Biochem. J. (1973) **136**, 455–465
Printed in Great Britain

Relationships Between Concentration of Hepatic Intermediary Metabolites and Induction of the Key Glycolytic Enzymes *in vivo*

By J. M. GUNN and C. B. TAYLOR
Department of Biochemistry, University of Sheffield, Sheffield S10 2TN, U.K.

(*Received* 2 *April* 1973)

1. The time-course for the induction of hepatic glucokinase, hexokinase, phosphofructokinase, liver-type and muscle-type pyruvate kinases in reponse to various diets and insulin has been investigated over the first 48h of change in both diabetic and non-diabetic rats. 2. The results are consistent with there being separate regulatory mechanisms for the induction of each of the three key enzymes, that is for glucokinase, phosphofructokinase and liver-type pyruvate kinase. 3. To investigate the possibility that induction of these enzymes is mediated through specific metabolites a full metabolite profile has been determined under conditions identical with those in the induction experiments and the results examined for correlations between metabolite concentrations and enzyme activities. 4. Several such relationships were detected and those between glucokinase activity and the phosphorylation state of the adenine nucleotides and between liver-type pyruvate kinase activity and the concentrations of dihydroxyacetone phosphate and pyruvate are discussed in relation to the concept of inducing metabolites. 5. It is suggested that the induction of glycolytic enzymes by insulin may be secondary to the changes in the concentration of specific hepatic metabolites brought about by the acute effects of the hormone. 6. The details of the metabolite concentrations in the various experimental states have been deposited as Supplementary Publication SUP 50021 at the British Library (Lending Division) (formerly the National Lending Library for Science and Technology), Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1973), **131**, 5.

The control of glycolysis in rat liver is usually explained in terms of the properties of three enzymes, hexokinase (ATP–D-hexose 6-phosphotransferase, EC 2.7.1.1), phosphofructokinase (ATP–D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) and pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40). All three are known to have isoenzymes (Walker, 1963; Vinuela *et al.*, 1963; Tanaka *et al.*, 1967; Taylor & Bew, 1970; Kemp, 1971) and, although little is known as yet about the inducibility of the individual forms of phosphofructokinase, the other two enzymes occur in the liver in what are essentially either constitutive forms, hexokinase and muscle-type pyruvate kinase, or adaptive forms, glucokinase and liver-type pyruvate kinase. Glucokinase, phosphofructokinase and liver-type pyruvate kinase activities are reported to fall in starvation and on a low-carbohydrate diet or in diabetes and conversely to rise on re-feeding the starved animal, particularly with a high-carbohydrate diet, and also on treating the diabetic rats with insulin (Sharma *et al.*, 1963; Salas *et al.*, 1963; Krebs & Eggleston, 1965; Weber & Singhal, 1965; Weber *et al.*, 1965; Tanaka *et al.*, 1967). In general, increases of enzyme activity in these experiments could be prevented by actinomycin D, indicating that protein synthesis and gene transcription may be involved.

The fact that the activities of these three enzymes all change in a similar sense in such experiments has given rise to the concept of synchronous control of their synthesis in a system analogous to the prokaryotic operon (Weber *et al.*, 1966*a*,*b*).

However, animals exhibit a degree of flexibility in controlling the amount of hepatic enzymes to cope with the influx of diverse dietary substrates which is difficult to reconcile with the view that the whole glycolytic function is turned on or off as a unit (Sillero *et al.*, 1969*b*; Tepperman & Tepperman, 1970). On the contrary, when presented with a substance entering at the triose level, some of which will be catabolized and some of which will be converted into glucose, the hepatic enzymes required for both processes are induced, that is, flux through a particular portion of a pathway appears to be followed by increased capacity of that part of the sequence. Such a system is more readily explained in terms of specific inducing metabolites controlling the amounts of particular enzymes than by a concerted action on the whole sequence by a hormone. Bailey *et al.* (1968) suggested that such a system was involved in the induction of hepatic pyruvate kinase and Sillero *et al.* (1969*b*), have invoked inducing metabolites to explain the parallel induction of glucokinase and pyruvate kinase in their experiments. However, as yet no

systematic search for inducing metabolities in hepatic carbohydrate metabolism has been reported.

In the present paper, therefore, we are concerned with three main problems, first, to assess the validity of the view that the three key enzymes of glycolysis are synchronously regulated, secondly, to determine the role of insulin in their induction and, thirdly, to test the hypothesis that these enzymes may be regulated by the cellular concentrations of specific inducing metabolites. To this end we have measured the time-course of changes in the activity of glucokinase, phosphofructokinase and liver-type pyruvate kinase during the transition from one steady state to another caused by various inducing stimuli. The inducing procedures were chosen to give increased carbon flux from hexose and triose either separately or simultaneously, that is, by feeding glucose, glycerol or sucrose. Such studies were carried out in both normal and diabetic rats and in addition the response to insulin was investigated in the diabetic animals. Throughout the studies full metabolite profiles were determined at each point for each inducing procedure and the data examined for correlations between metabolite concentrations and induction of enzymes.

Experimental

Animals

Male Wistar rats from the Sheffield University colony were used throughout. They were housed in groups of four in screen-battened cages without bedding material and in all experiments the initial weights were in the range 240–260g. The rats were kept at 25°C and 45% humidity on a cycle of alternating 12h periods of darkness and light with the light period starting at 09:00h. In all the dietary experiments described here zero time was 09:00h. Rats, which were made diabetic by intravenous injection of a fresh aqueous solution of alloxan monohydrate (35mg/kg), were used 2–4 weeks after injection, animals with blood glucose values less than 300mg % being rejected. Insulin was given in doses of 10i.u./100g body wt. every 3h during the light period starting at zero time, the last injection being given 3h before death.

Diets

Food was provided *ad libitum* to all but the starved animals. The stock diet was Oxoid 'Modified Diet 86' (Herbert C. Styles Ltd., Bewdley, Worcs., U.K.), the composition of which is: carbohydrate 62.8%, protein, 23%; fat, 4.4%; ash, 6.0%; fibre, 3.8%. A high-protein carbohydrate-free diet was prepared, the composition of which was: casein, 71.8%; gelatin, 3.2%; margarine, 18.3%; corn oil, 0.5%; cod liver oil, 0.5%; 'A' salts, 5%; 'B vitamins', 0.7%. In addition diets were prepared in which 30% glucose, sucrose or glycerol replaced 30% of the casein in the high-protein diet. The composition of the 'A' salts is given by Bartley *et al.* (1967). Constituents of the diets were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., except for the margarine, corn oil and cod liver oil, which were domestic varieties, and the 'B vitamins', which were provided by Marmite Yeast Extract, Marmite Ltd., Enfield, Middx., U.K.

Normal (non-diabetic) rats previously fed on the stock diet were starved for 72h then re-fed on either the high-protein diet or the 30%-glycerol diet and in addition two groups re-fed on the protein diet were given a 20% aqueous solution of either glucose or sucrose to drink. These latter animals are designated 'normal glucose fed' and 'normal sucrose fed' respectively. The rats were killed at intervals of 6, 12, 24 and 48h after re-feeding. Diabetic rats on the stock diet were starved for 24h, then re-fed on one of the diets containing protein, glycerol, glucose or sucrose. In addition the 'diabetic glucose fed' and 'diabetic sucrose fed' groups received 20% glucose or sucrose respectively instead of drinking water. The re-fed diabetic animals were killed either at 24h or 48h after the start of re-feeding. A group of diabetic rats fed on stock diet and treated with insulin were killed at 6, 12, 24 or 48h after the first injection. All rats fed on stock diet were killed between 08:00h and 10:00h. Metabolite and enzyme assays were not carried out on the same liver.

Chemicals

All reagents used were of analytical grade or the purest available. Enzymes, coenzymes and nucleotides were obtained from Boehringer Corp. (London) Ltd. (London W.5, U.K.). Ox insulin, potency 25i.u./mg, was obtained from BDH Chemicals Ltd.

Enzyme assays

These were carried out at 28°C in a final volume of 1.5ml, with a Gilford model 2000 absorbance recorder coupled to a Beckman DU spectrophotometer. In all cases the reaction was started by the addition of substrate after preincubation for 5min at 28°C. Glucokinase and hexokinase were assayed by the method of Novello *et al.* (1969); 6-phosphogluconate dehydrogenase was not added, a factor of 1.7 being employed instead (Sharma *et al.*, 1963). Blank rates were determined in the absence of ATP (Niemeyer *et al.*, 1967). Phosphofructokinase was assayed by the method of Opie & Newsholme (1967) on a '600*g* supernatant' and pyruvate kinase by the method of Bücher & Pfleiderer (1955) as modified by Taylor & Bailey (1967). Liver pyruvate kinase isoenzymes were assayed employing control serum and antiserum prepared in the rabbit against rat muscle

pyruvate kinase as outlined below. The enzyme unit used in the tables and figures is defined as the enzyme activity causing the transformation of 1 μmol of substrate in 1 min at 28°C.

Preparation of antiserum

Muscle-type pyruvate kinase required for raising antibody was prepared from rat muscle by the method of Tanaka *et al.* (1967). The final preparation gave an activity of 2700units/ml and a specific activity of 50units/mg. From the specific activity of the pure muscle enzyme (780units/mg at 37°C reported by Tanaka *et al.*, 1967) it can be calculated that there were about 7mg of enzyme/ml in the solution used here for antibody production. Equal volumes of the protein solution and Freund's complete adjuvant were thoroughly dispersed, by using a homogenizer, and 3.0ml of the emulsion was injected subcutaneously into a rabbit weighing 2kg. The injections were repeated at 7 and 28 days. At 14 days after the last injection, blood was taken from the marginal ear vein and serum prepared. Antibody activity was determined by titration against muscle-type pyruvate kinase solution of known activity. The antibody–antigen reaction was carried out at room temperature in small polythene centrifuge tubes with a total reaction volume of 0.1ml. After 15min the tubes were cooled on ice, centrifuged at 25000*g* and 4°C for 15min and the supernatant fluids assayed for pyruvate kinase. The antibody potency, expressed as units of pyruvate kinase activity inhibited by 1ml of serum, varied from 10 to 50 in the various preparations. Excess of antiserum completely inhibited muscle-type pyruvate kinase, but was without effect on a sample of purified liver-type pyruvate kinase. For the routine assay of liver- and muscle-type isoenzymes in liver samples essentially this same assay procedure was employed, the pyruvate kinase activity of each supernatant sample being determined both in the presence of a fivefold excess of anti-(muscle-type serum) and in the presence of control serum. The difference between the two values gives the muscle-type pyruvate kinase activity and the remainder is designated liver-type pyruvate kinase.

Extraction of liver samples

The rats were anaesthetized with ether and the liver samples clamped *in situ* by using tongs precooled in liquid N_2. Any unfrozen or slowly frozen tissue was trimmed off and the now brittle liver powdered in a precooled mortar under liquid N_2. Samples of the tissue (1.5–2g) were transferred to weighed tubes containing 2ml of frozen 25% (w/v) perchloric acid and reweighed. This was thawed, with mixing, in an ice – ethanol bath at about −7°C and then homogenized. After the addition of 2ml of ice-cold distilled water the contents were rehomogenized and centrifuged for 10min at 2000*g* and the precipitate was re-extracted with 2ml of ice-cold 3% (w/v) perchloric acid. The combined supernatant fluids were titrated to pH3–4 with ice-cold 20% (w/v) KOH and Methyl Red as indicator. After the tubes had stood on ice for at least 20min, potassium perchlorate was removed by centrifugation at 2000*g* for 10min. The supernatant fluids were used for metabolite assays on the same day.

Metabolite assays

These were carried out in a final volume of 2ml by using the Gilford model 2000 absorbance recorder coupled to a Beckman DU spectrophotometer or by using a Beckman DB spectrophotometer in conjunction with a Sargent model SR chart recorder. Particular assays were always carried out on the same instrument. Glycolytic intermediates, ATP, ADP and AMP were all assayed by the method of Cartier *et al.* (1967). The methods described by Bergmeyer (1963) were used for the assays of lactate, malate, α-glycerophosphate, 3-hydroxybutyrate, acetoacetate, glutamate, 2-oxoglutarate and isocitrate. Citrate was estimated by the method of Moellering & Gruber (1966). Fructose 1-phosphate was determined by using rat liver aldolase prepared by the method of Leutenhardt & Wolf (1955) modified to include a heat-treatment step (see Woods *et al.*, 1970). This assay was carried out by following the oxidation of NADH in the triethanolamine buffer (pH7.6) of Cartier *et al.* (1967) with liver aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase as auxiliary enzymes. Glycogen and P_i were determined chemically by the methods of Carroll *et al.* (1956) and Fiske & SubbaRow (1925) respectively and blood sugar by the method of Nelson (1944).

Results

The starting conditions in all experiments, that is starvation or diabetes, were chosen to give low initial activities of the enzymes that were then induced by procedures designed to increase glycolytic flux from either the hexose or triose level. Since dietary fructose and glycerol enter hepatic glycolysis via triose (Sillero *et al.*, 1969*a*), three different inducing systems were readily obtained by feeding either glucose or glycerol to study respectively induction by hexose or triose and by feeding sucrose, which may be regarded as a mixed inducer in that it will enter glycolysis partly as hexose and partly as triose. A fourth mode of induction was achieved by treating diabetic rats with insulin.

The changes in activities of the glycolytic enzymes measured during the dietary shifts in the non-diabetic

Table 1. *Changes in the activities of the key enzymes of hepatic glycolytic regulation in normal rats over the 48h time-courses of treatment*

Rats were starved for 3 days and then re-fed on one of the four experimental diets for the time-interval shown. Details of the diets are given in the Experimental section. Enzyme activities are expressed as units/g wet wt. of liver (means ± S.E.M.).

Group	No. of observations	Enzyme activity (units/g of liver; means ± S.E.M.) Glucokinase	Liver-type pyruvate kinase	Phosphofructokinase	Hexokinase	Muscle-type pyruvate kinase
*†Stock diet	28	1.27 ± 0.05	26.4 ± 1.15	1.54 ± 0.04	0.22 ± 0.01	5.3 ± 0.28
Starved 72h	16	0.32 ± 0.03	9.5 ± 0.54	1.20 ± 0.06	0.22 ± 0.01	4.0 ± 0.22
Re-fed protein 12h	4	0.22 ± 0.07	10.1 ± 0.48	0.91 ± 0.06	0.24 ± 0.02	4.8 ± 0.59
protein 24h	4	0.22 ± 0.07	6.1 ± 1.37	1.26 ± 0.11	0.25 ± 0.01	3.8 ± 0.52
protein 48h	8	0.27 ± 0.03	11.6 ± 1.06	1.19 ± 0.04	0.15 ± 0.003	3.7 ± 0.43
* glucose 6h	4	0.65 ± 0.01	7.7 ± 0.55	0.99 ± 0.04	0.15 ± 0.02	3.0 ± 0.20
* glucose 12h	8	0.84 ± 0.07	10.5 ± 0.83	0.91 ± 0.05	0.21 ± 0.01	4.6 ± 0.38
*† glucose 24h	8	0.70 ± 0.04	8.2 ± 0.66	1.07 ± 0.06	0.22 ± 0.01	4.6 ± 0.58
*† glucose 48h	8	1.66 ± 0.10	46.9 ± 6.29	1.17 ± 0.08	0.17 ± 0.01	7.1 ± 0.96
*† sucrose 6h	4	0.64 ± 0.05	11.8 ± 1.21	1.56 ± 0.04	0.16 ± 0.01	3.2 ± 0.50
*† sucrose 12h	8	0.73 ± 0.07	15.7 ± 1.84	0.92 ± 0.02	0.18 ± 0.01	4.6 ± 0.57
*† sucrose 24h	8	0.64 ± 0.05	18.2 ± 0.89	0.96 ± 0.08	0.24 ± 0.02	5.6 ± 0.59
*† sucrose 48h	8	1.12 ± 0.08	51.9 ± 5.67	1.36 ± 0.07	0.18 ± 0.01	7.5 ± 0.92
† glycerol 6h	4	0.47 ± 0.05	8.6 ± 0.80	0.99 ± 0.06	0.15 ± 0.003	3.2 ± 0.36
† glycerol 12h	8	0.38 ± 0.07	22.3 ± 1.85	0.94 ± 0.06	0.19 ± 0.01	4.2 ± 0.31
*† glycerol 24h	8	0.42 ± 0.05	20.2 ± 1.11	1.11 ± 0.04	0.20 ± 0.01	6.2 ± 0.55
*† glycerol 48h	8	0.78 ± 0.03	46.0 ± 2.57	1.12 ± 0.09	0.14 ± 0.01	6.8 ± 0.77

*, †, For explanation see Table 3.

rats are detailed in Table 1. The comparative aspects of the data for glucokinase and liver-type pyruvate kinase are seen in Fig. 1, in which the time-course of the percentage change in each enzyme is presented. Starvation for 3 days produced falls in glucokinase and liver-type pyruvate kinase activities to less than 25 and 35% respectively of their control values, and these lowered values are not raised by re-feeding the protein diet. The other three diets, glucose, glycerol and sucrose, all increased the activity of glucokinase and liver-type pyruvate kinase within the 48h duration of the experiments, but the time-course of induction varied for the different diets. Glucose and sucrose increased glucokinase activity within 12h compared with the 48h required by glycerol, whereas sucrose and glycerol increased liver-type pyruvate kinase activity within 12h compared with the 48h required by glucose. Fig. 1 also shows that when glucokinase and liver-type pyruvate kinase are induced within the first 24h the increase in activity is apparently not linear, but levels off before increasing again between 24 and 48h. Changes in the corresponding activities of hexokinase and muscle-type pyruvate kinase are, in general, on a smaller scale, in both relative and absolute terms, but are nevertheless substantial, particularly in the case of muscle-type pyruvate kinase.

Table 2 and Fig. 2 show the corresponding changes in these hepatic enzymes produced by insulin and the diets in diabetic rats. In these animals activities of glucokinase and liver-type pyruvate kinase were low, glucokinase especially so, and were not decreased further by starvation for 24h. Glucokinase activity was unaffected by any of the diets used, whereas liver-type pyruvate kinase activity was increased by sucrose and glycerol between 24h and 48h, but for any particular time-interval the increase was less than that produced by the same diet in the non-diabetic rats. The glucose diet, which caused a rise of liver-type pyruvate kinase activity in normal rats at 48h, did not affect this enzyme in diabetic animals. Insulin induced both glucokinase and liver-type pyruvate kinase, but whereas glucokinase activity had risen significantly at 6h, the increase in the pyruvate kinase activity did not occur until between 12 and 24h. Although some statistically significant changes in hexokinase and muscle-type pyruvate kinase activities are caused by insulin and the diets in the diabetic rats,

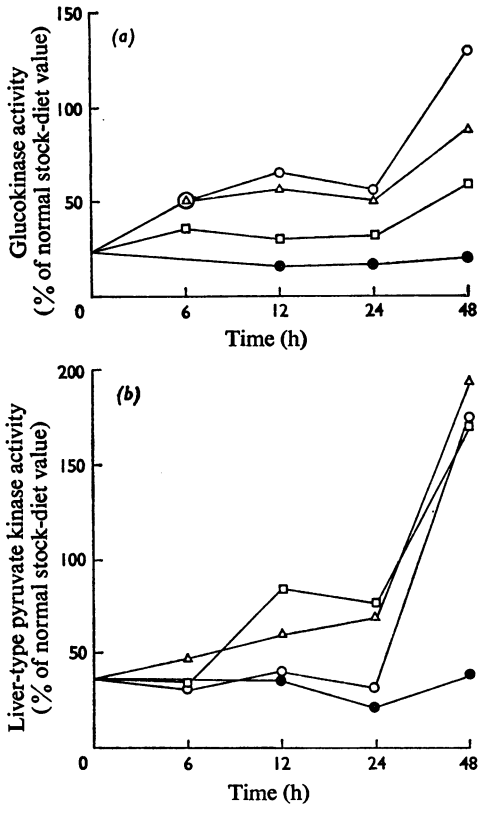

Fig. 1. *Effect of diets on the time-course of induction of hepatic glucokinase (a) and liver-type pyruvate kinase (b) in non-diabetic rats*

Rats previously fed on the stock diet were starved for 3 days and re-fed on diets containing either ○, glucose; △, sucrose; □, glycerol; ●, protein, *ad libitum*. Enzyme activities, expressed as a percentage of normal stock-diet values, are calculated from data in Table 1 and presented as the means only. Details of the diets are given in the Experimental section.

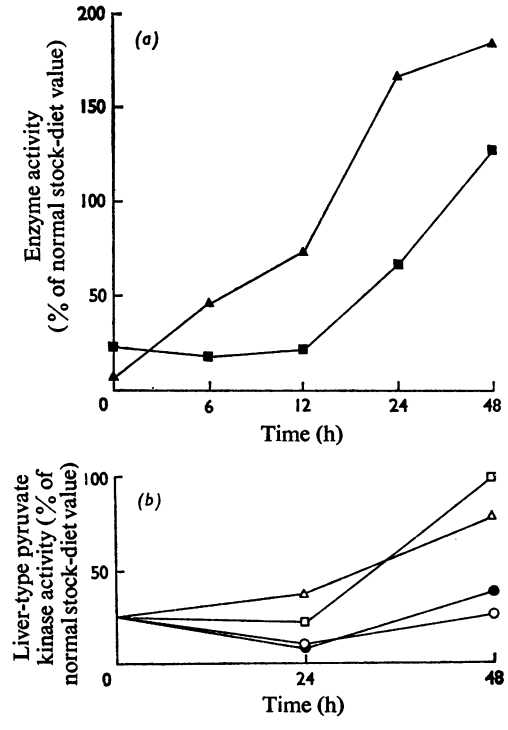

Fig. 2. *Effect of (a) insulin and (b) diets on the time-course of induction of hepatic glucokinase and liver-type pyruvate kinase in diabetic rats*

For the insulin time-course, rats fed on the stock diet *ad libitum* were injected at zero time with the hormone at a dose of 10i.u./100g body wt. as described in the Experimental section. ▲, Glucokinase; ■, liver-type pyruvate kinase. For the dietary time-courses, diabetic rats previously fed on the stock diet were starved for 24h and re-fed on diets containing either ○, glucose; △, sucrose; □, glycerol; or ●, protein, *ad libitum*. Enzyme activities, expressed as a percentage of normal stock-diet values, are calculated from the data in Tables 1 and 2 and presented as the means only. Details of the diets are given in the Experimental section.

these are much smaller than the corresponding changes in the non-diabetic animals.

Starving the non-diabetic rats for 3 days caused a fall of only 20% in hepatic phosphofructokinase activity and this lowered activity was not appreciably raised 48h after re-feeding with any of the four diets. In fact the changes that do occur are of the same order as those for the supposed constitutive enzymes hexokinase and muscle-type pyruvate kinase.

Hepatic metabolite concentrations

Changes in the concentration of hepatic metabolites occurring under the conditions used to investigate the time-course of changes in enzyme activity are presented in Supplementary Publication 50021. To facilitate detection of meaningful relationships between specific metabolites and the induction of a particular enzyme, the metabolite data for the 31 states studied was divided into two groups by designating each state as either 'inducing' or 'non-inducing' with respect to that enzyme (Table 3). In selecting the criteria upon which to make the division, it was deemed probable that changes in the concentration of inducing metabolites would be detectable before induction of the enzyme itself. Accordingly, the 'inducing' group comprises, first,

Table 2. *Changes in the activities of the key enzymes of hepatic glycolytic regulation in alloxan-diabetic rats over the 48h time-courses of treatment*

Diabetic rats were either treated with insulin or starved for 24h and re-fed on one of the four experimental diets for the time-intervals shown. Rats in the insulin groups were fed on the stock diet throughout. Details of the diets are given in the Experimental section. Enzyme activities are expressed as units/g wet wt. of liver (means±S.E.M.).

Group	No. of observations	Enzyme activity (units/g of liver; means±S.E.M.) Glucokinase	Liver-type pyruvate kinase	Phosphofructokinase	Hexokinase	Muscle-type pyruvate kinase
Stock diet	12	0.07±0.01	5.8±0.70	1.60±0.08	0.26±0.01	3.2±0.26
Starved 24h	4	0.06±0.01	7.4±0.41	1.51±0.04	0.22±0.01	3.7±0.47
Re-fed protein 24h	4	0.04±0.02	2.0±0.27	1.52±0.12	0.27±0.01	2.7±0.21
protein 48h	8	0.07±0.02	10.0±1.42	1.69±0.07	0.21±0.01	3.9±0.22
glucose 24h	4	0.07±0.02	2.8±0.55	1.77±0.08	0.29±0.03	2.7±0.40
glucose 48h	6	0.06±0.01	7.2±1.48	1.65±0.08	0.21±0.003	3.6±0.13
† sucrose 24h	4	0.06±0.02	9.3±0.81	1.67±0.11	0.27±0.01	3.5±0.37
† sucrose 48h	7	0.07±0.02	20.2±2.33	1.77±0.07	0.23±0.01	4.9±0.48
† glycerol 24h	4	0.03±0.003	6.3±1.27	1.55±0.06	0.24±0.02	3.7±0.14
† glycerol 48h	8	0.10±0.02	25.5±2.1	1.78±0.07	0.18±0.01	5.1±0.45
Stock diet						
* +insulin 6h	4	0.61±0.14	5.0±0.50	1.09±0.02	0.33±0.01	3.1±0.52
*† +insulin 12h	4	0.93±0.07	6.22±0.36	1.14±0.05	0.23±0.02	2.4±0.84
*† +insulin 24h	4	2.13±0.31	18.2±3.78	1.15±0.05	0.36±0.03	3.6±0.26
*† +insulin 48h	4	2.35±0.49	33.7±2.49	1.64±0.10	0.38±0.02	3.7±0.23

*, †, For explanation see Table 3.

the control (stock-diet) animals, because in these the activity of the enzyme in question is high, secondly, values from the time-course experiments for those time-intervals at which induction was significant, and, thirdly, values from the time-intervals immediately before those at which induction was significant. The 'non-inducing' group comprises the remainder, that is those states, such as starvation and diabetes, in which the activity of the enzyme is very low and those time-intervals in the feeding experiments at which it was deemed that induction was not occurring.

Table 3 shows a comparison of the mean values for the concentration of each metabolite in the 'inducing' and 'non-inducing' groups for the two cases in which the division of data was made upon the basis of the increase of either glucokinase or liver-type pyruvate kinase activity. It was hoped that this procedure would serve as a coarse test giving preliminary evidence of correlations and thus indicating areas which might fruitfully be investigated in more detail. Table 3 shows that the increase of glucokinase activity is accompanied by rises in the concentration of ATP, glycogen, fructose 1-phosphate, pyruvate, citrate, 2-oxoglutarate, as well as in the ratios for dihydroxyacetone phosphate/glyceraldehyde 3-phosphate and several of those calculated for the adenine nucleotides. Conversely, the increase was apparently accompanied by a fall in the concentration of ADP, AMP, P_i, blood glucose, glycerol 3-phosphate, 3-phosphoglyceric acid, acetoacetic acid and 3-hydroxybutyrate. There were also significant differences in all the ratios indicating the redox state of the cytosol, so that glucokinase induction apparently correlates with an increase in the ratios NAD^+/NADH and $NADP^+$/NADPH. In the same way an increase in liver-type pyruvate kinase is associated with an increase in several of the nucleotide ratios and glycogen, fructose 1,6-diphosphate, fructose 1-phosphate, glycerol 3-phosphate, dihydroxyacetone phosphate, pyruvate and 2-oxoglutarate, whereas there is a fall in the concentration of 3-hydroxybutyrate. As in the case of glucokinase, the increase in liver-type pyruvate kinase is accompanied by a rise in the $NADP^+$/NADPH ratio. For phosphofructokinase such a division of data into 'induced' and 'non-induced' states proved impossible, since activity varied within a very narrow range of values.

Supplementary information

The detailed data on metabolite concentrations in the various experimental states have been deposited with the National Lending Library for Science and

Table 3. *Comparative tabulation of metabolite data (see Supplementary Publication 50021) to show possible correlations between the activities of either glucokinase or liver type pyruvate kinase and hepatic metabolite concentrations*

Division of the data into 'induced' and 'non-induced' groups was performed as described in the text. Animal states marked* in Tables 1 and 2 comprise the glucokinase 'induced' group; those marked† comprise the liver-type pyruvate kinase 'induced' group. The results for glycogen are expressed as mg/g wet wt. of liver, for blood glucose as mg/100ml of blood and for the other metabolites as nmol/g wet wt. of liver (means ± S.E.M.). ‡ indicates significant differences: P values <0.05 (Student's t test, 'induced' versus 'non-induced', degrees of freedom 29). Equilibrium constants for lactate dehydrogenase, glycerophosphate dehydrogenase, 3-hydroxybutyrate dehydrogenase, 'malic' enzyme and isocitrate dehydrogenase used in calculating the free $[NAD(P)^+]/[NAD(P)H]$ ratios are taken from Krebs & Veech (1969) and Greenbaum *et al.* (1971).

	Glucokinase		Liver-type pyruvate kinase	
	'induced'	'induced'	'induced'	'non-induced'
ATP	2007±42	‡1693±69	1886±57	1789±88
ADP	814±47	‡1041±41	899±53	976±48
AMP	392±34	‡563±21	456±35	513±34
[ATP]+[ADP]+[AMP]	3210±63	3308±74	3253±54	3278±93
P_i	3610±195	‡4995±150	4173±255	4535±218
[ATP]/[ADP]	2.56±0.19	‡1.59±0.1	2.33±0.19	‡1.71±0.12
[ATP]/[ADP][P_i]	0.75±0.09	‡0.32±0.03	0.65±0.09	‡0.36±0.03
[ATP]/[AMP]	5.92±0.45	‡3.04±0.13	4.85±0.43	‡3.07±0.16
[ATP]/[AMP][P_i]	1.54±0.18	‡0.61±0.04	1.35±0.18	‡0.64±0.04
[ATP]+[AMP]/[ADP]^2	1.31±0.12	‡0.89±0.10	1.17±0.12	1.01±0.12
Energy charge	0.75±0.013	‡0.66±0.009	0.73±0.14	‡0.67±0.01
[Dihydroxyacetone phosphate]/[glyceraldehyde phosphate]	2.94±0.26	‡2.03±0.19	2.72±0.25	2.13±0.03
[Pyruvate]/[phosphoenolpyruvate]	1.91±0.36	1.11±0.24	1.76±0.31	1.13±0.27
$[NAD^+]/[NADH]$(lactate dehydrogenase)	1113±93	‡614±86	984±100	686±107
$[NAD^+][NADH]$(glycerophosphate dehydrogenase)	1938±175	‡1110±100	1572±197	1415±117
$[NAD^+]/[NADH]$(3-hydroxybutyrate dehydrogenase)	11.09±0.66	10.86±0.53	11.19±0.57	10.56±0.60
$[NADP^+]/[NADPH]$('malic' enzyme)	0.011±0.001	‡0.006±0.001	0.010±0.001	‡0.006±0.001
$[NADP^+]/[NADPH]$(isocitrate dehydrogenase)	0.009±0.001	‡0.003±0.001	0.008±0.001	‡0.003±0.001
Glycogen	48.3±5.0	‡15.9±2.3	40.0±5.7	‡21.3±4.0
Blood glucose	93±4.7	‡241±38	149±28	197±41
Glucose 1-phosphate	13±1.2	12±1.1	13±1.0	11±1.4
Glucose 6-phosphate	229±15	253±19	239±16	244±20
Fructose 6-phosphate	36±2.6	38±5.3	40±4.5	33±3.3
Fructose 1,6-diphosphate	20±2.8	15±1.1	16±0.9	‡13±1.0
Fructose 1-phosphate	391±64	‡220±42	378±58	‡198±37
Glycerophosphate	332±27	‡533±83	519±75	‡325±25
Dihydroxyacetone phosphate	35±3.4	28±2.0	35±2.4	‡26±2.5
Glyceraldehyde phosphate	12±0.5	13±0.8	14±0.7	12±0.7
3-Phosphoglycerate	233±14	‡300±20	268±20	266±19
2-Phosphoglycerate	32±2.4	37±3.5	36±3.1	31±3.0
Phosphoenolpyruvate	93±9.0	118±13	101±11	112±13
Pyruvate	141±12	‡100±9.5	136±10	‡97±11
Lactate	1246±101	1453±139	1406±128	1280±113
Citrate	390±16	‡307±22	361±20	327±23
Isocitrate	21±1.6	25±1.7	22±1.5	25±2.0
2-Oxoglutarate	147±16	‡59±4.8	126±16	‡68±9.1
Glutamate	3376±421	2946±393	3061±381	3304±444
Malate	487±44	518±29	506±39	498±31
Acetoacetate	107±12	‡267±35	151±24	243±43
3-Hydroxybutyrate	189±17	‡506±38	269±38	‡468±45

Technology, Boston Spa, York LS23 7BQ, U.K. for storage on microfiche (Supplementary Publication SUP 50021).

The information comprises: (1) the hepatic metabolite concentrations, adenylate and redox ratios listed in the first column of Table 3 for each of the dietary and hormonal states listed in the first column of Tables 1 and 2; (2) tables giving, for liver in each of these states, the wet weight, percentage of body weight, dry weight, total protein, supernatant protein, RNA and DNA.

Discussion

Induction of glucokinase, phosphofructokinase and liver-type pyruvate kinase

These studies indicate, first, that when glucokinase and liver-type pyruvate kinase are both induced by

a particular procedure, that is by re-feeding glucose, sucrose or glycerol to non-diabetic rats or by insulin treatment of diabetic rats, the time-course of induction differs in such a way that substrate entering glycolysis at the hexose level induces glucokinase before liver-type pyruvate kinase, whereas substrate entering glycolysis through triose induces liver-type pyruvate kinase before glucokinase. With sucrose, which enters at both the hexose and triose levels, the induction of the two enzymes is simultaneous. Thus the temporal order in which these enzymes are induced can be readily reversed by altering the experimental conditions. Secondly, although glucokinase is only induced in the presence of insulin, we find, in agreement with the results of Takeda *et al.* (1967) and Sillero *et al.* (1969*b*), who measured total pyruvate kinase activity, that liver-type pyruvate kinase can be induced by diet in diabetic rats but only by substrates entering glycolysis at the triose level, that is, by sucrose or glycerol. Thirdly, the activity of phosphofructokinase, in contrast with the other two enzymes, alters little in these experiments, being decreased only moderately in starvation and scarcely at all in diabetes, suggesting that phosphofructokinase should not be regarded as a chronic regulator of glycolysis.

In contrast with the view the glucokinase, phosphofructokinase and pyruvate kinase are regulated as a 'functional genome unit' and that insulin acts by switching this on (Weber *et al.*, 1966*a*,*b*), the independent variations in the activities of these three enzymes suggests they are separately controlled. Although insulin appears always to be involved in glucokinase induction, the data are more consistent with liver-type pyruvate kinase being induced by a specific metabolite, as was suggested by Bailey *et al.* (1968) to explain why dietary fructose is a more effective inducer of hepatic pyruvate kinase than either starch or glucose. This hypothetical metabolite must be common to the metabolic pathways of glucose, fructose and glycerol and would therefore be expected to occur on the glycolytic sequence at, or below, the triose level. It is possible that there are separate mechanisms for the induction of liver-type pyruvate kinase by insulin and the metabolite, as was suggested by Weber (1969), but all our observations are consistent with the simpler view that the primary effect of the hormone is to increase the flux through glycolysis by inducing glucokinase and so to cause a change in the same hypothetical metabolite that is responsible for the induction by triose (Sillero *et al.*, 1969*b*).

Metabolite changes during the induction of glycolytic enzymes

Certain gross features are apparent in the changes of metabolite pattern caused by the experimental procedures and the most obvious of these relate to the adenylate ratios, redox ratios and glycogen.

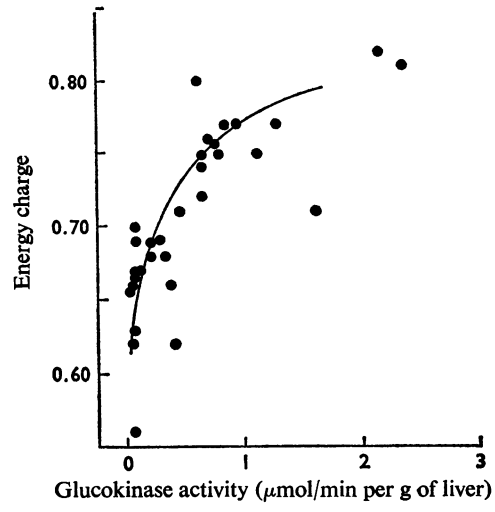

Fig. 3. *Relationship between glucokinase activity and the hepatic energy charge*

The energy charge is given by [ATP]+½[ADP]/[ATP]+[ADP]+[AMP]. All 31 experimental animal states are included.

It can be seen from Table 3 that an increase in glucokinase activity is accompanied by changes in all the adenylate ratios, each indicating an increase in the phosphorylation state of the liver. This relationship is made more explicit in Fig. 3, which shows glucokinase activity for each of the 31 states plotted against the energy charge ([ATP]+½[ADP]/[ATP]+[ADP]+[AMP]; Atkinson & Walton, 1967; Atkinson, 1968). Glucokinase activity is clearly correlated with the energy status of the cell. Table 3 also indicates a positive correlation between glucokinase activity and the ratio [dihydroxyacetone phosphate]/[glyceraldehyde 3-phosphate], which has been shown by Greenbaum *et al.* (1971) to be an index of glycolytic flux. It is also clear that induction of glucokinase is associated with an increase in the oxidation state of the cytosol as indicated by the values for the various redox couples listed in Table 3. These results must therefore be interpreted as showing that glycolytic flux, the phosphorylation state, the redox state and glucokinase activity all increase together. The relationship between the redox couples and the adenylate ratios, an increase in the oxidation state being associated with an increase in the degree of phosphorylation, is in agreement with the results of Veech *et al.* (1970).

Glucokinase and glycogen both decrease in starvation or diabetes and remain low on re-feeding any

of the diets to diabetic rats or on re-feeding the high-protein diet to normal rats. Only four conditions produced an increase in glucokinase activity (re-feeding glucose, sucrose or glycerol to normal rats and insulin treatment of diabetic rats) and only these four conditions produced an increase in glycogen at 24h. This correlation is consistent with the observations of Potter *et al.* (1966) that glycogen and glucokinase undergo similar diurnal variations but slightly out of phase. It is difficult to envisage how glycogen might be implicated in enzyme induction and it is probably better to regard this correlation as a consequence rather than the cause of glucokinase induction, particularly since the relationship holds only for a limited period of time. The good correlation between glucokinase induction and energy

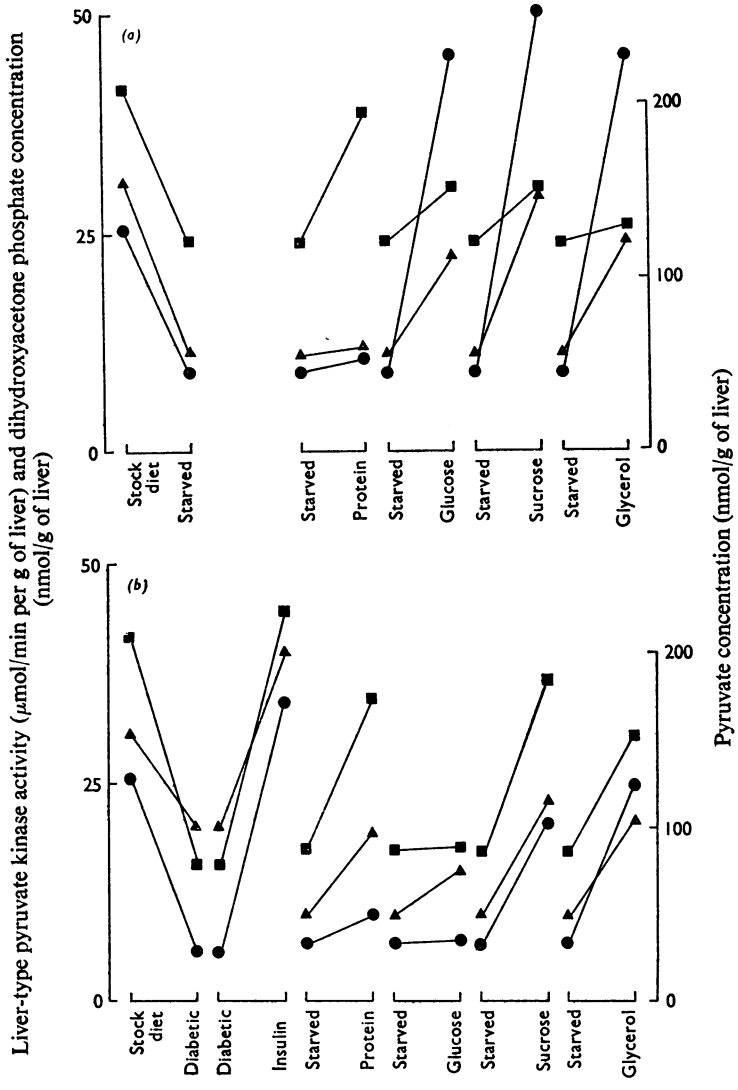

Fig. 4. *Relationship between liver-type pyruvate kinase activity and hepatic dihydroxyacetone phosphate and pyruvate concentrations for (a) normal and (b) diabetic rats*

Only the 48h dietary and insulin values are presented. Experimental conditions were as for Figs. 1 and 2. The left-hand ordinate shows the scale for dihydroxyacetone phosphate concentration (■) and liver-type pyruvate kinase activity (●); the right-hand ordinate gives the scale for pyruvate concentration (▲).

charge is of interest in connexion with Atkinson's (1969) suggestion that energy charge might be a factor in regulating rates of production of the glycolytic enzymes.

One notable correlation which appears to apply to liver-type pyruvate kinase but not to glucokinase is that with dihydroxyacetone phosphate concentration. The relationship is made more explicit by Fig. 4, which shows that liver-type pyruvate kinase activity alters in consonance with the concentration of dihydroxyacetone phosphate for the steady-state transitions studied, namely stock diet to starved, stock diet to diabetic and diabetic to insulin-treated. Further, on re-feeding the diets to starved animals there is an increase in both parameters for glucose, sucrose and glycerol in normal rats and for sucrose and glycerol in diabetic rats, whereas on re-feeding glucose to starved diabetic rats both the enzyme and metabolite remain at the values in starved rats. The relatively low concentration of dihydroxyacetone phosphate at 48h in conjunction with supra-normal enzyme activities noted in the feeding experiments might be explained as indicating that the transition from one steady state to another is accompanied by the restoration of most of the components of the system to their original values, that is the changes in enzyme activity are the response to perturbations in a homeostatic system which operates to maintain constant amounts of certain critical metabolites. Before this the dihydroxyacetone phosphate concentrations at 6, 12 and 24h were higher and, as might be expected of an inducing agent, the metabolite concentration was often elevated before an increase in enzyme activity could be detected. It is tempting therefore to postulate dihydroxyacetone phosphate as the inducing agent for liver-type pyruvate kinase. Its position in glycolysis renders it highly suitable to fulfil the role of feed-forward inducer of this enzyme, and, since it is the first common metabolite in the main hepatic pathways of fructose, glycerol and glucose metabolism, it would provide a ready explanation of much of the data on dietary induction of liver-type pyruvate kinase in normal and diabetic rats.

This correlation fails in one specific case, namely when the starved animals are re-fed with protein. Here, as shown in Fig. 4, there is a marked increase in dihydroxyacetone phosphate at 48h without a concomitant rise in the liver-type pyruvate kinase activity. Unless a specific inhibition of the induction by the protein diet is postulated this finding argues against dihydroxyacetone phosphate being the inducing agent. A further consideration of the data in Table 3 suggests pyruvate as a possible correlating metabolite and in Fig. 4 the 48h values for pyruvate concentration are superimposed upon those for dihydroxyacetone phosphate and liver-type pyruvate kinase. In fact the parallelism between pyruvate and the enzyme for these major states is even better than for dihydroxyacetone phosphate and in particular the relationship holds for the protein-fed animals. Since dihydroxyacetone phosphate and pyruvate concentrations act as indices of glycolytic flux (Greenbaum *et al.*, 1971), both would appear teleologically to be plausible inducers of liver-type pyruvate kinase. The dihydroxyacetone phosphate would constitute a simple feed-forward induction, the use of which is readily perceived. Induction by pyruvate is conceptually more difficult to accept, since that would constitute a positive feed-back system, but a switch mechanism of this kind cannot be ruled out for the control of this complex metabolic crossroads for glycolysis and gluconeogenesis.

Interpretation of these data cannot yield definitive conclusions because of the difficulty in distinguishing cause from effect. Moreover, the nature of the increase in the enzyme activities recorded here is uncertain, for although there is ample evidence in the literature that such changes are blocked by cycloheximide and actinomycin D, suggesting involvement of enzyme synthesis, alterations in degradation rates or in the equilibrium between active and inactive forms of the enzymes cannot be ruled out. However, certain tentative suggestions may be made upon which to base further investigation. First, the chronic effects of insulin on hepatic carbohydrate metabolism may be indirect and occur through acute effects on metabolite concentrations; secondly, the regulation of glucokinase and liver-type pyruvate kinase by diet may be mediated through specific hepatic glycolytic intermediates; thirdly, the correlations between liver-type pyruvate kinase, dihydroxyacetone phosphate and pyruvate may be indicative of a role for these metabolites in regulating that enzyme.

The authors are indebted to the Medical Research Council for the research studentship awarded to J. M. G.

References

Atkinson, D. E. (1968) *Biochemistry* **7**, 4030–4045

Atkinson, D. E. (1969) in *Exploitable Molecular Mechanisms and Neoplasia*, p. 412, Williams and Wilkins Co., Baltimore

Atkinson, D. E. & Walton, G. M. (1967) *J. Biol. Chem.* **242**, 3239–3241

Bailey, E., Taylor, C. B. & Bartley, W. (1968) *Nature (London)* **217**, 471–472

Bartley, W., Dean, B., Taylor, C. B. & Bailey, E. (1967) *Biochem. J.* **103**, 550–555

Bergmeyer, H. U. (1963) *Methods of Enzymatic Analysis*, pp. 215–459, Academic Press Inc., New York

Bücher, T. & Pfleiderer, G. (1955) *Methods Enzymol.* **1**, 435–440

Carroll, N. V., Longley, R. W. & Roe, J. H. (1956) *J. Biol. Chem.* **220**, 583–593

Cartier, P., Leroux, J.-P. & Temkine, H. (1967) *Ann. Biol. Clin.* **25**, 791–813

Fiske, C. H. & SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375–400

Greenbaum, A. L., Gumaa, K. A. & McLean, P. (1971) *Arch. Biochem. Biophys.* **143**, 617–663
Kemp, R. G. (1971) *J. Biol. Chem.* **246**, 245–252
Krebs, H. A. & Eggleston, L. V. (1965) *Biochem. J.* **94**, 3c–4c
Krebs, H. A. & Veech, R. L. (1969) *Advan. Enzyme Regul.* **7**, 397–413
Leutenhardt, F. & Wolf, H. P. (1955) *Methods Enzymol.* **1**, 320–322
Moellering, H. & Gruber, W. (1966) *Anal. Biochem.* **17**, 369–376
Nelson, N. (1944) *J. Biol. Chem.* **153**, 375–380
Niemeyer, H., Perez, N. & Codeceo, R. (1967) *J. Biol. Chem.* **242**, 860–864
Novello, F., Gumaa, K. A. & McLean, P. (1969) *Biochem. J.* **111**, 713–725
Opie, L. H. & Newsholme, E. A. (1967) *Biochem. J.* **103**, 391–399
Potter, V. R., Gebert, R. A. & Pitot, H. C. (1966) *Advan. Enzyme Regul.* **4**, 247–265
Salas, M., Vinuela, E. & Sols, A. (1963) *J. Biol. Chem.* **238**, 3535–3538
Sharma, C., Manjeshwar, R. & Weinhouse, S. (1963) *J. Biol. Chem.* **238**, 3840–3845
Sillero, M. A. G., Sillero, A. & Sols, A. (1969*a*) *Eur. J. Biochem.* **10**, 345–350
Sillero, A., Sillero, M. A. G. & Sols, A. (1969*b*) *Eur. J. Biochem.* **10**, 351–354
Takeda, Y., Inoue, H., Honjo, K., Tanioka, H. & Daikuhara, Y. (1967) *Biochim. Biophys. Acta* **136**, 214–222
Tanaka, T., Harano, Y., Sue, F. & Morimura, H. (1967) *J. Biochem. (Tokyo)* **62**, 71–91
Taylor, C. B. & Bailey, E. (1967) *Biochem. J.* **102**, 32c–33c
Taylor, C. B. & Bew, M. (1970) *Biochem. J.* **119**, 797–798
Tepperman, J. & Tepperman, H. M. (1970) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **29**, 1284–1293
Veech, R. L., Raijman, L. & Krebs, H. A. (1970) *Biochem. J.* **117**, 499–503
Vinuela, E., Salas, M. & Sols, A. (1963) *J. Biol. Chem.* **238**, PC 1175–PC 1176
Walker, D. G. (1963) *Biochim. Biophys. Acta* **77**, 209–226
Weber, G. (1969) *Advan. Enzyme Regul.* **7**, 15–40
Weber, G. & Singhal, R. L. (1965) *Life Sci.* **4**, 1993–2002
Weber, G., Stamm, N. B. & Fisher, E. A. (1965) *Science* **149**, 65–67
Weber, G., Singhal, R. L., Stamm, N. B. & Lea, M. A. & Fisher, E. A. (1966*a*) *Advan. Enzyme Regul.* **4**, 59–81
Weber, G., Lea, N. A., Fisher, E. A. & Stamm, N. B. (1966*b*) *Enzymol. Biol. Clin.* **7**, 11–24
Woods, H. F., Eggleston, L. V. & Krebs, H. A. (1970) *Biochem. J.* **119**, 501–510