Biochem. J. (1974) **144**, 149–160
Printed in Great Britain

Enzyme Synthesis in the Regulation of Hepatic 'Malic' Enzyme Activity

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(*Received* 15 *May* 1974)

A homogeneous preparation of 'malic' enzyme (EC 1.1.1.40) from livers of thyroxine-treated rats was used to prepare in rabbits an antiserum to the enzyme that reacts monospecifically with the 'malic' enzyme in livers of rats in several physiological states. Changes in enzyme activity resulting from modification of the state of the animal are hence due to an altered amount of enzyme protein. The antiserum has been used to precipitate out 'malic' enzyme from heat-treated supernatant preparations of livers from both adult and neonatal rats, in a number of physiological conditions, that had been injected 30min earlier with L-[4,5-^{3}H]leucine. The low incorporations of radioactivity into the immunoprecipitable enzyme have permitted the qualitative conclusion that changed enzyme activity in adult rats arises mainly from alterations in the rate of enzyme synthesis. The marked increase in 'malic' enzyme activity that occurs naturally or as a result of thyroxine treatment of the weanling rat is likewise due to a marked increase in the rate of enzyme synthesis possibly associated with a concurrent diminished rate of enzyme degradation.

An important role of hepatic 'malic' enzyme [L-malate–$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.40] in the conversion of carbohydrate into fat in the adult rat has been well established by studies of its adaptation under a number of differing physiological conditions. Observations include the rise in 'malic' enzyme activity that follows the administration of a high-carbohydrate, low-fat diet and/or of thyroxine (Wise & Ball, 1964; Tarentino *et al.*, 1966) and a lowering of activity in starvation and in the alloxan-diabetic animal (Shrago *et al.*, 1963; Wise & Ball, 1964). Its function in certain situations has, however, been questioned (Kornacker & Ball, 1965; Wada *et al.*, 1968; Frenkel *et al.*, 1972).

The enzyme first appears in significant quantities in the developing rat liver at that stage in postnatal life around 18 days after birth when the thyroid gland becomes active (Beltz & Reineke, 1968) and when there is an increased metabolic emphasis on lipogenesis that is associated with the transition from the high-fat milk diet to the solid high-carbohydrate diet at weaning. The importance of thyroxine in the initial development of rat hepatic 'malic' enzyme and the subsequent involvement of the diet in determining the final activity has been indicated by the studies of Hemon (1968), Vernon & Walker (1968*a*,*b*), Lockwood *et al.* (1970) and Greengard & Jamdar (1971). There are similarities between the factors that cause increases in the activity of 'malic' enzyme in the adult rat liver and its appearance in the weanling rat.

The work reported in this paper was aimed to establish the role of changing rates of enzyme protein synthesis on the observed changes in enzymic activity associated with both contrived and natural developmental modifications of the physiological environment. The preparation of an antiserum to rat liver 'malic' enzyme is described. This is first characterized and subsequently used to study the incorporation *in vivo* of [^{3}H]leucine into 'malic' enzyme by specific precipitation of the enzyme as enzyme–anti-enzyme complex from heat-treated liver supernatant preparations. The results indicate that markedly changing rates of enzyme synthesis are largely responsible for the changes in hepatic 'malic' enzyme activity observed both during adaptation in the adult rat and during development. A preliminary report of this work has been given (Murphy & Walker, 1972).

Materials and Methods

Animals

The rats were of a Wistar albino strain. Those used as the source of liver for purification of 'malic' enzyme were aged 35–40 days, of either sex, and were fed *ad libitum* for 5 days before being killed, on a high-carbohydrate diet similar in composition to that containing sucrose described by Walker & Eaton (1967) and supplemented with powdered thyroxine at an approximate dosage of 1mg/rat per day.

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In the studies on the rate of synthesis of 'malic' enzyme, animals aged 30–35 days (about 100g normal weight) that had been weaned at 21 days were used; they have an hepatic 'malic' enzyme specific activity around or just above that of more mature adult animals. Alloxan-diabetic rats were prepared by administration of a single intraperitoneal dose of alloxan (22mg/100g body wt. in 0.1M-sodium citrate buffer, pH5.2) to animals previously starved overnight; rats with blood glucose concentrations greater than 400mg/100ml of blood at 11 days after injection were used. The thyroxine-treated rats received an intraperitoneal injection of sodium L-thyroxine dissolved in 0.9% NaCl at pH8.5 at 12 noon daily for the designated length of treatment (see the Results section). The normal, alloxan-diabetic and thyroxine-treated animals were all fed *ad lib* on a pelleted diet [Oxoid pasteurized breeding diet; Herbert Styles (Bewdley) Ltd., Bewdley, Shropshire, U.K.]. Rats starved for 48h were kept (at 28–30°C) in wire-bottomed metabolism cages and allowed water *ad lib*.

For the investigation of the normal and thyroxine-stimulated development of the enzyme in the livers of suckling and weaned rats an initial study of enzymic activity in animals aged 5–40 days at 5-day intervals was made. Litters of ten young rats were divided at random into two groups. One group was treated by intraperitoneal injection of sodium L-thyroxine in 0.9% NaCl, pH8.5, at a dose of 50μg/100g body wt., given daily at 12 noon for 3 days, i.e. 69, 45 and 21h before death; the other group was untreated. The whole litter remained with the mother up to 21 days of age, when they were weaned. There was no difference in total weight gain between the treated and untreated groups of animals either before or after weaning.

The incorporation of [^{3}H]leucine into 'malic' enzyme was studied in normal and thyroxine-treated rats of 9, 15 and 23 days of age that had been treated in an identical fashion to those above. The early-weaned animals were removed from the mother at 16 days and given a crumbled form of the manufactured rat diet. This group lost weight for about 2 days after weaning, but after a further 2 days weight gain became normal. Weaning on to an artificial solid milk diet was effected by the procedure described by Vernon & Walker (1968*b*), by using the diet described by Pinto & Bartley (1967), until the animals were 30 days old. During this time the gain in body weight was similar to normal, although the animals appeared somewhat obese.

Assay procedures

Homogenates of liver tissue were prepared in 0.25M-sucrose at the concentrations given in the Results section. The medium was adjusted to pH7.2 with 0.002M-potassium phosphate (final concn.) in the preparations used in immunological studies. Supernatant fractions were prepared by centrifugation at 105000*g* in an MSE Super-Speed 50 centrifuge for 1h.

The concentration of protein in this supernatant fraction and during the early stages in the purification of 'malic' enzyme was determined by a biuret method (Gornall *et al.*, 1949). Protein present in solutions from later stages in the purification, in sucrose-density-gradient fractions and in antigen–antibody precipitates was assayed by the method of Lowry *et al.* (1951) with dried bovine albumin [Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, U.K.] as the standard.

$NADP^+$-specific malate dehydrogenase ('malic' enzyme) activity was assayed at 26°C by a slight modification of the system used by Hsu & Lardy (1967); the incubation medium contained 80mM-triethanolamine–HCl buffer, pH7.4, 2mM-$MnCl_2$, 2mM-potassium L-malate and 0.5mM-$NADP^+$. Studies at pH6.5 and pH8.4 were performed at the same concentration of imidazole and glycine buffers respectively. Enzyme activity is expressed as μmol of NADPH produced/min per mg of protein or per g of tissue. Kinetic data on initial velocity against malate concentrations for crude 'malic' enzyme preparations were analysed by means of a FORTRAN programme that gave an estimate of the K_m for malate from an unweighted linear least-squares regression analysis of the Lineweaver–Burk double-reciprocal plot and from a non-linear unweighted fit of observed rates to the Michaelis–Menten hyperbolic curve.

Purification of 'malic' enzyme and tests for homogeneity

The enzyme was prepared from the livers of rats fed on a high-sucrose, thyroxine-supplemented diet, by the method of Hsu & Lardy (1967) for pigeon liver 'malic' enzyme, but with a few modifications that resulted in consistently higher yields. A typical purification scheme is shown in Table 1. All steps were performed in the presence of 0.01M-2-mercaptoethanol unless otherwise stated. The heat treatment was carried out at pH6.0 and this was followed by 20–30% and 21–35% (v/v) ethanol fractionations. The enzyme was further purified by dialysis of the 60%-satd.-$(NH_4)_2SO_4$ supernatant against 75%-satd. $(NH_4)_2SO_4$ for 3h. The material that precipitated was redissolved and chromatographed on DEAE-cellulose and finally purified by chromatography on agarose (Bio-Gel A-1.5m; 100–200mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.). This was performed in a column (2.5cm×95cm) equilibrated with 0.03M-Tris–HCl buffer, pH7.7, containing 0.02M-magnesium acetate and 0.001M-dithioerythritol. 'Malic'

Table 1. *Purification of NADP$^+$-specific malate dehydrogenase from rat liver*

Summary of a typical purification from 125g of liver from hyperthyroid rats, as described in the text. Enzyme activity units and specific activity are defined in the Materials and Methods section.

Stage	Description	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)
1	Supernatant fraction of liver homogenate	265	9800	3010	0.31	100
2	Supernatant after heat treatment	222	2885	2290	0.80	76
3	First ethanol fractionation (20–33%)	145	666	1950	2.9	65
4	Second ethanol fractionation (21–35%) after dialysis	35	123	695	5.7	23
5	$(NH_4)_2SO_4$ fractionation (60–75%-satd.)	2.1	34	488	14.4	16
6	Eluate from DEAE-cellulose column	3.5	13.5	269	19.9	9
7	Eluate from agarose column	40.5	8.4	194	23.0	6.5

enzyme activity was eluted as a symmetrical peak in a few fractions that were found to contain constant specific activity; the fractions containing high enzyme activity were pooled and concentrated by vacuum dialysis to approx. 3–4mg of protein/ml. Higher protein concentrations could not be achieved without causing precipitation of the enzyme.

Sucrose-density-gradient centrifugation of purified hepatic 'malic' enzyme, used as a method of assessing the homogeneity of the preparation, was carried out as described by Martin & Ames (1961). Polyacrylamide-gel electrophoresis was carried out on 5% (w/v) gel slabs (Akroyd, 1967) and discs (Davis, 1964) in the presence of 0.01M-Tris–glycine buffer, pH8.6, or 0.05M-sodium acetate buffer, pH4.5. Electrophoresis in a pH-gradient gel was performed by using a modification of the method of Awdeh *et al.* (1968). After establishment of the gradient and application of samples, the gels (3%, w/v) containing 2% (w/v) Ampholine, pH3–10 (LKB Instruments Ltd., Croydon, U.K.), were electrophoresed for 21–23h at 200–300V, at a starting current of 15mA. The gel was then cut into strips for staining.

Protein in slab and disc gels was detected by staining with Amido Black, but in pH-gradient gels with Bromophenol Blue (Awdeh *et al.*, 1968). 'Malic' enzyme activity in the gels was detected by a procedure adapted from the histochemical method of Henderson (1966).

Preparation of and studies on anti-('malic' enzyme)

Preparations of 'malic' enzyme were emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Chicago, Ill., U.S.A.) and injected into the foot-pads of New Zealand White rabbits. A primary injection of 2mg of enzyme protein was given in the left pad and a secondary injection of 3mg in the right pad 21 days later. Test bleedings at intervals after the second injection indicated a high titre of antibody 3 weeks later and about 50ml of blood was withdrawn from an ear vein at weekly intervals for a further 5 weeks. Serum was prepared as described by Chase (1967), pooled and dialysed against 0.15M-NaCl and then stored at −15°C. Before use, thawed serum was centrifuged at 10000*g* for 30min to remove any precipitated or fatty material.

Ouchterlony double-diffusion analyses and quantitative precipitin titrations were carried out as described by Ouchterlony (1964) and by Kabat & Mayer (1961) respectively.

Molecular-weight determinations by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis were performed as described by Weber & Osborn (1969). Radioactively labelled 'malic' enzyme antigen–antibody precipitates and control precipitates (prepared with normal rabbit serum) were dissociated and solubilized by incubating in 4% (w/v) sodium dodecyl sulphate, 4M-urea, 1% (v/v) 2-mercaptoethanol, all in 0.01M-sodium phosphate buffer, pH7.2, at 100°C for 1min and then at 37°C overnight. Polypeptide markers of known molecular weight prepared as a mixture of commercially available purified proteins, together with purified rabbit immunoglobulin G (a gift from Dr. L. Mole, Department of Biochemistry, University of Oxford) were dissociated under identical conditions. After electrophoresis in 0.1M-sodium phosphate buffer, pH7.2, containing 0.1% (w/v) sodium dodecyl sulphate for 8h at 8–9mA per gel, protein in the electrophoretograms was detected by staining with Coomassie Blue. The position of the 'malic' enzyme protein was located by comparison of the staining pattern of samples of the antigen–antibody complex with those of the control and immunoglobulin G preparations and by cutting out the protein bands, dissolving them in 0.5ml of 30% (v/v) H_2O_2 at 37°C for 24h and counting the radioactivity after the addition of 5ml of Unisolve I scintillation fluid (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.).

Radioisotope-incorporation studies and calculation of relative rates of 'malic' enzyme synthesis

Both adult and young rats were injected intraperitoneally with 0.2mCi of L-[4,5-^{3}H]leucine, specific radioactivity 15Ci/mmol (The Radiochemical Centre, Amersham, U.K.)/100g body wt. at 30min before death. Supernatant preparations from 25% (w/v) liver homogenates were prepared and samples were taken for measurement of the incorporation of radioactivity into total soluble protein. This was precipitated initially from 0.2ml portions, in triplicate, of supernatants with 10% (w/v) trichloroacetic acid. The precipitates were treated to remove nucleic acid and lipid material by washing three times with 5ml of cold 5% (w/v) trichloroacetic acid, then with 5ml of 5% trichloroacetic acid at 90°C for 15min, then with 5ml of aq. 95% (v/v) ethanol containing 1% (w/v) potassium acetate, then twice with 5ml volumes of ethanol–ether–chloroform (2:2:1, by vol.) and finally twice with 5ml portions of ether. The precipitates were then dissolved in 1ml of 1M-Hyamine in methanol (Koch–Light) at 60°C (over 1–2h) and transferred to scintillation vials containing 10ml of toluene scintillation fluid [4g of 2,5-diphenyloxazole and 100mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre] for measurement of radioactivity. Liquid-scintillation counting was carried out as soon as possible, because of a slow increase in colour due to a reaction between the Hyamine and protein, in a Nuclear–Chicago Automatic Counter no. 725. Corrections for efficiency of counting were made by the channels-ratio method, which had previously been shown to be suitable for this system.

Before treatment with the antibody to precipitate specifically the 'malic' enzyme, liver supernatants were treated by incubation at 37°C for 1h in the presence of 0.02M-$MgCl_2$ followed by centrifugation to remove any denatured protein. Enzymic activity was unchanged by this heat treatment, but considerable non-specific precipitation at a later stage was thus prevented. The treated supernatants were made 0.09M with respect to NaCl and divided between three types of incubations, each of which was performed either in duplicate or in triplicate: (A) antigen–antibody precipitation; (B) controls to measure non-specific precipitation; (C) controls to measure non-specific adsorption. The volume of the enzyme samples was 3–4ml. A heat-treated (as above) supernatant preparation from the livers of rats that had been fed on a high-sucrose, thyroxine-containing diet, i.e. from animals with high 'malic' enzyme activity, was added to bring the total enzyme activity to 5 units in incubations A and B and to 10 units in incubation C. The supernatants were then incubated with (A) 0.2ml of anti-('malic' enzyme) serum, or (B) with 0.2ml of control serum or (C) with 0.4ml of anti-('malic' enzyme) serum, in a total volume of 8.0ml at 37°C for 30min and at 4°C for 16h. The amount of antiserum used in this standard procedure is approximately twice that required to neutralize the total enzyme activity in the incubations, as determined by a standard quantitative precipitin test (see the Results section). The resulting precipitates were collected by centrifugation and samples of the supernatant fluids were examined to verify either the presence of the initial 'malic' enzyme activity or the absence of 'malic' enzyme activity as appropriate. The precipitates were washed first with 5ml and then with two further 3ml portions of cold 0.15M-NaCl adjusted to pH7.2 with phosphate buffer, drained and dissolved in 0.5ml of 1M-Hyamine in methanol at 60°C. Radioactivity was measured by liquid-scintillation counting as above.

The radioactivity in incubation A was corrected for the non-specific precipitation of radioactivity given by incubation B and for the non-specific adsorption due to added unlabelled enzyme (Cho-Chung & Pitot, 1968); the latter is given by the difference in radioactivity in incubation C and incubation A, which is a measure of the non-specific adsorption of radioactivity on to the complex formed between 5 units of unlabelled enzyme and antiserum. In the Results quoted below the latter correction for non-specific adsorption was around 5% and always less than 10% of the total counts in incubation A; the correction given by incubation B varied from less than 10%, when the amounts of radioactivity incorporated in incubation A and the rates of enzyme synthesis were high (e.g. in hyperthyroid animals), to a figure not over 35% when the incorporations and rates of synthesis were low (e.g. in diabetic and suckling animals).

Results

Nature of 'malic' enzyme in livers of normal and hyperthyroid rats

Because it was intended to use the livers of hyperthyroid rats for the preparation of the pure enzyme required to make an antibody to it, some non-immunological tests on the common identity of the enzyme in the normal and hyperthyroid states were performed. (*a*) Portions of a supernatant preparation of normal rat liver containing 1.08units of enzyme activity/ml were mixed in seven different proportions ranging from 1:10 to 5:1 (v/v) with portions of a similar preparation from hyperthyroid rat liver (1.34units/ml). The activities determined, after suitable dilution, were not significantly different from the calculated values. (*b*) The apparent K_m values for malate were determined at pH7.4 and at four concentrations of Mn^{2+} (0.5, 1, 5 and 10mM) on a

supernatant preparation of liver from a normal and a hyperthyroid rat and on a mixture of the two preparations containing equal enzymic activities of each. No significant differences between the results were found; the mean value of 20 results, each of which was the average of three determinations, was 0.35±0.04mM, which in turn compares favourably with that determined on the pure enzyme, 0.32mM-malate. (*c*) The relative activities of the crude enzyme from livers of normal and hyperthyroid rats, or a mixture of equal activities of the two preparations, were found to be identical at three different pH values (6.5, 7.4 and 8.4) each in the presence of 0.1, 1.0, 10 or 100mM-malate. (*d*) Supernatants and heat-treated supernatant preparations from livers of normal and hyperthyroid rats and mixtures of the two showed identical migration of the component staining for 'malic' enzyme activity after electrophoresis at pH8.6 in 5% (w/v) polyacrylamide gel. The 'malic' enzyme band corresponded to the single protein band obtained after electrophoresis of the purified rat liver enzyme under the same conditions.

Further evidence for the identical nature of normal and hyperthyroid rat liver 'malic' enzyme comes from the similarity of their immunological reaction with the specific anti-('malic' enzyme) serum described below.

Homogeneity of the purified 'malic' enzyme

The 'malic' enzyme preparation from hyperthyroid rat liver, as eluted from the Bio-Gel column (Table 1) and concentrated to 4mg of protein/ml, was found to sediment as a single symmetrical peak during density-gradient ultracentrifugation. Samples of the purified enzyme (0.5 and 1.0mg) were analysed by centrifugation at 130000*g* for 18h in a gradient of 4.6ml of 5–20% (w/v) sucrose in 0.05M-Tris–HCl buffer, pH7.4 (Martin & Ames, 1961). On collection of 9-drop fractions the enzyme was found to be present in three or four fractions at constant specific activity.

Samples (25–200μg) of the purified enzyme showed a single band that stained for both protein and enzymic activity after electrophoresis at pH8.6 in polyacrylamide gel. Isoelectric focusing of freshly purified enzyme on polyacrylamide gel containing Ampholine, pH range 3–10, also gave a single protein component having 'malic' enzyme activity. It was noted that if the enzyme was first left at 4°C for 5 days or more, then two or three close blurred protein bands were detectable after isoelectric focusing, all having 'malic' enzyme activity; these bands arising during aging of the preparation possibly relate to those observed after electrophoresis in a vertical starch gel by Li *et al.* (1970), which result from the fact that the enzyme is an isologous tetramer (Li, 1972).

Gel electrophoresis in the presence of sodium dodecyl sulphate revealed a single band indicating a subunit molecular weight of 61000 (cf. Saito & Tomita, 1973).

With the Ouchterlony double-diffusion technique, the reaction of differing concentrations of purified 'malic' enzyme against a crude anti-('malic' enzyme) serum (prepared by injection of a rabbit with a partially purified preparation of the enzyme and which gave numerous precipitin bands when made to react with crude enzyme preparations) resulted in a single precipitin band. No such band was detectable when the purified enzyme was diffused against antisera raised to either normal or hyperthyroid crude rat liver supernatant preparations. The antiserum raised to the purified 'malic' enzyme (see below) gave a single precipitin band on double diffusion or immunoelectrophoresis at pH8.6 against the original antigen or another purified enzyme preparation. The band was identical with the single band obtained by diffusion of this anti-('malic' enzyme) serum against a variety of 'malic' enzyme preparations of differing specific activity, including samples from a number of purification stages (Table 1) and material from both normal and hyperthyroid rat liver. The antiserum obtained by re-injection of purified 'malic' enzyme into the same rabbits 5 months after the initial immunization programme showed behaviour during double diffusion that was identical with that of the original antiserum with both crude and purified 'malic' enzyme preparations. Quantitative analysis of the precipitation reaction between purified rat liver 'malic' enzyme and its specific antiserum gave a curve corresponding very closely to those obtained for various crude preparations of the enzyme having a range of specific activities (see Fig. 1 and below).

On no occasion did we observe a faint second band during double immunodiffusion, as has been described by Saito & Tomita (1973). The differences between our results and theirs may be due to a number of factors such as the detailed nature of the antigen or of the antibody. If under different conditions such a second band is obtainable then we must be dealing here with the major band, which, according to Saito & Tomita (1973), is the one that adapts to different physiological conditions. These authors also state that their second faint band can be detected by diffusion of their antisera with supernatants of other tissues. An antiserum produced in our preliminary studies against a slightly impure preparation of hepatic 'malic' enzyme that has a specific activity of 16units/mg was found to react immunologically with kidney and brown adipose tissue, and in addition it gave a faint second band with spleen, kidney, intestinal mucosa and cardiac muscle. However, in agreement with Isohashi *et al.* (1971) we observed no reactivity

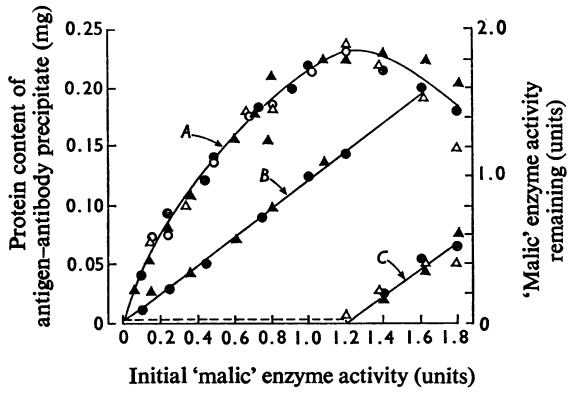

Fig. 1. *Quantitative precipitin curve and estimation of equivalence zone for anti-('malic' enzyme) serum preparation*

Full experimental details are given in the text. The antiserum (25 μl) or control serum was incubated with liver supernatant preparation from rats in various physiological conditions, as below, in a total volume of 2ml. After standing, the antigen–antibody and control precipitates were collected by centrifugation, washed and the quantity of protein in each was determined. The protein contents of the control precipitates were subtracted from the corresponding values for the antigen–antibody precipitates. All the supernatants, after removal of the precipitates, were assayed for enzyme activity. Curve *A*: protein content of antigen–antibody precipitate (corrected). Curve *B*: enzyme activity in supernatants after incubation with control (non-immune) serum. Curve *C*: enzyme activity in supernatants after incubation with anti-('malic' enzyme) serum. ○, Normal adult rat liver (0.03 unit of 'malic' enzyme activity/mg of protein); ●, normal 25-day-old rat liver (0.015unit/mg); ▲, hyperthyroid rat liver (0.2unit/mg); △, liver from adult rats fed on the high-sucrose diet (0.06unit/mg).

(immunoprecipitation) between the antiserum to the liver enzyme and the enzyme in white adipose tissue.

Characterization of the antiserum to purified 'malic' enzyme

The antibody raised against purified rat hepatic 'malic' enzyme showed only one precipitin line with crude preparations of the enzyme from normal and hyperthyroid animals, or a mixture of the two, after Ouchterlony double-diffusion analysis or by immunoelectrophoresis at pH 8.6. The reaction varied in strength according to the specific activity of the particular sample of 'malic' enzyme. Titering out of the antigen or antibody sources, i.e. serial dilution of one reactant and diffusion against a constant amount of the other, thus achieving a range of antigen/antibody ratios within which the optimum for precipitation should occur, revealed no further precipitin reaction. Diffusion of crude liver preparations against serum from unimmunized rabbits gave no detectable reaction. Quantitative measurement of the protein content of 'malic' enzyme antigen–antibody complexes prepared from the reaction of the specific antiserum with preparations of enzyme antigen from normal and hyperthyroid rat liver showed that the peak of complex precipitation and the enzyme–antiserum equivalence zone with respect to inhibition of 'malic' enzyme activity were identical for both these enzyme sources and for the purified enzyme (Fig. 1). This indicates that the antibody precipitated an immunologically identical protein in each case. The changes in enzyme activity resulting from alteration of physiological status, such as high-sucrose feeding or treatment with thyroxine, are therefore due to changes in the amount of the same enzyme protein. A similar conclusion has been reported by Isohashi *et al.* (1971).

Dissociation of representative specimens of 'malic' enzyme–antibody precipitates, prepared from a variety of enzyme sources, with sodium dodecyl sulphate followed by electrophoresis in the presence of this agent always resulted in three major protein components being detectable, two of which corresponded to the light and heavy chains of the γ-globulin antibody (purified rabbit immunoglobulin G fraction). The remaining band was attributed to the 'malic' enzyme antigen, and its common identity in all cases is a further indication of the specificity of the antiserum and of the identical nature of the 'malic' enzyme protein from the livers of animals under different physiological conditions. Further confirmation of this was obtained by dissociation with sodium dodecyl sulphate and electrophoresis of antigen–antibody precipitates from [^{3}H]leucine-labelled liver supernatant preparations from radioisotope incorporation studies *in vivo* (see below); the third band attributed above to 'malic' enzyme was found to contain the majority of the radioactivity detectable in the protein bands detected in the gels. The small amount of remaining radioactivity could be accounted for as that detectable in 'control' gels of that material precipitated non-specifically from the labelled liver supernatant preparations and further treated similarly. The actual number of d.p.m. associated with this latter material varied considerably according to the 'malic' enzyme activity of the preparation; it was higher when a large volume of low-specific-activity supernatant was involved in the standard precipitin reaction (see the Materials and Methods section). All the evidence indicated that the control incubations, which were used to apply corrections as described in the Materials and Methods section, accounted satisfactorily for non-specific precipitation and adsorption.

Table 2. *Rate of synthesis of hepatic 'malic' enzyme in adult rats in various states*

Enzyme activity and the incorporation of L-[4,5-^{3}H]leucine into total soluble protein and immunoprecipitable enzyme was measured as described in the text. The corrections applied are given in the Materials and Methods section. The relative rates of enzyme synthesis are expressed as two parameters as discussed in the text.

Expt. no.	Treatment of animals	No. of animals	Mean body wt. (g)	Mean liver wt. (g)	Total liver soluble protein (mg/rat)	Total enzyme activity (units/rat)	$10^3\times$ Specific enzyme activity (units/mg of soluble protein)	Incorporation of radioactivity into: Soluble protein ($10^{-2}\times$ d.p.m./min per 100mg of soluble protein) (*a*)	Incorporation of radioactivity into: 'Malic' enzyme (d.p.m./min per 100mg of soluble protein) (*b*)	Incorporation of radioactivity into: Total 'malic' enzyme (d.p.m./min per rat) (*c*)	Relative rate of 'malic' enzyme synthesis: $10^4\times\frac{b}{a}$	Relative rate of 'malic' enzyme synthesis: $10^4\times\frac{c}{a}$
1	Normal	3	90	4.4	297	7.7	25.9	670	115	350	17.2	52.0
2	Normal	3	99	4.7	363	8.4	23.2	650	64	230	9.8	35.4
3	Thyroxine (100μg/100g body wt.) at 45h and 21h before expt.	3	87	3.8	285	13.3	46.7	690	180	530	26.0	77.0
4	Thyroxine (50μg/100g body wt.) daily for 9 days before expt.	3	101	4.6	383	49.3	129	770	460	1750	59.8	227
5	Thyroxine (50μg/100g body wt.) daily for 9 days before expt.	3	99	4.6	338	45.7	135	1110	370	1230	33.3	111
6	Alloxan-diabetic	3	42	2.4	183	0.77	4.2	265	21	39	7.9	14.7
7	Alloxan-diabetic	4	58	3.3	262	1.65	6.3	295	12	32	4.1	10.8
8	Starved for 48h	5	68	2.6	199	3.7	18.6	980	55	109	5.6	11.1

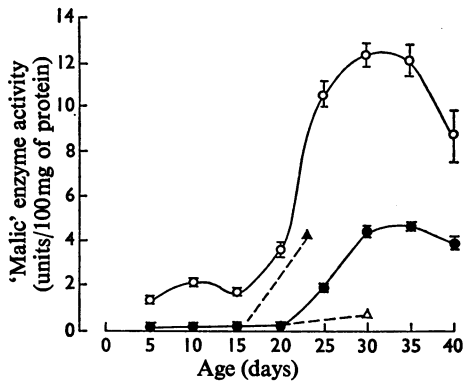

Fig. 2. *Development of hepatic 'malic' enzyme in normal and thyroxine-treated rats*

Animals were weaned and/or treated with thyroxine as described in the text. Enzyme activity was assayed as described in the Materials and Methods section. Each point is the mean of assays on three animals. The vertical bar represents ±1 S.E.M. when large enough to record. ●, Normal animals weaned; ○, animals treated for 3 days with thyroxine before being killed; ▲, animals weaned earlier than normal at 16 days; △, animals weaned on to solid milk diet.

Rates of synthesis of hepatic 'malic' enzyme

(*a*) *Adult rats under different physiological conditions.* The incorporation of L-[4,5-^{3}H]leucine into immunoprecipitable 'malic' enzyme protein relative to the incorporation into total hepatic soluble protein was compared for female rats, aged 30–35 days, in each of four physiological conditions: normal fed; starved for 48h; treated with thyroxine; alloxan-diabetic.

Table 2 shows that treatment with thyroxine for 9 days gave an approximately fourfold rise in the incorporation of the [^{3}H]leucine into 'malic' enzyme protein and a sixfold rise in specific activity of the enzyme compared with the normal animal. Alloxan-diabetes and starvation resulted in a three- to four-fold decrease in the rate of synthesis of 'malic' enzyme associated with lower enzyme activities.

(*b*) *Postnatal rats during the suckling–weaning transition period.* When supernatants from the livers of neonatal rats were incubated alone with the anti-('malic' enzyme) serum or in the presence of added adult rat liver preparation of high 'malic' enzyme activity (i.e. as in the standard precipitin reaction procedure described in the Materials and Methods section), all the enzyme activity present was inhibited, suggesting that the very low activity of 'malic' enzyme in neonatal liver is precipitated by the antiserum and is hence identical with adult enzyme. The standard type of incorporation procedures used for adult animals was therefore applicable for studies in the development period. However, these latter supernatants showed no detectable immunodiffusion reaction with the antiserum, owing to the very small quantity of antigen present.

The developmental profile of the enzyme for our colony of rats was established in order to permit selection of those ages of animals for further study that were likely to provide the most pertinent information about any changes in the relative rates of enzyme synthesis (Fig. 2).

Table 3 indicates that both normal 9- and 15-day-old unweaned animals (Expts. 1, 2 and 3) showed a small but significant incorporation of L-[4,5-^{3}H]-leucine into the specific enzyme protein, standard corrections having been applied, during the first 30min after administration of label. The rate of synthesis of immunoprecipitable material only increased around twofold as a result of thyroxine administration to otherwise similar animals (Expt. 4), although this was associated with a substantially increased 'malic' enzyme activity. After weaning for 2 days the rate of synthesis of 'malic' enzyme had increased markedly in the liver of 23-day-old rats (Expt. 5). A further sevenfold rise in specific activity as a result of thyroxine administration at this age (Expt. 7) was accompanied by a fivefold rise in the rate of synthesis of enzyme protein relative to the normal animal. Early weaning at 16 days (Expt. 6) resulted in a fourfold increase in 'malic' enzyme specific activity and a small rise in the rate of enzyme synthesis. Animals still effectively unweaned at 30 days after maintenance on a solid diet of similar composition to rat milk, i.e. given a high-fat diet (Expt. 8), had considerably lower activities of 'malic' enzyme that were similar to the activity in animals starved for 48h at the same age (Table 2). The rates of [^{3}H]leucine incorporation in these two latter situations were also similar and were considerably less than the rate for normal animals at this age.

Discussion

Experiments have been described that establish, on the basis of certain kinetic parameters such as apparent K_m values and pH effects, the identical nature of 'malic' enzyme in the livers of normal and hyperthyroid rats; this was confirmed by the immunological evidence presented. A measured increase in enzymic activity in a tissue preparation therefore represents a proportional increase in enzyme content. Preparation of the 'malic' enzyme for use as an antigen from the livers of hyperthyroid animals was also thus justified.

The monospecificity of the rabbit antiserum raised against such a homogeneous preparation of

Table 3. *Rate of synthesis of hepatic 'malic' enzyme in neonatal and weanling rats of various ages and treated in various ways*

Enzyme activity and the incorporation of L-[4,5-^{3}H]leucine into total soluble protein and immunoprecipitable enzyme was measured as described in the text. The corrections applied are given in the Materials and Methods section. The relative rates of enzyme synthesis are expressed as two parameters as discussed in the text.

Expt. no.	Treatment of animals	Age of animals (days)	No. of animals	Mean body wt. (g)	Mean liver wt. (g)	Total liver soluble protein (mg/rat)	Total enzyme activity (units/rat)	$10^3\times$ Specific enzyme activity (units/mg of soluble protein)	Incorporation of [^{3}H]leucine into: Soluble protein ($10^{-2}\times$ d.p.m./min per 100mg of soluble protein) (*a*)	Incorporation of [^{3}H]leucine into: 'Malic' enzyme (d.p.m./min per 100mg of soluble protein) (*b*)	Incorporation of [^{3}H]leucine into: Total 'malic' enzyme (d.p.m./min per rat) (*c*)	Relative rate of 'malic' enzyme synthesis: $10^4\times\frac{b}{a}$	Relative rate of 'malic' enzyme synthesis: $10^4\times\frac{c}{a}$
1	Unweaned, normal	9	17	19.2	0.58	37.0	0.038	1.03	1000	87	32	8.7	3.2
2	Unweaned, normal	15	10	32.0	0.94	61.5	0.065	1.10	1000	102	63	10.2	6.3
3	Unweaned, normal	15	10	36.0	1.09	70.0	0.053	0.72	1290	50	32	3.9	2.5
4	Unweaned, thyroxine (50μg/100g body wt.) at 69, 45 and 21h before expt.	15	10	32.8	0.89	57.2	0.79	13.8	1450	175	100	12.0	6.9
5	Weaned at 21 days	23	10	49.4	1.8	112	1.07	9.6	1070	138	150	12.9	14.0
6	Weaned at 16 days	23	8	46.0	2.0	141	5.68	40.0	1060	153	220	14.4	20.7
7	Weaned at 21 days; thyroxine (50μg/100g body wt.) at 69, 45 and 21h before expt.	23	6	50.7	1.7	122	7.67	63.0	1590	1050	1290	66.0	81.0
8	Weaned on to a solid milk diet	30	3	89.0	4.3	298	2.40	8.0	850	32	96	3.8	11.3

rat liver 'malic' enzyme was established by means of Ouchterlony diffusion and immunodiffusion procedures and by quantitative titration of the antiserum with the purified 'malic' enzyme and with that present in crude extracts of livers from animals under various physiological conditions. Electrophoretic examination of the enzyme–antiserum complexes after dissociation with sodium dodecyl sulphate indicated that just one protein species had been specifically precipitated from crude liver supernatants by the antiserum. Appreciable interference was observed because of non-specific precipitation only in the situation when a large amount of crude material was required owing to its low enzymic specific activity, but suitable corrections could be applied. The antiserum was thus judged to be suitable for use in the subsequent radioisotope-incorporation studies.

Schimke (1964) argued that [^{14}C]guanidinoarginine is the most suitable amino acid for studies on amino acid incorporation into protein in ureotelic species, but our preliminary studies revealed that the rate of arginine breakdown was too rapid to permit sufficient incorporation into 'malic' enzyme protein even when maximum labelling of the total soluble protein might be expected. The use of [^{3}H]leucine results in a less accurate measurement because of reutilization (Arias *et al.*, 1969; Kuehl & Sumsion, 1970). Further, it proved necessary to use comparatively large amounts of radioactivity in order to obtain a sufficient incorporation (see Tables 2 and 3) because of the rather low turnover rate of the enzyme (Tarentino *et al.*, 1966) and the low absolute amount of enzyme protein in liver. Although this had the advantage of minimizing any disturbance of the free amino acid pool size (Neuberger & Richards, 1964) it did not make measurements of rates of degradation practicable; higher initial radioactivities would have been excessively costly. Further studies therefore had to be restricted to the measurement of rates of synthesis only, which requires a measurement of incorporation after a single time-period.

The general theories available to describe the changes in enzyme concentration during adaptation (e.g. Segal & Kim, 1963) assume that the rate of synthesis of an enzyme changes to a new value as a result of a stimulus and then remains the same throughout the period of adaptation and into the final new steady-state situation, an assumption that may not always be valid (Haining, 1970). In the new steady state

$$K_s - K_d E = 0$$

where K_s is the rate constant for enzyme synthesis (activity units·time^{-1}·mass^{-1}), K_d is the rate constant for enzyme degradation (time^{-1}) and E is the enzyme content (activity units·mass^{-1}). In the studies reported here a new true steady state is never reached, unlike in the studies on which the above type of theory is based. Hence our results for starved and alloxan-diabetic animals represent the situation at a certain stage in a continuing adaptive process. In particular with our work, an approach to a steady state is never likely to be reached in animals that are growing at the remarkable rate exhibited by neonatal rats. Only in the cases of the treatment of weanling animals with thyroxine for 9 days (Tarentino *et al.*, 1966) and of feeding over a period of a high-fat diet may the adaptations be approaching something like a steady-state situation.

Koch (1962) showed theoretically that the above equation can be modified by a factor to permit the rate of growth to be taken into account. Schimke & Doyle (1970) advocated from more practical considerations that the rates of synthesis should be calculated from the amount of radioactivity incorporated from the precursor pool into the specific protein expressed relative to that incorporated into unit total protein. This allows for any difference in the size of the precursor pools available for protein synthesis, assuming that all proteins have access to the same amino acid pool, and for variations in the doses of radioisotope administered. Such a procedure has been used in the calculations in Tables 2 and 3.

The pattern of results for the young adult rats (Table 2) indicates that the measured rates of enzyme synthesis parallel to a first approximation the observed enzymic activity under several nutritional and hormonal states. This conclusion has also been made by Gibson *et al.* (1972) on the basis of changes of 'malic' enzyme activity resulting from changed nutritional status and on evidence of a similar type to the present study. They found their higher values of K_d in situations of net enzyme formation. For the weanling animal, in which there is a natural rise in plasma thyroxine at about 20 days (Beltz & Reineke, 1968), the marked increases in enzyme activity after administration of thyroxine are also associated with significant increases in the measured rate of enzyme synthesis (Table 3). These increases were, in the cases of both normal weaning and the thyroxine-treated animals, approximately 3.5- and 1.5-fold greater than the rate in the controls respectively, but resulted in much greater increases in enzymic activities. The qualitative implication is that, under these conditions in the developing rat, there may well be a concurrent low rate of enzyme degradation but, as noted above, the lack of steady-state conditions seems to us not to permit quantification of this possibility on the basis of the present results. Philippidis *et al.* (1972) have demonstrated an apparent absence of degradation of phosphoenolpyruvate carboxykinase (EC 4.1.1.32) in the neonatal rat liver at the time of the marked increase in activity

of this enzyme. In a parallel study on chick liver, Silpananta & Goodridge (1971) recorded a 50-fold increase in the rate of synthesis of 'malic' enzyme when neonatal chicks were first fed; the $t_{\frac{1}{2}}$ for the enzyme degradation in the unfed animals was very high, at 350h.

The demonstration of a significant incorporation of radioactivity into 'malic' enzyme of apparent low activity in the suckling rat not only contributes to the low relative increase in synthetic rates noted in the previous paragraph but also raises the possibility that there exists some immunoprecipitable but enzymically inactive precursor form of 'malic' enzyme in the young animals. Such a general possibility was postulated by Moog (1966) and demonstrated for phosphoenolpyruvate carboxykinase in foetal rat liver (Philippidis *et al.*, 1972). The low radioactivity incorporations obtainable for 'malic' enzyme plus the comparatively large corrections that have to be applied in such situations have not permitted us to clarify this point satisfactorily to date, but the amount of such inactive enzyme must be small, and inactivation of the latter could not account for the subsequent large enzymic activities.

On general grounds, Greengard (1970) considers that enzymic differentiation is essentially a problem of changed rates of enzyme synthesis; an alternative situation, where the change is from one where the rates of synthesis and degradation were essentially equal and the enzyme was virtually absent to one in which a lowered rate of enzyme degradation resulted in appearance of the enzyme, seems inherently unlikely. The possibility of the initial activation of a small amount of a precursor form of enzyme followed by synthesis of new enzyme in response to hormonal and dietary stimuli (Greengard & Jamdar, 1971) is more attractive. The mechanisms involved in the regulation of synthesis of 'malic' enzyme and other hepatic enzymes concerned in lipogenesis from glucose in response to nutritional and other factors represent unsolved problems (e.g. Greengard, 1971; Gibson *et al.*, 1972; Philippidis *et al.*, 1972; Gunn & Taylor, 1973).

We are grateful to the Medical Research Council for the award of a Training Studentship to G. M., and to that body, the British Nutrition Foundation and the Wellcome Trust for help towards the costs.

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