A study of properties and abundance of the components of liver carnitine palmitoyltransferases in mitochondrial inner and outer membranes

Effects of hypothyroidism, fasting and a ketotic diabetic state

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1. Liver mitochondrial outer and inner membranes were isolated from normal, 48 h-fasted, streptozotocin-diabetic and hypothyroid rats. 2. Relative to membrane protein, fasting and diabetes substantially increased the activity of carnitine palmitoyltransferase (CPT) in outer membranes. Inner-membrane CPT specific activity was only slightly altered, being increased in diabetes and decreased in hypothyroidism. Abundance of an inner-membrane M_r -68 000 polypeptide that cross-reacted with an anti-CPT serum was significantly increased in diabetes and hypothyroidism. Relative to inner-membrane CPT activity, this cross-reactivity was increased by 37% in diabetes and by 400% in hypothyroidism, suggesting modification of the intrinsic activity of the CPT in these states. 3. CPT in outer membranes was inhibitable by malonyl-CoA, whereas inner-membrane CPT was insensitive to malonyl-CoA. Fasting and diabetes increased the IC_{50} (concentration of malonyl-CoA causing 50% inhibition) for outer-membrane CPT, whereas the IC_{50} was decreased in hypothyroidism. 4. Binding of [^{14}C]malonyl-CoA was observed with both outer and inner membranes and was fitted to two-site models in each case. Fasting, diabetes and hypothyroidism changed the K_D for binding at the higher-affinity site in outer membranes in a manner that correlated closely with changes in IC_{50} for inhibition of outer-membrane CPT by malonyl-CoA. Fasting and diabetes increased the abundance of this outer-membrane high-affinity malonyl-CoA-binding site, whereas hypothyroidism decreased its abundance.

INTRODUCTION

Utilization of long-chain fatty acids by mitochondria requires the participation of carnitine palmitoyltransferases (CPTs). Two forms, CPT₁ and CPT₂, have been localized to liver mitochondrial outer and inner membranes respectively [1–3]. CPT₁ is inhibited by malonyl-CoA in many mammalian tissues [4,5], whereas CPT₂ *in situ* appears to be insensitive to physiological concentrations of this ligand [6–8]. A high-affinity binding site for malonyl-CoA has been recognized in intact mitochondria from rat liver, heart or skeletal muscle [9,10] and in rat liver mitochondrial outer membranes [11,12]. This binding site can be discriminated kinetically [13,14] and topographically [1] from the catalytic site of CPT₁. Despite the claim [15] that these regulatory and catalytic sites reside on the same M_r -94 000 polypeptide, there is evidence accumulating that the catalytic and malonyl-CoA-binding entities reside on separate polypeptides. This conclusion rests on studies employing radiation-inactivation analysis [11], anti-CPT sera [2] and separation of components by chromatography [16] or detergent extraction [3,17].

Rat CPT₂, which appears to be readily solubilizable with detergents [18], is generally accepted as being represented by a polypeptide of M_r 68 000–69 000 on SDS/PAGE [2,8,15,16,18]. A minimum M_r of CPT₂ of approx. 70 000 is also apparent from radiation-inactivation analysis [19]. CPT₁ has not yet been purified. However, a minimum M_r for CPT₁ of 97 000 was estimated by Zammit *et al.* by radiation-inactivation analysis [19], although subsequently these workers have provided a revised estimate of M_r = 83 000 [11].

Rat liver mitochondrial CPT₁ shows substantial changes with physiological state. As measured *in situ* in intact mitochondria,

overt CPT specific activity is increased in fasting [10,20–22], in diabetes [23] and in hyperthyroidism [24], and is decreased in hypothyroidism [10,24–26] and by oestrogen therapy [27]. More recent studies have shown that the up-regulation of CPT₁ activity in fasting or diabetes is also observed with isolated mitochondrial outer membranes [12,28,29]. The molecular mechanisms underlying these activity changes are still unclear. Where estimated, latent (CPT₂) activity shows less adaptivity [20,25,30]. However, this conclusion has been reached indirectly, and is not based on direct assay of CPT₂ in isolated mitochondrial inner membranes. Changes in the specific activity of CPT₁ with physiological states are often accompanied by changes in sensitivity to the inhibitor malonyl-CoA [21,22,24,31–35]. Analysis of [^{14}C]malonyl-CoA binding to intact liver mitochondria did not reveal any effect of physiological state on the affinity or maximal extent of malonyl-CoA binding [10]. However, complex binding profiles are observed with whole mitochondria [10,36]. Furthermore, it is reasonable to expect some binding of malonyl-CoA to proteins in regions of the mitochondria other than the outer membrane. Such extraneous binding would complicate analyses and may mask changes in the properties of the functional malonyl-CoA-binding component of CPT₁ in the outer membrane.

This study had two main objectives. The first was to establish by direct assay of isolated inner membranes whether CPT₂ activity/abundance really is invariant with physiological state. The second was to examine the characteristics of [^{14}C]malonyl-CoA binding to outer membranes in these states, with a view to attempting correlations with alterations in sensitivity of CPT₁ to inhibition by malonyl-CoA. At the same time, we have also characterized extensive binding of malonyl-CoA to inner membranes. Near to the conclusion of the present work,

Kolodziej & Zammit [12] published a study of malonyl-CoA binding to liver outer membranes in the fasted state. Our study complements and extends these previous findings.

MATERIALS AND METHODS

Chemicals and reagents

These were obtained and treated as in [10,25,37]. Peroxidase-conjugated anti-rabbit IgG (raised in goat) was from ICN Immunobiologicals (Lisle, IL, U.S.A.). Anti-rabbit IgG was labelled with ^{125}I as in [38]. Antiserum to rat liver CPT was raised in rabbits [2].

Animals

Sprague-Dawley rats (7–8 weeks old) bred at University College London were maintained at 21 °C on a 13 h-light/11 h-dark cycle, with light from 06:00 h to 19:00 h. The diet was Rat & Mouse No. 3 Breeding Diet (Special Diet Services, Witham, Essex, U.K.), which contained (by wt.) 21% digestible crude protein, 4% digestible crude oil and 39% starches and sugars. Normal animals weighed 248 ± 6 g ($n = 40$). Hypothyroid animals which had been maintained for 4 weeks previously on a low-iodine version of the No. 3 diet together with 0.01% (w/v) 6-n-propyl-2-thiouracil in the drinking water [26] weighed 137 ± 4 g ($n = 40$). Fasting was induced by removal of food for 48 h. Diabetes was induced by subcutaneous injection of streptozotocin (150 mg/kg) dissolved in 0.3 ml of 50 mM-sodium citrate buffer (pH 4.0) containing 0.15 M-NaCl. At the time of injection these rats weighed 273 ± 6 g ($n = 25$); 48 h later at the time of experimentation, they weighed 232 ± 6 g ($n = 25$) and had plasma glucose concentrations of 29 ± 1 mM. All diabetic animals showed a strongly positive (8–16 mmol/litre) 'Ketostix' test for urinary ketones, and in all cases severe hyperlipidaemia was visible.

Isolation of mitochondrial membranes

Liver mitochondria were obtained and then treated and subfractionated to obtain outer and inner membranes as described in [2]. These were then stored at -70 °C until used.

Analytical methods

CPT was assayed at 25 °C in a final volume of 1.0 ml. Reactions were initiated by addition of 0.4 mM-L-carnitine or L-[Me - ^3H]carnitine as appropriate. The spectrophotometric assay contained 150 mM-sucrose, 60 mM-KCl, 25 mM-Tris/HCl buffer (pH 6.8), 1 mM-EDTA, 0.1 mM-4,4'-dithiodipyridine, fatty-acid-poor BSA (1.3 mg/ml) and 40 μM -palmitoyl-CoA. The reaction was monitored at 324 nm [2]. The radiochemical assay of CPT contained similar components, except that 4,4'-dithiodipyridine was omitted and 1 mM-dithiothreitol was included. Incorporation of [^3H]carnitine into palmitoylcarnitine was detected as described in [25].

Succinate dehydrogenase and rotenone-insensitive NADH-cytochrome *c* reductase were assayed as described in [39] and [40] respectively.

Binding of [^{14}C]malonyl-CoA was measured by a filtration assay. Unless stated otherwise, portions of inner and outer membranes were incubated on ice in a final volume of 1.0 ml of medium containing 150 mM-sucrose, 60 mM-KCl, 25 mM-Tris/HCl buffer (pH 6.8), 1 mM-EDTA, 1 mM-dithiothreitol and fatty-acid-poor BSA (1.3 mg/ml). [^{14}C]Malonyl-CoA was added over the range 1–2000 nM. After 20 min, bound and free ligand were separated by filtration under vacuum through Whatman GF/B glass-fibre filters on a programmable Brandel cell harvester. The filters were washed with 2×5 ml of the same

Table 1. Enzyme activities in mitochondrial inner and outer membranes

Activities are expressed as nmol/min per mg of protein; '% inhibition' refers to the effect of 100 μM -malonyl-CoA; IC_{50} values relate to inhibition of CPT by malonyl-CoA in the spectrophotometric assay. Methods A and B for calculation of IC_{50} are described in the text. $n = 4$ in every case.

Condition of rats	Mitochondrial membrane	Succinate dehydrogenase activity	NADH-cytochrome <i>c</i> reductase activity	CPT (radiochemical)		CPT (spectrophotometric)		IC_{50} values (μM)	
				Activity	% inhibition	Activity	% inhibition	Method A	Method B
Normal	Outer	0.9 ± 0.4	825 ± 64	6.1 ± 0.8	97 ± 1	4.2 ± 0.7	84 ± 5	2.5	1.4
	Inner	40 ± 7	108 ± 7	9.3 ± 0.2	$+3 \pm 4$	—	—	—	—
Hypothyroid	Outer	0.5 ± 0.1	$2316 \pm 90^{\text{D}}$	8.9 ± 1.4	$90 \pm 2^{\text{A}}$	4.7 ± 0.5	80 ± 5	1.0	0.7
	Inner	57 ± 3	106 ± 7	$8.3 \pm 0.3^{\text{A}}$	1 ± 2	—	—	—	—
Diabetic	Outer	1.8 ± 0.1	$536 \pm 60^{\text{B}}$	$26.5 \pm 3.1^{\text{D}}$	94 ± 1	$19.4 \pm 2^{\text{D}}$	78 ± 2	8.7	4.7
	Inner	59 ± 2	80 ± 8	$10.6 \pm 0.4^{\text{A}}$	$+3 \pm 4$	—	—	—	—
Fasted	Outer	0.8 ± 0.3	715 ± 38	$21.0 \pm 1.7^{\text{D}}$	$84 \pm 5^{\text{A}}$	$15.5 \pm 1.2^{\text{D}}$	$67 \pm 2^{\text{B}}$	10.0	4.8
	Inner	53 ± 3	110 ± 11	10.3 ± 1.1	2 ± 4	—	—	—	—

medium (ice-cold) before being taken for scintillation counting. Concentrations of inner-membrane protein in these assays were ($\mu\text{g/ml}$): normal, 230 ± 30 ; hypothyroid, 320 ± 30 ; fasted, 280 ± 40 ; diabetic, 280 ± 8 . At the lowest tested concentration of [^{14}C]malonyl-CoA (1 nM), the percentage of ligand bound to inner membranes ranged from 6% in hypothyroidism up to 31% in fasting. Concentrations of outer-membrane protein in the binding assays were ($\mu\text{g/ml}$): normal, 140 ± 10 ; hypothyroid, 130 ± 6 ; fasted, 140 ± 7 ; diabetic, 130 ± 9 . At 1 nM-malonyl-CoA the percentage of ligand bound to outer membranes was: normal, 51%; hypothyroid, 40%; fasted, 54%; diabetic, 59%. In the normal and hypothyroid states, some further measurements were made in which the volume, quantities of components and amount of outer-membrane protein were increased 3-fold in order to be able to estimate binding with 0.4–0.8 nM-malonyl-CoA. Even under these conditions < 80% of the ligand was bound at the lowest tested concentration, and the recorded d.p.m. value was always at least twice the background. To analyse saturation binding, the background was first subtracted (an overall mean value of 24 d.p.m.) to give a value for bound ligand. In turn, this value was subtracted from the total in order to calculate the free [malonyl-CoA]. Analysis of binding was by a non-linear least-squares curve-fitting procedure using a search-type minimization method [41]. On input of ‘bound’ values as means $\pm$ S.E.M. and ‘free’ values as means, the program provided estimates of K_D and B_{max} . As suggested by [42], no correction for ‘non-specific binding’ was used.

For immunoblotting, polypeptides were separated by SDS/PAGE in gels containing 10% (w/v) acrylamide and 0.27% (w/v) bisacrylamide. After electroblotting on to nitrocellulose sheets, blocking with gelatin (3%, w/v) and reaction with anti-CPT serum, cross-reacting bands were made visible and quantified by using a mixture of anti-rabbit IgG conjugated with peroxidase and ^{125}I -labelled anti-rabbit IgG. The M_r -68 000 band was cut out for γ -radiation counting. Equivalent-sized unstained regions of the nitrocellulose sheet were counted for radioactivity to determine background, which was subtracted from all values. Blots were normalized relative to each other by comparison against a standard 50 μg of total mitochondrial membrane protein from normal rats, which was run every time.

Protein was measured [43] with BSA as a standard.

Statistical methods

All values are means $\pm$ S.E.M. Where bars are not shown in Figures, these lie within the symbol. Significance of differences

was assessed by Student’s *t* test for unpaired samples: throughout, $^{\text{A}}P < 0.05$, $^{\text{B}}P < 0.02$, $^{\text{C}}P < 0.01$, $^{\text{D}}P < 0.001$. Statistical analysis to assess appropriateness of one- or two-site models for [^{14}C]malonyl-CoA binding was performed by a partial F test [44].

RESULTS AND DISCUSSION

Cross-contamination between the two membrane preparations was assessed by the distribution of succinate dehydrogenase and rotenone-insensitive NADH-cytochrome *c* reductase (Table 1). Cross-contamination of outer membrane by inner membrane was 0.9%, 1.5%, 2.2% and 3.1% in the hypothyroid, fasted, normal and diabetic states respectively. Cross-contamination of inner membranes by outer membranes was more extensive, and was 5%, 13%, 15% and 15% for the hypothyroid, normal, fasted and diabetic states respectively.

Inhibition of CPT by malonyl-CoA

This is summarized in Table 1. In all four states outer-membrane CPT was extensively inhibitable by malonyl-CoA.

Fig. 1. Quantitative immunoblotting of mitochondrial inner-membrane proteins

Rats: $\circ$, normal; $\square$, hypothyroid; $\blacksquare$, diabetic; $\bullet$, fasted. In all cases $n = 4$, and linear correlation coefficients (r) were > 0.990 . Gradients of these lines are shown in Table 2.

Table 2. Reaction of anti-CPT serum with mitochondrial outer and inner membranes

Values for immunoreactivity were derived from gradients of plots of ^{125}I in the M_r -68 000 band versus amount of protein electrophoresed (Fig. 1). CPT activities refer to the radiochemical assay (Table 1). $n = 4$ in every case.

Condition of rats	Mitochondrial membrane	^{125}I d.p.m. in M_r -68 000 band per 100 μg of membrane protein (<i>A</i>)	Ratio
			$\frac{A}{\text{CPT activity (nmol/min per mg of protein)}}$
Normal	Outer	1404 ± 285	213 ± 17
	Inner	9258 ± 1339	995 ± 145
Hypothyroid	Outer	1095 ± 56	$128 \pm 14^{\text{C}}$
	Inner	$33371 \pm 2373^{\text{D}}$	$4064 \pm 440^{\text{D}}$
Diabetic	Outer	1076 ± 208	$45 \pm 14^{\text{D}}$
	Inner	$14581 \pm 496^{\text{C}}$	$1371 \pm 39^{\text{A}}$
Fasted	Outer	1050 ± 99	$51 \pm 8^{\text{D}}$
	Inner	9680 ± 383	974 ± 107

Table 3. K_D and B_{max} values for binding of [14 C]malonyl-CoA to outer and inner membranes

The values are computed from the binding curves shown in Figs. 2 and 4. All of the binding curves were fitted to a two-site model of binding, in preference to a one-site model ($P < 0.005$, by the F test). Values of K_D are expressed as nM and B_{max} as pmol/mg of membrane protein. N, H, D and F indicate normal, hypothyroid, diabetic and fasted states respectively.

	Outer membrane				Inner membrane			
	K_{D1}	$B_{max.1}$	K_{D2}	$B_{max.2}$	K_{D1}	$B_{max.1}$	K_{D2}	$B_{max.2}$
N	0.57 ± 0.17	6.0 ± 0.4	425 ± 45	31.8 ± 1.9	12.0 ± 1.7	14.1 ± 0.8	935 ± 247	21.5 ± 1.7
H	0.37 ± 0.03	4.9 ± 0.08^A	833 ± 30^D	31.9 ± 0.4	23.4 ± 6.9	4.0 ± 0.5^D	800 ± 100	26.7 ± 0.8^A
D	1.6 ± 0.3^A	25.3 ± 1.4^D	198 ± 56^B	24.9 ± 1.4^A	7.0 ± 0.9^A	7.1 ± 0.5^D	212 ± 18^A	17.0 ± 0.4^A
F	1.9 ± 0.2^C	19.7 ± 0.5^D	503 ± 45	39.4 ± 2.9	3.9 ± 0.9^C	6.1 ± 0.7^D	139 ± 11^B	27.1 ± 0.5^B

Fig. 2. Binding of [14 C]malonyl-CoA to mitochondrial outer membranes

Rats: (a) normal (○); (b) hypothyroid (●); (c) fasted (□); (d) diabetic (■). $n = 4$ in every case.

Fig. 3. Scatchard plots for binding of [^{14}C]malonyl-CoA to outer membranes

Rats: (a) normal ($\circ$); diabetic ($\blacksquare$); (b) hypothyroid ($\bullet$); fasted ($\square$). The broken lines are fits to the computed values of K_{D1} and $B_{\max,1}$ which are shown in Table 3.

This was seen with both the radiochemical and the 4,4'-dithiodipyridine-linked spectrophotometric assays, although the latter gave a lower estimate for the maximum inhibitability of the enzyme. Presumably this is because thiol-group reagents interfere with malonyl-CoA binding [10] and decrease sensitivity of CPT₁ to malonyl-CoA [45]. We have attempted to minimize this limitation by making the assays at pH 6.8 [2]. Both assays revealed a highly significant increase in outer-membrane CPT specific activity in fasting and diabetes, in accord with [10,12,20–23,28,29]. By contrast, inner-membrane CPT specific activity was only slightly decreased and increased in hypothyroidism and diabetes respectively. One observation not in accord with previous findings was the lack of effect of hypothyroidism on outer-membrane CPT specific activity. Our laboratory has demonstrated an 80% decrease in overt CPT activity relative to total mitochondrial protein in rats on the low-iodine-diet/propylthiouracil feeding regime [10,26]. Likewise, decreases of 50–60% have been observed in thyroidectomized rats [25] or in rats treated with propylthiouracil alone [24]. We suggest two possible explanations for this anomaly: first, that the decrease in CPT₁ activity observed with whole mitochondria might be due to a relatively non-persistent modification that relaxes during the lengthy isolation of outer membranes; second,

that hypothyroidism may cause a selective loss of other proteins normally present in the outer-membrane preparation (i.e. either intrinsic to the outer membrane or contaminants from other cellular membranes). It is noteworthy that outer-membrane CPT activity was decreased by 50–60% relative to rotenone-insensitive NADH-cytochrome *c* reductase activity in this state. IC_{50} values (concn. giving 50% inhibition) for malonyl-CoA were calculated in two separate ways. Method A is a simple reading-off of the [malonyl-CoA] that gives 50% of the activity in the absence of malonyl-CoA. Method B used the linear plot $(\% \text{ inhibition})^{-1}$ versus $([\text{malonyl-CoA}])^{-1}$, as first used in [31] and elaborated by [46]. This method makes allowance for the presence in the membrane preparations of any CPT that is non-inhibitable by malonyl-CoA. Methods A and B were in qualitative agreement (Table 1). As found previously with intact mitochondria [21,24,25,30–33] or isolated outer membranes [12,29], fasting decreased sensitivity of outer-membrane CPT₁ to malonyl-CoA. The streptozotocin dose of 150 mg/kg resulted in diabetic ketosis and, in accord with other studies with ketotic rats [22,35], decreased sensitivity to malonyl-CoA. In agreement with [24], sensitivity to malonyl-CoA was increased in the propylthiouracil-treated rat.

Immunoblotting experiments

The same membrane preparations were immunoblotted to quantify the abundance of the M_r -68 000 cross-reactive band. Fig. 1 shows that the measured signals were linear with amount of inner-membrane protein. The gradients of these lines were therefore taken as indices of the abundance of M_r -68 000 cross-reacting protein(s). Table 2 shows that hypothyroidism, and to a lesser extent diabetes, significantly increased the abundance of inner-membrane M_r -68 000 cross-reactive protein relative to CPT₂ activity. The implication is that if all or most of the inner-membrane M_r -68 000 band represents CPT₂ protein, the enzyme's intrinsic activity must be subject to modification with physiological state. Table 2 shows that the anti-CPT serum also recognized a M_r -68 000 band in outer membranes. As discussed previously [2], this is not simply due to cross-contamination by CPT₂ from the inner membrane. The cross-reactivity of this outer-membrane band relative to CPT activity was less than in inner membranes, and there was no apparent correlation between outer-membrane M_r -68 000 immunoreactivity and CPT₁ activity in the different states. Any similarity between CPT₁ and CPT₂ is at present a matter of conjecture. However, the demonstration that inner-membrane CPT₂ can be made to interact productively with malonyl-CoA-binding material from outer membranes [3,17] implies that the two forms of CPT may have some common structural features.

Measurements of [^{14}C]malonyl-CoA binding

Although different from physiological conditions, we deliberately chose to make these measurements at pH 6.8. In part this was to provide comparability with the CPT-inhibition studies (Table 1). Also, we have considerably more confidence in the filtration assay at pH 6.8 than at pH 7.4, because tighter binding is encountered [13]. As expected, therefore, K_{D1} values shown in Table 3 are some 15–50-fold lower than those reported for an outer-membrane binding site at pH 7.4 by Kolodziej & Zammit [12]. At pH 6.8, binding to outer membranes clearly resolved into two classes of sites (Figs. 2 and 3; Table 3). This finding also differs from that of Kolodziej & Zammit [12], who proposed that outer-membrane binding at pH 7.4 is described by only one class of saturable site plus an apparently non-saturable component. Again, we attribute this apparent discrepancy to differences in pH between the two studies. We are satisfied that the higher-affinity site observed here (Table 3) is the same as that reported

Fig. 4. Binding of [^{14}C]malonyl-CoA to mitochondrial inner membranes

Rats: (a) normal ($\circ$); (b) hypothyroid ($\bullet$); (c) fasted ($\square$); (d) diabetic ($\blacksquare$). $n = 4$ in every case.

in [12], since in both studies its abundance ($B_{\text{max},1}$ in Table 3) was significantly increased in fasting. By contrast, the $B_{\text{max},2}$ for binding to outer membranes was unchanged (Table 3). In turn, the outer-membrane high-affinity site observed in [12] appears to have very similar binding characteristics to the site which was studied in [11], using radiation-inactivation analysis, on the presumption that it represented the malonyl-CoA-binding component of CPT₁.

Our findings at pH 6.8 also differ qualitatively from those in [12] in that we have observed a significant increase in $K_{\text{D}1}$ for malonyl-CoA binding in fasting and diabetes (Table 3). In addition, there was a small but non-significant decrease in the outer-membrane $K_{\text{D}1}$ value in hypothyroidism. The most striking feature of these changes in outer-membrane $K_{\text{D}1}$ values is that they paralleled ($r = 0.999$) changes in IC_{50} for inhibition of outer-membrane CPT₁ by malonyl-CoA (Table 1). If this high-affinity site represents the malonyl-CoA-binding entity of CPT₁, these alterations in its binding properties would contribute to changes in sensitivity of the enzyme to malonyl-CoA in various

physiological states. This conclusion in no way precludes other physiological modifications in the CPT₁ system, e.g. suggested alteration in transmission of conformational changes between the malonyl-CoA-binding and the catalytic entities of CPT₁ [12]. It should be pointed out that the IC_{50} values (μM range) are substantially greater than the $K_{\text{D}1}$ values (nM range). This apparent discrepancy is reconciled by the fact that palmitoyl-CoA has, of necessity, to be included in the assay of CPT. This metabolite increases the K_{D} for binding of malonyl-CoA [10,12,13] and is also suggested to negate transmission of information from the binding entity to the catalytic entity of CPT₁ [47].

We observed that $B_{\text{max},1}$ for binding to outer membranes was significantly increased in fasting and diabetes, suggesting that decreased sensitivity to CPT₁ to malonyl-CoA observed in these two states is not due to decreased abundance of the malonyl-CoA-binding entity of the enzyme. It may be questioned why the abundance of this component should increase. We advance as a possible explanation that this would permit a more rapid

subsequent restoration of sensitivity to malonyl-CoA on refeeding or insulin administration respectively. For example, Grantham & Zammit [48] observed that the approx. 2-fold increase in hepatic CPT₁ activity occurring on fasting for 48 h is barely reversed on subsequent refeeding for 24 h. By contrast, in the same refeeding period the CPT₁ showed a substantial regaining of inhibitability by malonyl-CoA [48].

As shown in Table 3, the values of K_{D2} and $B_{max,2}$ in outer membranes decreased in diabetes, whereas the K_{D2} value increased in hypothyroidism. At present we have no suggestions as to the identity of this low-affinity site or regarding the physiological significance of these changes.

Fig. 4 shows that inner membranes also bound [¹⁴C]malonyl-CoA. The overall abundance of sites ($B_{max,1} + B_{max,2}$) per mg of protein was comparable with that in outer membranes. Again the binding profiles were represented by a two-site model (Table 3). The K_{D1} at the higher-affinity site was somewhat higher than that in outer membranes, whereas the K_{D2} was roughly comparable with that of the low-affinity site in outer membranes. At present it is not possible to tell what proportion of these inner-membrane sites would be accessible to malonyl-CoA applied to the outside of intact mitochondria or to assign physiological relevance to changes in K_D or B_{max} with physiological state. Bird & Saggerson [10] did not observe any effects of fasting or other physiological states on the computed K_D and B_{max} of high-affinity sites for malonyl-CoA in intact liver mitochondria. If even some of the inner-membrane binding sites are accessible to the ligand in intact mitochondria, it is clear that the changes reported here for K_{D1} and $B_{max,1}$ in the outer membrane could easily have been obscured in the earlier analysis of [10].

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