogen store exhibits wide fluctuations in well-fed animals. The final phosphoenolpyruvate carboxykinase activity was very similar in all cases, and somewhat lower than the normal values obtained in intact rats (20.2 ± 0.5) (Table 2).

Concentrations of glucose in the perfusate lower than the physiological range were obtained in different ways: changing the medium once (at 70 min) or twice (at 30 and 60 min), adding glucose oxidase (50 mg) at the beginning of the perfusion, or both. The changing of the medium leads to varying concen-

trations of circulating glucose, always under 3 mm. The addition of glucose oxidase leads to the consumption of most of the glucose in the perfusate, the resulting concentration of glucose being about 0.5 mm (Table 2). Glucose oxidase did not produce any significant alteration in the properties of the perfusate, which possesses a high catalase activity. The concentration of haemoglobin was the same both before and after the experiments.

The glycogen content in all of these conditions was quite variable also, oscillating between 0 and 120 µmol/g wet wt. These smaller values were expected because of the loss of glucose from the liver, induced by the low concentration of circulating glucose. The values of phosphoenolpyruvate carboxykinase activity were, in all cases, significantly higher ($P < 0.001$) than those obtained from livers perfused without addition or in the presence of 10 and 17.3 mm-glucose (Table 2). Therefore these results clearly indicate that phosphoenolpyruvate carboxykinase activity is enhanced *in vitro* under 'hypoglycaemic' conditions. Lactate dehydrogenase, fructose diphosphatase and glucose 6-phosphatase were determined simultaneously and no increase was found in their activities (Table 3).

Table 3. Lactate dehydrogenase, fructose diphosphatase and glucose 6-phosphatase activities in livers of fed rats after perfusion with glucose oxidase

Lactate dehydrogenase activity is expressed in μmol of pyruvate transformed/min per mg of protein at 25°C . Fructose diphosphatase activity is expressed in nmol of fructose diphosphate transformed/min per mg of protein at 22°C . Glucose 6-phosphatase activity is expressed in μmol of phosphate formed/min per g wet wt. of liver at 37°C . The measurements were made after 130 min of perfusion. The concentration of glucose oxidase was 50mg in the total perfusion medium. The results are means $\pm$ s.e.m. Statistical significance of differences compared with the controls: $*P < 0.05$.

Additions to the perfusion medium	No. of experiments	Lactate dehydrogenase activity	Fructose diphosphatase activity	Glucose 6-phosphatase activity
None	3	0.51 ± 0.06	34.80 ± 1.94	8.64 ± 0.28
Glucose oxidase	4	0.47 ± 0.03	30.37 ± 3.10	$6.82 \pm 0.43^*$

Involvement of cyclic AMP in the stimulation of phosphoenolpyruvate carboxykinase by 'hypoglycaemia' in perfused rat liver

As shown in Table 2, when rats were previously treated with cycloheximide and actinomycin D, the values of phosphoenolpyruvate carboxykinase activity after perfusion in the presence of glucose oxidase or after changing the medium remained low. Therefore the increase in activity obtained by hypoglycaemic conditions can be attributed to the synthesis of the enzyme *de novo*. A direct effect of glucose oxidase on phosphoenolpyruvate carboxykinase activity is unlikely, because the increase in activity is obtained also by 'hypoglycaemia' without addition of the enzyme. Moreover, the activity of phosphoenolpyruvate carboxykinase after perfusion with gluconate or H_2O_2 (the products of the glucose oxidase reaction) was the same as in control experiments.

Since cycloheximide and actinomycin D inhibit the stimulation of liver phosphoenolpyruvate carboxykinase by dibutyryl cyclic AMP injection (Wicks *et al.*, 1972), it seemed likely that the enhancement of phosphoenolpyruvate carboxykinase activity brought about by 'hypoglycaemia' was mediated by cyclic AMP. When added *in vitro*, imidazole is known to enhance the activity of phosphodiesterase, leading to lower concentrations of cyclic AMP (Nair, 1966; Cheung, 1967). To obtain some data on the action of cyclic AMP in the induction of liver phosphoenolpyruvate carboxykinase synthesis, livers of fed rats were perfused in the presence of glucose oxidase (50mg) and 40mM-imidazole. Under these conditions the phosphoenolpyruvate carboxykinase activities were not increased, in spite of the low concentrations of circulating glucose (Table 2).

Moreover, when only dibutyryl cyclic AMP was added to the perfusion medium, an enhancement of phosphoenolpyruvate carboxykinase activity, very similar to that produced by 'hypoglycaemia', was found (Table 4).

With the aim of confirming the involvement of cyclic AMP changes in the stimulation of phosphoenolpyruvate carboxykinase activity under 'hypo-

Table 4. Time-course of the effect of dibutyryl cyclic AMP on phosphoenolpyruvate carboxykinase in perfused rat liver

Livers of well-fed rats were perfused for 120 min. Dibutyryl cyclic AMP was added to the perfusion medium at 28 min, reaching a concentration of $30 \mu\text{M}$. Phosphoenolpyruvate carboxykinase activity was measured in sequential liver samples taken at the times given in the Table. Glucose is given as μmol in the total perfusion medium. Enzyme activity is expressed in nmol of oxaloacetate formed at $30^{\circ}\text{C}/\text{min}$ per mg of protein. The results are means $\pm$ s.e.m. for four experiments.

Time (min)	Glucose found	Enzyme activity
30	535 ± 40	14.8 ± 1.2
60	710 ± 36	18.4 ± 1.7
120	950 ± 32	24.9 ± 2.6

Fig. 2. Time-course of the effect of the low concentration of circulating glucose (O) on cyclic AMP concentration (Δ) and phosphoenolpyruvate carboxykinase activity ($\bullet$) in perfused rat liver

Glucose oxidase (50mg) was added after 40min of perfusion. The results are means of four experiments. The s.e.m. values are indicated by error bars.

glycaemic' conditions, the intracellular concentration of the cyclic nucleotide was measured under the same conditions. As shown in Fig. 2, the concentration of glucose in the medium drastically decreases after the addition of glucose oxidase after 40 min of perfusion, whereas phosphoenolpyruvate carboxykinase activity and the concentration of cyclic AMP clearly increase.

Discussion

The time-course of the effect of nicotinic acid treatment on hepatic glycogen content and phosphoenolpyruvate carboxykinase activity was very similar to that obtained by Foster *et al.* (1966) in intact rats, in which renal loss of glucose was caused by administration of phlorrhizin. The blood glucose concentration was also very similar, with no significant decrease after the second hour of the experiment; this is most likely due to the rapid mobilization of liver glycogen and, to some extent, the switch-on of gluconeogenesis.

Wicks *et al.* (1972) reported that the injection of dibutyl cyclic AMP and theophylline to normal rats leads to a rapid enhancement of phosphoenolpyruvate carboxykinase activity (resulting from an increase in the rate of the synthesis of enzyme protein) and an equally rapid depletion of liver glycogen. These authors suggested that both phenomena share a common intermediary, possibly protein kinase, which activates glycogen phosphorylase and may induce phosphoenolpyruvate carboxykinase. Since the enhancement of phosphoenolpyruvate carboxykinase activity in our experimental conditions is apparently due to the synthesis of enzyme *de novo*, it seems probable that in this case, as well as in rats treated with phlorrhizin (Foster *et al.*, 1966), the mobilization of glycogen and the induction of phosphoenolpyruvate carboxykinase are also produced by means of protein kinase, in response to a rise in hepatic cyclic AMP concentration.

With regard to the experiments with actinomycin D, Wicks & McKibbin (1972) have shown that the early (1 h) increase in phosphoenolpyruvate carboxykinase synthesis, induced by dibutyl cyclic AMP in Rember H 35 cells, is not prevented by actinomycin D. However, it must be taken into account that, under our experimental conditions, the measurements of enzyme activity were carried out 5½ h after antibiotic administration, when a very low concentration of specific enzyme template is expected to exist.

The metabolic link between nicotinic acid administration and cyclic AMP increase in liver is not clear. A direct positive effect is unlikely, because nicotinic acid seems to inhibit adenylate cyclase in adipose tissue (Butcher *et al.*, 1968; Kupiecki & Marshall, 1968; Peterson *et al.*, 1968) and probably in kidney cortex (Issekutz, 1971). Moreover, the addition of nicotinic acid (1 mM) to the perfusion medium does

not increase the phosphoenolpyruvate carboxykinase activity in liver from well fed rats (F. J. Moreno, L. Sánchez-Urrutia, J. M. Medina, F. Sánchez-Medina & F. Mayor, unpublished work). Since treatment with phlorrhizin brings about a similar response, it seems more likely that the increase in cyclic AMP in liver is mediated by hormones in response to a slight hypoglycaemia.

In rats treated with nicotinic acid the gluconeogenic capacity of livers (as measured by conversion of lactate into glucose) is low and considerably below the values found in rats starved for 48 h. This is in spite of the fact that glycogen is almost fully depleted and phosphoenolpyruvate carboxykinase is induced to the same extent as that occurring after 48 h of starvation. These results were not unexpected, as they are in good agreement with those obtained by Ross *et al.* (1967) in rats previously treated with phlorrhizin and glucagon, a treatment which produces a response similar to that of nicotinic acid.

As has been pointed out above, the stimulation of phosphoenolpyruvate carboxykinase activity and glycogen depletion which take place after nicotinic acid treatment are not two interrelated processes but two independent phenomena which are very probably influenced by hormonal factors. The experiments carried out in perfused liver (not under hormonal control) seem to corroborate this assumption. Actually, the increase in phosphoenolpyruvate carboxykinase activity which takes place in these conditions after 'hypoglycaemia' is not related to the glycogen content because under each hypoglycaemic condition tested the enzymic activities were constant, whereas the values of glycogen exhibited wide fluctuations. On the other hand, the phosphoenolpyruvate carboxykinase activities were always high when the circulating glucose concentration was low.

The stimulation of phosphoenolpyruvate carboxykinase activity after 'hypoglycaemic' conditions is most probably mediated by cyclic AMP. The rise in enzymic activity results from an increase in the rate of synthesis of protein *de novo*, as indicated by the data obtained with rats previously treated with cycloheximide or actinomycin D, and it is well established that cyclic AMP stimulates phosphoenolpyruvate carboxykinase activity by increasing the concentration of enzyme protein (Wicks *et al.*, 1972).

On the other hand, the addition of dibutyl cyclic AMP to the medium in normal perfusions produces an enhancement of phosphoenolpyruvate carboxykinase activity similar to that promoted by 'hypoglycaemia'. In addition, there is no rise in enzymic activity under 'hypoglycaemic' conditions when imidazole, a known activator of phosphodiesterase, is added to the perfusate. Lastly, the increase in the phosphoenolpyruvate carboxykinase activity which takes place in 'hypoglycaemia' is accompanied by a sharp rise in the hepatic concentration of cyclic AMP.

Since the hepatic concentration of cyclic AMP found when the concentration of glucose in the perfusate is almost physiological is similar to that which occurs *in vivo* under normal conditions, it can be reasonably assumed that the presence of adequate concentrations of glucose in the perfusate prevents the rise in basal cyclic AMP concentration. Therefore the lack of these concentrations of glucose would lead to a rapid increase in the cellular concentration of cyclic nucleotide and subsequent accelerated rates of phosphoenolpyruvate carboxykinase synthesis. It is noteworthy that the presence of glucose in the perfusion medium does not overcome the induction of phosphoenolpyruvate carboxykinase activity by dibutyryl cyclic AMP. These results are in agreement with those obtained in rats starved for 60h by Treadow & Khairallah (1972), who showed that glucose represses phosphoenolpyruvate carboxykinase activity, but that dibutyryl cyclic AMP can reverse this repression *in vivo*. More recently, Tilghman *et al.* (1974) have found that the administration of dibutyryl cyclic AMP to 20h-starved rats blocks the deinduction of phosphoenolpyruvate carboxykinase brought about by refeeding glucose.

As for the significance of the enhancement of phosphoenolpyruvate carboxykinase activity found during 'hypoglycaemia' *in vitro*, it must be pointed out that such an increase takes place in only 130 min, whereas the doubling of enzyme activity by nicotinic acid treatment requires 5h. On the other hand, the stimulation by 'hypoglycaemia' of phosphoenolpyruvate carboxykinase activity is specific, since the other enzymes tested do not change (lactate dehydrogenase, fructose diphosphatase) or are even slightly decreased (glucose 6-phosphatase). The significance *in vivo* of this regulatory mechanism of phosphoenolpyruvate carboxykinase may be questioned because of the rather low concentration of circulating glucose needed to bring it about. The hormonal control which seems to operate so precisely in rats treated with nicotinic acid is likely to be the main control system in physiological circumstances, overriding the primary role of glucose itself. Nevertheless, it could be important in other animals exhibiting wider oscillations in their glycaemia or in certain pathological situations.

In conclusion, our findings seem to support the postulate that liver phosphoenolpyruvate carboxykinase is regulated through hormonal factors in physiological circumstances in such a way that major alterations in the consumption of glucose by peripheral tissues apparently do not effect the concentration of blood glucose. Under these conditions, hepatic glycogen content and phosphoenolpyruvate carboxykinase activity are inversely related. On the other hand, in the absence of hormonal factors, the regulation of phosphoenolpyruvate carboxykinase can be accomplished by glucose itself, a deficiency resulting

in the induction of the enzyme. Under these conditions, there is no inverse relationship between hepatic glycogen concentration and phosphoenolpyruvate carboxykinase activity.

The agent able to unify the control scheme in both hormonal and non-hormonal regulation of phosphoenolpyruvate carboxykinase appears to be cyclic AMP. It can be assumed that the control of phosphoenolpyruvate carboxykinase through cyclic AMP was first brought about by glucose in the primitive organisms. The same mechanism would be utilized afterwards by hormones to achieve its efficient and elaborated regulatory role.

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