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Effects of diet and of alloxan-diabetes on the activity of branched-chain 2-oxo acid dehydrogenase complex and of activator protein in rat tissues

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The total activities (sum of active and inactive forms) of branched-chain 2-oxo acid dehydrogenase complex in tissues of normal rats fed on a standard diet were (unit/g wet wt.): liver, 0.82; kidney, 0.77; heart, 0.57; hindlimb skeletal muscles, 0.034. Total activity was decreased in liver by 9%- or 0%-casein diets and by 48h starvation, but not by alloxan-diabetes. Total activities were unchanged in kidney and heart. The amount of active form of the complex (in unit/g wet wt. and as % of total) in tissues of normal rats fed on standard diet was: liver, 0.45, 55%; kidney, 0.55, 71%; heart, 0.03, 5%; skeletal muscle <0.007, $<20\%$ (below lower limit of assay). The concentration of the active form of the complex was decreased in liver and kidney, but not in heart, by low-protein diets, 48h starvation and alloxan-diabetes. In heart muscle alloxan-diabetes increased the concentration of active complex. The concentration of activator protein (which activates phosphorylated complex without dephosphorylation) in liver and kidney was decreased by 70–90% by low-protein diets and 48h starvation. Alloxan-diabetes decreased activator protein in liver, but not in kidney. Evidence is given that in tissues of rats fed on a normal diet approx. 70% of whole-body active branched chain complex is in the liver and that the major change in activity occasioned by low-protein diets is also in the liver.

In animal tissues degradation of branched-chain amino acids is initiated after transamination (a reversible reaction) by oxidative decarboxylation of branched-chain keto acids to acyl-CoA. This non-reversible reaction is catalysed by the mitochondrial branched-chain 2-oxo acid dehydrogenase complex (abbreviated here to 'branched-chain complex'). A single complex oxidizes the three naturally occurring ketoacids ketoleucine (4-methyl-2-oxopentanoate), ketoisoleucine (L-3-methyl-2-oxopentanoate) and ketovaline (3-methyl-2-oxobutyrate) (Pettit *et al.*, 1978; Parker & Randle, 1978; Heffelfinger *et al.*, 1983).

Purified branched-chain complexes from ox kidney, rabbit liver and rat kidney are inactivated by phosphorylation of serine residues on the α-chain of the decarboxylase component by a kinase intrinsic to the complex (Fatania *et al.*, 1981; Lau *et al.*, 1982; Paxton & Harris, 1982; Odessey, 1982; Cook *et al.*, 1983). Dephosphorylation and re-activation are catalysed by a mitochondrial phosphatase requiring Mg^{2+} (Fatania *et al.*, 1983). This interconversion has been shown also in complex from rat heart and skeletal muscles, rat kidney and rat liver (Parker & Randle, 1978, 1980; Odessey, 1980; Lau *et al.*, 1981). Phosphorylated complex is also re-activated without measurable dephosphorylation by a protein fraction present in liver and kidney mitochondria, and named 'activator protein' (Fatania *et al.*, 1982; Lau, 1983). Activator protein has no effect on dephosphorylated complex and has not been detected in heart or skeletal-muscle mitochondria.

Reversible phosphorylation of branched-chain complex presumably adjusts the rate of degradation of branched-chain keto-acids in relation to dietary intake. Activator protein is assumed to facilitate oxidation of branched-chain keto acids in liver and kidney relative to that in muscles (Randle *et al.*, 1984). The purpose of the present study was to develop reliable methods for the assay of branched-chain complex (active form, inactive form) and activator protein in rat tissues and to investigate effects of diet and of alloxan-diabetes. Because the total concentration of branched-chain complex in rat tissues is low, it was necessary to concentrate the complex before spectrophotometric assay. This has been achieved by isolating complex in mitochondria, under conditions which block interconversion of active and inactive forms.

This has the advantage of avoiding losses owing to proteolysis or to fractionation when the complex is concentrated from tissue extracts in which mitochondria have been disrupted. Gillim *et al.* (1983) have described the effects of diet on concentrations of active and inactive forms of branched-chain complex, with freeze-clamped tissue samples. However, that study overlooked the role of activator protein in liver and kidney, and evidence is given here that their recovery of complex was incomplete and that some conversion of inactive complex into active complex may have occurred before assay. The present study provides, for the first time, evidence for important effects of diet on the activity of activator protein.

Experimental

Materials

Details of source, feeding (standard diet), starvation and induction of alloxan-diabetes in male albino Wistar rats (220–350g) are given in Kerbey *et al.* (1977). Special diets containing 80% casein, 9% casein and 0% casein were provided by Special Diet Services Ltd., Witham, Essex, U.K.; the formulae were as given by Wohlhueter & Harper (1970). The diets were given in powdered form *ad libitum* for a period of 10-14 days. Weight gain on the 80%-casein diet did not differ significantly from that on standard diet (results not shown). Rats on the 0%-casein diet lost weight (-8.6 ± 0.77% over 7 days; mean ± S.E.M.). Rats on the 9%-casein diet gained weight, but the weight gain was significantly less than on standard or 80%-casein diets (results not shown).

Sources of other chemicals and biochemicals were as given by Parker & Randle (1978, 1980) and Coore *et al.* (1971).

Mitochondria

All operations were at 0°C. Rat liver and kidney mitochondria were prepared by the method of Hogeboom *et al.* (1948) in 0.25M-sucrose/5mM-Tris/HCl/2mM-EGTA, pH7.5. Rat heart mitochondria were prepared either with Nagarse (Chappell & Hansford, 1971) or in the absence of Nagarse by the use of a Polytron homogenizer as described by Kerbey *et al.* (1976). Skeletal-muscle mitochondria were prepared by the method of Chappell & Perry (1954). For assay of active complex and total complex (sum of active and inactive forms), tissues were removed and homogenized in the appropriate medium, either in the presence or in the absence of ketoleucine (5mM) and NaF (100mM). This procedure took approx. 15s. Heart mitochondria were isolated without washing (10min at 800*g*, 10min at 10000*g*). Skeletal-muscle mitochondria were resuspended once and again centrifuged twice (10min at 800*g*, 10min at 10000*g*). Liver and kidney mitochondria were resuspended twice and sedimented on each occasion at 10000*g* for 10min.

Extracts

For assay of branched-chain complex and citrate synthase, mitochondria isolated with NaF and ketoleucine were freed of supernatant and frozen immediately (liquid N_2). Mitochondria isolated in the absence of NaF and ketoleucine were taken up either in sucrose medium (see the preceding section) or (skeletal muscle) in KCl medium (see Kerbey *et al.*, 1976). Conversion of inactive complex into active complex was effected by incubation in KCl medium (2mg of mitochondrial protein/ml) at 30°C for 30min. Control experiments showed that maximum activities were achieved in 20min (heart, liver and kidney) or 30min (skeletal muscle). Mitochondria were then sedimented (Eppendorf 3200 centrifuge), and the supernatant was aspirated, frozen in liquid N_2 and stored in liquid N_2 before extraction and assay. For assay of activity in mitochondria as made, mitochondria were added to KCl medium and sedimented immediately. For assay of branched-chain complex and citrate synthase, extracts were prepared by freezing and thawing (three times) in 30mM-potassium phosphate/5mM-EGTA/2mM-dithiothreitol/1% (v/v) ox serum/0.2mM-thiamin pyrophosphate, pH7.4, as described by Lau *et al.* (1981).

For assay of activator protein, extracts of liver and kidney mitochondria (50mg of protein/ml) were prepared in extraction buffer (as above, but minus thiamin pyrophosphate) containing in addition 10mM-benzamidine, 1mM-phenylmethanesulphonyl fluoride and 1% (v/v) ethanol. The extracts were sedimented for 2h at 150000*g* and the supernatant (containing activator protein) was aspirated.

Whole-tissue citrate synthase was assayed as in Kerbey *et al.* (1976).

Analytical methods

Mitchondrial protein was assayed by the method of Gornall *et al.* (1949). Branched-chain complex was assayed by the method of Lau *et al.* (1981), with 1mg of mitochondrial protein per assay. Citrate synthase was assayed by the method of Srere *et al.* (1963) as modified by Coore *et al.* (1971).

Activator protein was assayed by the degree of reactivation of phosphorylated (inactive) ox kidney branched-chain complex as described by Fatania *et al.* (1982). Initial assays used an amount of supernatant fraction equivalent to 2mg of mitochondrial protein. The amount of supernatant

added in the assay was then adjusted either to give approx. 50% re-activation of phosphorylated complex (50 μl for standard or 80%-casein diets) or up to 200 μl for low-protein diets, starvation, and diabetes. Activation by activator protein conforms to Michaelis-Menten kinetics (Fatania *et al.*, 1982).

Procedure and calculations

Experiments were completed in a single day, i.e. mitochondria were prepared and incubated, extracts prepared and assays performed. Activities of branched-chain complex and citrate synthase were calculated per mg of mitochondrial protein, and the ratio of munits of branched-chain complex/unit of citrate synthase was computed. One unit of enzyme forms 1 μmol of product/min at 30°C. Activities of activator protein were calculated as $K_{0.5}$, the amount (mg) of mitochondrial protein required to give 50% re-activation of phosphorylated complex in the assay. The relative activity was then computed from the formula: $(100K^1_{0.5}/K^2_{0.5})$ where $K^1_{0.5}$ is the $K_{0.5}$ for normal rats on standard diet and $K^2_{0.5}$ is the $K_{0.5}$ for the second group (i.e. changed diet or alloxan-diabetes).

Results and discussion

Effect of method of preparation of mitochondria on the concentration of branched-chain complex

Heart and skeletal-muscle mitochondria may be prepared either by using proteinases (Nagarse or trypsin) or by mechanical disruption in their absence. The methods have been compared for two reasons. In heart mitochondria prepared with Nagarse total activity of branched-chain complex is variable and can range from 1–4 munits/mg of mitochondrial protein (results not shown; see also Parker & Randle, 1978, 1980; Lau *et al.*, 1981). It seemed possible that different degrees of inactivation by Nagarse may be responsible. In the present study NaF and ketoleucine were used to inhibit reversible phosphorylation of the complex, and speed was essential. Preparation of mitochondria is quicker in the absence of Nagarse.

The results of this comparison are shown in Table 1. The total activity of branched-chain complex in heart mitochondria prepared without Nagarse (Polytron) was approx. 3-fold greater than in heart mitochondria prepared with Nagarse. The proportion of active complex in freshly prepared mitochondria was unaffected (25 ± 3%, Nagarse; 21 ± 2%, Polytron; means ± S.E.M. for nine observations). In Table 1 concentrations of branched-chain complex are given per unit of citrate synthase. Activities in munits/mg of mitochondrial protein (means ± S.E.M., nine observations) were 5.8 ± 0.15 (Polytron) and 2.3 ± 0.2 (Nagarse). The activity of citrate synthase was unaffected by the method of preparation (results not shown). It is known from other studies that the total activity of pyruvate dehydrogenase complex is not affected by Nagarse [cf. Kerbey *et al.* (1976) and Hutson & Randle (1978)]. It is suggested that Nagarse may inactivate branched-chain complex in heart mito-

Table 1. *Activity of branched-chain 2-oxo acid dehydrogenase complex in rat heart mitochondria; effect of method of preparation and of fluoride (100 mM) + ketoleucine (5 mM)*

Rat heart mitochondria were prepared in sucrose medium either with Nagarse or by mechanical disruption without Nagarse (Polytron). In Expt. 3(*b*) sucrose medium contained, in addition, 100 mM-NaF and 5 mM-ketoleucine. Mitochondria were added to KCl medium (2 mg of mitochondrial protein/ml) and, except in Expts. 2(*b*) and 2(*c*), were separated by centrifugation either immediately (as made) or after 10 min of incubation at 30°C (no substrate or 5 mM-succinate). In studies on the effect of storage [Expts. 2(*b*) and 2(*c*)] mitochondria were kept in KCl medium for 90 min at 0°C before centrifugation, with [Expt. 2(*c*)] or without [Expt. 2(*b*)] addition of NaF (final concn. 100 mM) plus ketoleucine (final concn. 5 mM). After centrifugation, supernatant was aspirated, pellets were frozen, and extracts were prepared and assayed for branched-chain complex and citrate synthase activities. For details of media and assays, see the Experimental section. Values are means ± S.E.M. for six to nine observations. *$P < 0.01$ for effect of Nagarse; †$P < 0.01$ for effect of NaF + ketoleucine; ‡$P < 0.05$ for effect of storage; for other differences within columns $P > 0.05$.

Expt. no.	Preparation method	Storage and/or addition of NaF + ketoleucine	Branched-chain complex activity (munits/unit of citrate synthase)		
			No substrate	As made	5 mM-succinate
1(*a*)	Nagarse	None	2.0 ± 0.22*	0.49 ± 0.06*	–
(*b*)	Polytron	None	7.2 ± 0.61	1.52 ± 0.15	–
2(*a*)	Nagarse	None	2.0 ± 0.22*	0.49 ± 0.06*	0.55 ± 0.04
(*b*)	Nagarse	90 min at 0°C	1.9 ± 0.15	0.62 ± 0.06	0.35 ± 0.07‡
(*c*)	Nagarse	NaF + ketoleucine; 90 min at 0°C	1.8 ± 0.14	0.64 ± 0.05	0.65 ± 0.04†
3(*a*)	Polytron	None	5.0 ± 0.24	2.05 ± 0.11	–
(*b*)	Polytron	None: NaF + ketoleucine during preparation	–	0.29 ± 0.03†	–

chondria but that citrate synthase and pyruvate dehydrogenase complex are unaffected. The total activity of branched-chain complex in heart mitochondria prepared without Nagarse corresponds to a tissue activity of approx. 600munits/g wet wt. (Table 5). This is substantially higher than the values of <300munits/g wet wt. observed previously (Parker & Randle, 1980; Waymack *et al.*, 1980; Gillim *et al.*, 1983). The total activity of branched-chain complex in skeletal-muscle mitochondria prepared without proteinases was also much greater than in mitochondria prepared with trypsin (results not shown).

Efficacy of fluoride plus ketoleucine in preventing interconversion of active and inactive forms of branched-chain complex

The branched-chain dehydrogenase kinase reaction is inhibited by ketoleucine (Parker & Randle, 1978; Lau *et al.*, 1982) and the phosphatase reaction is inhibited by EDTA (removal of Mg^{2+}) and by fluoride (Fatania *et al.*, 1983). In heart mitochondria the K_i for ketoleucine is approx. 125μM (Randle *et al.*, 1981); the concentration in the present study was $40 \times K_i$. The efficacy of fluoride as an inhibitor of the phosphatase reaction has been shown in liver mitochondria from rats fed on a 0%-casein diet by incubation in KCl medium for 30min at 30°C with or without 25mM-NaF. In the presence of NaF there was no significant change in branched-chain complex activity during incubation (change in activity -4.9 ± 4.5munits/unit of citrate synthase; mean ± S.E.M. for four observations). In the absence of NaF, activity increased (change in activity $+7.7 \pm 1.9$munits/units of citrate synthase; mean ± S.E.M. for 18 observations: $P<0.01$ for difference from zero; $P<0.02$ for effect of NaF). The concentration of NaF used in the present study was 100mM. It was shown by control experiments with extracts of heart mitochondria or purified bovine complex that the concentration of NaF in the cuvette has no effect on the activity of branched-chain complex in the spectrophotometric assay (results not shown).

The efficacy of a combination of fluoride and ketoleucine to prevent interconversion of active and inactive forms of branched-chain complex in isolated heart mitochondria was tested in Expt. 2 (Table 1). Activity of branched-chain complex was measured in extracts of mitochondria separated and frozen immediately or after 90min of storage at 0°C. These conditions (90min at 0°C) cover, with time to spare, those that pertained during the isolation of mitochondria in the present study. In freshly prepared mitochondria or in mitochondria incubated for 30min at 30°C in KCl medium (to effect conversion of inactive complex into active complex), the presence of ketoleucine plus fluoride, during storage, had no effect on branched-chain complex activity. In mitochondria incubated for 10min at 30°C in the presence of 5mM-succinate, complex activity fell during storage at 0°C, and this fall was prevented by ketoleucine plus fluoride. As shown in Table 1 (Expt. 3) and in Tables 2 and 4, the activity of branched-chain complex in heart and liver mitochondria isolated in the presence of ketoleucine plus fluoride was lower than in the same mitochondria isolated in their absence. This indicates that conversion of inactive into active complex occurs during isolation of mitochondria, but not on storage, and that this is prevented by inhibition of the phosphatase reaction with NaF.

Activator protein in liver and kidney mitochondria

The effects of diet and of alloxan-diabetes on the activity of activator protein in liver are shown in column 5 of Table 2. Details of the calculation are given in the Experimental section; for normal rats on the standard diet, mitochondrial supernatant fraction equivalent to 1.8 ± 0.25mg of mitochondrial protein/ml was required for 50% reactivation of phosphorylated complex in the assay. Activator protein was decreased in activity to approx. 10% of the control by low-protein diets and to approx. 30% of the control by starvation (48h) or alloxan-diabetes.

The effects of diet and of alloxan-diabetes on the activity of activator protein in rat kidney mitochondria are shown in the last column of Table 3. For normal rats on a standard diet, mitochondrial supernatant fraction equivalent to 5.4 ± 1.1mg of mitochondrial protein/ml was required for 50% reactivation of phosphorylated complex in the assay. The activity in kidney mitochondria was thus lower than in liver mitochondria. The effects of diet were qualitatively similar in the two tissues, but alloxan-diabetes had no effect on activator protein in kidney, whereas it decreased activity in liver.

Branched-chain complex in liver and kidney mitochondria

Columns 1 and 2 of Table 2 show total activities of branched chain complex in liver mitochondria. These are maximum activities obtained after incubation of liver mitochondria for 30min in the absence of respiratory substrate. The evidence that the increase in activity during incubation results from dephosphorylation of phosphorylated complex is as follows. The conversion was inhibited by fluoride (see above), which inhibits dephosphorylation (Fatania *et al.*, 1983); it displayed a progress curve (results not shown) unlike that for re-activation by activator protein (Fatania *et al.*, 1982); and after conversion activator protein is ineffec-

Table 2. *Activity of branched-chain 2-oxo acid dehydrogenase complex and of activator protein in rat liver mitochondria; effect of diet and of alloxan-diabetes*

The procedure for assay of branched-chain complex (total activity and active form) was as in the legend to Table 4, except that liver was disrupted with a Potter–Elvehjem homogenizer. Activities of citrate synthase averaged 75 munits/mg of mitochondrial protein, and there was no significant effects of diet or diabetes. For assay of activator protein, separate batches of mitochondria were prepared in sucrose medium without NaF and ketoleucine. For further details of methods and procedure, and for details of calculation of activator-protein concentration, see the Experimental section. $K_{0.5}$ for activator protein in normal rats on standard diet was 1.8 mg of mitochondrial protein/ml. Results are means ± S.E.M. for the numbers of observations (livers) shown in parentheses. *$P<0.01$, †$P<0.05$ for difference from normal or 80%-casein diet within columns; for other differences from normal or 80%-casein diets within columns $P>0.05$. ‡$P<0.01$ for effect of NaF + ketoleucine. §$P<0.01$ for difference between standard and 80%-casein diets.

	Branched-chain complex activity				
	Total (active + inactive forms)		Active form (% of total), with NaF + ketoleucine:		
Rat (diet)	(munits/mg of protein) [1]	(munits/unit of citrate synthase) [2]	Absent [3]	Present [4]	Activator protein (% of control) [5]
Normal (standard)	6.8 ± 0.14 (12)	100 ± 2.7 (6)	73 ± 2 (6)	55 ± 4.6‡ (6)	100 ± 14 (16)
Normal (80%-casein)	8.0 ± 0.41 (20)	96 ± 3.0 (20)	73 ± 2 (12)	30 ± 1.5‡§ (10)	98 ± 12 (10)
Normal (9%-casein)	3.3 ± 0.25* (19)	39 ± 2.5* (19)	30 ± 1* (12)	14 ± 1.0*‡	10 ± 1* (10)
Normal (0%-casein)	2.2 ± 0.28* (42)	31 ± 3.5* (21)	41 ± 1* (18)	29 ± 3.3*‡ (4)	9 ± 1* (8)
Normal (48h-starved)	5.2 ± 0.30* (28)	75 ± 3.4* (28)	61 ± 3* (7)	10 ± 0.7*‡ (4)	28 ± 4* (6)
Diabetic (standard)	5.9 ± 0.26* (5)	85 ± 5.4† (5)	40 ± 2* (6)	19 ± 0.7*‡ (8)	30 ± 2* (6)

tive (Fatania *et al.*, 1982). Total activity of complex in normal rats on a standard diet was 6.8 ± 0.14 munits/mg of mitochondrial protein. Comparable values were seen with 80%-casein diet (Table 2) and in previous studies by Lau *et al.* (1981). Total activity of complex was decreased to approx. one-third by low-protein diets (0 or 9% casein) and to approx. 80% by starvation (48h) or alloxan-diabetes. The evidence that the decrease in activity occasioned by diet or diabetes is due to a decrease in total activity and not to incomplete conversion of inactive to active complex is as follows. The time course of conversion was not influenced by diet or by diabetes (results not shown). Similar values for total activity were obtained when conversion was effected by inhibiting the kinase reaction with 5 mM-ketoleucine or with 1 μM-carbonyl cyanide *m*-chlorophenylhydrazone (results not shown).

Columns 3 and 4 of Table 2 show the proportion of complex in the active form in liver mitochondria isolated in the absence (column 3) or presence (column 4) of 100 mM-NaF and 5 mM-ketoleucine. Regardless of diet or type or rat (i.e. normal or diabetic), the proportion of active complex was lowered by NaF and ketoleucine, presumably because NaF inhibited dephosphorylation of the complex during isolation of mitochondria. The proportion of complex in the active form was decreased to approx. 20–50% of the control (normal rat, standard diet) by low-protein diets and by diabetes (Table 2, column 4, results with NaF + ketoleucine). It is not known whether this difference in the proportion of active complex is due to differences in the activity of activator protein (see column 5 of Table 2), or to differences in the proportions of phosphorylated and dephosphorylated complexes, or to both. We have been unable as yet to devise a reliable method of separating complex from activator protein with full recovery. The results in Table 5 show (regardless of mechanism) that low-protein diets, starvation and diabetes decreased the measured activity of branched-chain complex in liver 80–90%. The true change in activity *in vivo* is not known accurately, because of the complex kinetic relationships between re-activation by activator protein and the relative concentrations of activator protein and of phosphorylated branched-chain complex (Fatania *et al.*, 1982).

Table 3 shows activities of branched-chain complex in kidney mitochondria. Total activity (column 1) was 5.5 ± 0.4 munits/mg of mitochondrial protein in normal rats on a standard diet. Comparable values were seen with 80%-casein diet and by Lau *et al.* (1981). There were no consistent effects of low-protein diets, starvation or diabetes on total activity when discrepancies between activities per mg of mitochondrial protein and activities per unit of citrate synthase were taken into account (cf. columns 1 and 2). The reason for these discrepancies is not known. Total activity in

Table 3. *Activity of branched-chain 2-oxo acid dehydrogenase complex and of activator protein in rat kidney mitochondria; effect of diet and of alloxan-diabetes*

The procedure for assay of branched-chain complex (total activity and active form) was as in the legend to Table 4, except that kidney was disrupted with a Potter–Elvehjem homogenizer. Activities of citrate synthase averaged 157munits/mg of mitochondrial protein, and there was no significant effects of diet or diabetes. For assay of activator protein, separate batches of mitochondria were prepared in sucrose medium without NaF and ketoleucine. For further details of methods and procedure, and for details of calculation of activator-protein concentration, see the Experimental section. $K_{0.5}$ for activator protein in normal rats on standard diet was 1.8mg of mitochondrial protein/ml. Results are means ± S.E.M. for the numbers of observations (kidneys) shown in parentheses. *$P<0.01$, for difference from normal or 80%-casein diet within columns; for other differences from normal or 80%-casein diet within columns $P>0.05$. †$P<0.01$ for effect of NaF + ketoleucine. ‡$P<0.01$ for difference between standard and 80%-casein diets.

	Branched-chain complex activity				
	Total (active + inactive forms)		Active form (% of total), with NaF + ketoleucine:		
Rat (diet)	(munits/mg of protein) [1]	(munits/unit of citrate synthase) [2]	Absent [3]	Present [4]	Activator protein (% of control) [5]
Normal (standard)	5.5 ± 0.45 (18)	29 ± 2.7 (18)	63 ± 6.5	71 ± 4.9	100 ± 21 (6)
Normal (80%-casein)	4.7 ± 0.17 (12)	33 ± 2.4 (12)	67 ± 6.5	47 ± 3.8‡	173 ± 27 (6)
Normal (9%-casein)	3.9 ± 0.17* (10)	29 ± 1.3 (10)	41 ± 3.4*	21 ± 2.8*†	30 ± 5* (6)
Normal (0%-casein)	3.9 ± 0.25* (7)	25 ± 1.4 (7)	30 ± 8.1*	26 ± 5.9*	14 ± 4* (6)
Normal (48h-starved)	4.4 ± 0.39 (14)	23 ± 1.4* (14)	33 ± 2.9*	32 ± 2.6*	21 ± 4* (6)
Diabetic (standard)	4.4 ± 0.47 (9)	31 ± 2.9 (9)	50 ± 5.6	33 ± 5.7*	155 ± 26 (6)

kidney on a fresh-weight basis (Table 5) was not lowered by low-protein diets, starvation or diabetes, and in this respect kidney differed from liver.

The proportion of complex in the active form in kidney mitochondria was influenced less than in liver by inclusion of NaF + ketoleucine in the isolation medium (cf. columns 3 and 4 in Tables 2 and 3). As in liver, the measured proportion of complex in the active form was decreased relative to the control (normal rats, standard diet) by low-protein diets, starvation and alloxan-diabetes (by 60–70%). As in liver, it is not known whether these differences are due to alterations in the activity of activator protein or to altered proportions of phosphorylated and dephosphorylated complexes, or to both. The results in Table 5 show (regardless of mechanism) that low-protein diets, starvation and diabetes decreased the measured activity of branched-chain complex in kidney by approx. 50–70%.

In both liver and kidney mitochondria the concentration of the active form of the complex was lower in rats fed on a 80%-casein diet than in rats fed on the standard diet. The reason for this is not known; the protein content of the standard diet (approx. 17%, w/w) was lower than that of the 80%-casein diet. The effect of 0- or 9%-casein diets to decrease the activity of branched-chain complex is present regardless of whether activities with standard or 80%-casein diets are used as controls.

The total activities of branched-chain complex (normal rat, standard diet) in liver (820munits/g wet wt.) and in kidney (770munits/g wet wt.) are higher than the values obtained by Gillim *et al.* (1983) (liver, 612; kidney, 341). The effects of diet and diabetes on total activity in the two studies were broadly similar, except that Gillim *et al.* (1983) observed no effect of starvation in liver. The proportion of complex in the active form in liver and kidney in the present study was lower than found by Gillim *et al.* (1983), who also failed to observe an effect of starvation in liver and kidney and of streptozotocin-diabetes in the kidney. It is suggested that the higher activities of total complex in the present study may be due to greater recovery of complex when this is concentrated by isolation in mitochondria. The lower proportion of active complex is attributed to the use of fluoride to prevent dephosphorylation of phosphorylated complex in the present study.

Branched-chain complex in rat heart and skeletal-muscle mitochondria

Activator protein has not been detected in rat heart or skeletal-muscle mitochondria (Fatania *et al.*, 1982). As shown in Table 4 (columns 1 and 2) and Table 5, total activity of branched-chain complex in rat heart mitochondria and in rat heart was not changed by diet, but may have been decreased by alloxan-diabetes. The proportion of complex in the active form *in vivo* (i.e. in mitochondria isolated in the presence of NaF + ketoleucine) is shown in column 4 of Table 3. In normal rats on standard diet, approx. 5% of complex was in the

Table 4. *Activity of branched-chain 2-oxo acid dehydrogenase complex in rat heart mitochondria; effect of diet and of alloxan-diabetes*

Rat hearts were disrupted in sucrose medium (Polytron homogenizer), and two batches of mitochondria were prepared with or without addition of NaF (to 100mM) and ketoleucine (to 5mM). Mitochondrial pellets prepared with NaF and ketoleucine were frozen immediately and processed for assay of branched-chain complex (active form) and citrate synthase. Mitochondria prepared without NaF and ketoleucine were added to KCl medium (2mg of protein/ml) and either centrifuged immediately (active form) or after incubation for 30min at 30°C to effect conversion of inactive complex into active complex (total complex). Pellets were then processed for assay of branched-chain complex and citrate synthase. The activity of citrate synthase averaged 1.1 units/mg of mitochondrial protein, and there were no significant effects of diet or diabetes. For further details of methods and procedure, see the Experimental section. Results are means ± S.E.M. for the numbers of observations (hearts) shown in parentheses. *$P<0.01$ for difference from normal (standard or 80%-casein diets) within columns; for other differences within columns $P>0.05$. †$P<0.01$ for effect of NaF + ketoleucine.

	Branched-chain complex activity in mitochondria			
	Total (active + inactive forms)		Active form (% of total), with NaF + ketoleucine:	
Rat (diet)	(munits/mg of mitochondrial protein) [1]	(munits/unit of citrate synthase) [2]	Absent [3]	Present [4]
Normal (standard) (12)	6.14 ± 0.23	6.14 ± 0.36	29 ± 2.3	5.3 ± 0.46†
Normal (80%-casein) (11)	6.69 ± 0.29	5.72 ± 0.26	24 ± 3.5	8.9 ± 1.78†
Normal (9%-casein) (10)	6.15 ± 0.50	5.86 ± 0.49	30 ± 2.3	10.9 ± 1.93†
Normal (0%-casein) (7)	6.63 ± 0.22	6.63 ± 0.20	34 ± 1.1	6.0 ± 2.20†
Normal (48h-starved) (5)	5.92 ± 0.16	5.53 ± 0.18	31 ± 2.4	7.2 ± 0.99†
Diabetic (standard) (8)	5.86 ± 0.54	4.51 ± 0.34*	51 ± 5.9*	19.0 ± 3.81*†

Table 5. *Effects of diet and alloxan-diabetes on activities of branched-chain complex in rat liver, kidney and heart*

The values for branched-chain complex activity are calculated from mitochondrial activities (in munits/unit of citrate synthase) taken from Tables 2–4, and the whole-tissue concentrations of citrate synthase, which were (units/g wet wt.; means ± S.E.M.; numbers of observations): liver 8.2 ± 0.67 (34), kidney 26.5 ± 0.72 (20) and heart 92.8 ± 2.4 (18). There were no significant effects of diet or diabetes on the whole-tissue concentration of citrate synthase (results not shown). *$P<0.01$ for difference from standard or 80%-casein diets within columns. †$P<0.01$ for difference between standard and 80%-casein diets within columns. For other differences within columns $P>0.05$.

	Branched-chain complex (unit/g wet wt. of tissue) in:					
	Liver		Kidney		Heart	
Rat (diet)	Total [1]	Active form [2]	Total [3]	Active form [4]	Total [5]	Active form [6]
Normal (standard)	0.82 ± 0.06	0.45 ± 0.03	0.77 ± 0.07	0.55 ± 0.05	0.57 ± 0.04	0.03 ± 0.003
Normal (80%-casein)	0.79 ± 0.07	0.24 ± 0.03†	0.87 ± 0.07	0.41 ± 0.03†	0.53 ± 0.03	0.04 ± 0.010
Normal (9%-casein)	0.32 ± 0.04*	0.04 ± 0.01*	0.77 ± 0.04	0.16 ± 0.02*	0.57 ± 0.04	0.06 ± 0.01
Normal (0%-casein)	0.25 ± 0.05*	0.07 ± 0.02*	0.66 ± 0.03	0.17 ± 0.04*	0.62 ± 0.03	0.04 ± 0.02
Normal (48h-starved)	0.61 ± 0.04*	0.06 ± 0.01*	0.61 ± 0.02	0.19 ± 0.01*	0.55 ± 0.02	0.04 ± 0.005
Alloxan-diabetic (standard)	0.70 ± 0.06	0.13 ± 0.02*	0.82 ± 0.05	0.27 ± 0.04*	0.42 ± 0.02*	0.08 ± 0.01*

active form, in agreement with results of Parker & Randle (1980) and Waymack *et al.* (1980). The proportion is lower than the value of 48% obtained by Gillim *et al.* (1983), who extracted and processed the complex in the absence of fluoride. Because of the low proportion of active complex, the activities approached the lower limit of assay. The only change detected was an approx. 4-fold increase with diabetes. In mitochondria isolated in the absence of NaF + ketoleucine the proportion of active complex was increased (2–6-fold), presumably as a result of dephosphorylation in the absence of NaF.

In rat skeletal-muscle (hindlimb) mitochondria the total activity of branched-chain complex was 1.8munits/mg of mitochondrial protein, or 2.4munits/unit of citrate synthase (mean ± S.E.M. for nine observations; normal rats, standard diet). Less than 20% of the complex was in the active form (the lower limit of the assay). The activity of

the active form of the complex was too low for reliable assay, and because of this we have been unable to obtain reliable estimates of the effects of diet or diabetes. The total activity per unit weight of hindlimb muscle was 34munits/g wet wt. The concentration of mitochondria, based on citrate synthase, was 18mg of mitochondrial protein/g wet wt. We have been unable to detect branched-chain complex in skeletal-muscle mitochondria prepared with trypsin by the method of Fuller & Randle (1984).

General discussion and conclusions

In the present study branched-chain complex in rat tissues has been concentrated before assay by isolation within mitochondria. This has the advantage that endogenous branched-chain dehydrogenase phosphate phosphatase may be used to convert phosphorylated complex into dephosphorylated complex for assay of total complex. The active form of the complex *in vivo* was assayed after isolation of mitochondria in the presence of NaF and ketoleucine to inhibit interconversion of dephosphorylated and phosphorylated complexes. Tissue concentrations of complex have been computed by reference to mitochondrial and tissue concentrations of citrate synthase. Two problems of methodology have not been fully resolved. A more sensitive method of assay of branched-chain complex is needed for assay of the low concentrations of active complex in heart and skeletal-muscle mitochondria. In liver and kidney mitochondria a satisfactory method of separating complex and activator protein with full recovery has yet to be devised.

Low-protein diets decreased the total activity of branched-chain complex (i.e. the sum of active and inactive forms) in liver, decreased the concentration of the active form of the complex in liver and kidney, and decreased the activity of activator protein in liver and kidney. Overall the effect of low-protein diets was to decrease the activity of the complex relative to standard diet by 85% or greater in liver, and by approx. 70% in kidney. The corresponding decreases relative to 80%-casein diets were approx. 70% (liver) and 60% (kidney). These changes may be expected to decrease substantially the rate of degradation of branched-chain amino acids by liver and kidneys of rats in which dietary protein content was much decreased (9% casein) or absent (0% casein). The mechanisms are complex and involve decreased concentrations of total complex (liver) and of activator protein (liver, kidney), possibly as a result of decreased biosynthesis and/or enhanced degradation. Increased inactivation of the complex by phosphorylation may also be involved in liver and kidney, but this has yet to be established unequivocally, because of possible interference by activator protein in the assay.

In animals on standard or 80%-casein diets a substantial proportion of branched-chain complex was in the inactive (phosphorylated) form in heart (>90%) and skeletal muscle (>80%). There is therefore the potential in these tissues for much higher rates of degradation of branched-chain amino acids than is normally utilized. This surplus capacity is presumably essential in view of the known toxicity of excessive accumulation of branched-chain amino and keto acids. This has been shown most clearly in man in Maple Syrup Urine Disease. In this inborn error of metabolism, degradation of branched-chain keto acids is impaired because the K_m for branched-chain keto acids of branched-chain complex is increased substantially (Chuang *et al.*, 1981). In the absence of treatment by dietary restriction of branched-chain amino acids, affected infants rarely survive beyond the age of 2 years. Evidence for toxicity of excess of branched-chain amino acids in rats has been given by Harper *et al.* (1970).

Wohlhueter & Harper (1970) first showed that the activity of branched-chain complex in rat liver is decreased by low-protein diets. Their assays were made in liver homogenates and before the discovery of reversible phosphorylation and of activator protein. The results of the present study and of that of Gillim *et al.* (1983) show that the total concentration of branched complex is decreased by low-protein diets in liver but not in kidney or heart. The present study shows further that the proportion and concentration of complex in the active form is decreased in liver and kidney. In these tissues the decreased concentration of activator protein may contribute importantly to the decreased proportion and concentration of active complex, and the role of phosphorylation has yet to be established precisely.

The effects of starvation (48h) were qualitatively similar to those of low-protein diet in liver and in kidney. In heart muscle, starvation had no significant effect on the total activity or the concentration of the active form of the complex. The effects of alloxan-diabetes showed some important differences from those of starvation or low-protein diets. In liver, alloxan-diabetes had no effect on the total activity of complex, whereas this was decreased by starvation and low-protein diets. In kidney alloxan-diabetes did not decrease the activity of activator protein. In heart muscle alloxan-diabetes increased the concentrations of the active form of the complex, whereas starvation and low-protein diets were without effect. The biochemical mechanisms that may mediate effects of low-protein diet,

starvation and diabetes on the activity of branched-chain complex in tissues are not known.

In the rat there is evidence that transamination of branched-chain amino acids is predominantly extrahepatic and that branched-chain α-keto acids are released from extrahepatic tissues and oxidized in the liver (for reviews see Krebs & Lund, 1977; Livesey & Lund, 1980; Randle *et al.*, 1984). An important question is whether degradation of branched-chain α-keto acids occurs predominantly in muscles or in liver. In a 300g rat on standard diet, activity of branched-chain complex in liver is approx. 8 μmol/min under $V_{max.}$ conditions at 38°C (assuming 10g of liver and Q_{10} of 2). In muscles the corresponding activity is <2 μmol/min (assuming 135g of muscle, and average values of 20mg of mitochondrial protein/g of muscle, <0.36 munit of active complex/mg of mitochondrial protein and Q_{10} of 2). In kidney the corresponding activity is approx. 2 μmol/min (assuming 2g of kidney and Q_{10} of 2). On the basis of this calculation, approximately two thirds of active complex is hepatic, suggesting a major role of the liver in the degradation of branched-chain α-keto acids. This view may be supported by the observed effects of low-protein diets. By the same calculations low-protein diet decreased activity in liver from approx. 8 to 1 μmol/min, and in kidney from approx. 2 to 0.6 μmol/min. In muscles the corresponding values are not known, but the decrease (if any) can be no more than 2 μmol/min. Therefore approx. 70% of the decrease in activity occasioned by low-protein diets may be hepatic.

Further consideration of this problem is hampered by lack of knowledge of the mitochondrial concentration of branched-chain α-keto acids, the contribution to regulation of end-product inhibition (Danner *et al.*, 1978; Parker & Randle, 1978), and the lack of accurate information about rates of degradation of branched-chain amino acids in the rat *in vivo* and effects of low-protein diets.

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