BBA 45968

THE SENSITIVITY OF DICARBOXYLATE ANION EXCHANGE REACTIONS TO TRANSPORT INHIBITORS IN RAT-LIVER MITOCHONDRIA

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(Received February 9th, 1970)

SUMMARY

Rat-liver mitochondria were loaded with ^{14}C-labelled succinate, L-malate or malonate. Exchange of the labelled material with unlabelled dicarboxylate anions and P_i was investigated at 0°. Inhibitors of the dicarboxylate anion transporting system completely inhibited the exchanges when used at high concentrations. The exchange of the labelled material with P_i was more sensitive to inhibition by 2-*n*-butylmalonate than the exchange with dicarboxylate anions. The rates of exchange and the concentration dependence of these rates were determined using the 'inhibitor stop' technique.

INTRODUCTION

It has now been established that rat-liver mitochondria possess a transporting system for L-malate which catalyses an exchange of L-malate for P_i (refs. 1–5). This transporting system is inhibited by 2-butylmalonate[2,3], 2-phenylsuccinate[6,3] and *p*-iodobenzylmalonate[7]. It was originally proposed that this system could catalyse the exchange of succinate for L-malate[1] and the oxidation of succinate was found to be inhibited by 2-butylmalonate[3,7,8]. It has recently been reported, however, that dicarboxylate/dicarboxylate anion exchanges in rat-liver mitochondria at 20° are insensitive to 2-butylmalonate[4] at low concentrations (5 mM), and the existence of a separate transporting system catalysing such an exchange has been proposed. At low temperatures, these exchanges were found to be inhibited by 2-butylmalonate when used at high concentrations (25 mM). The kinetics of the dicarboxylate/dicarboxylate exchange reactions are discussed.

METHODS AND MATERIALS

Mitochondria were prepared as described previously[2]. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was provided by Dr. P. G. Heytler. Butylmalonic acid was obtained from the City Chemical Corp., New York, and *p*-iodobenzylmalonic acid was purchased from K. and K., Rare Chemicals, Plainview, N.Y. 2-*n*-Pentylmalonic acid was prepared by alkaline hydrolysis of the diethyl ester

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

followed by acidification, diethyl ether extraction and recrystalisation from ethanol. Isotopically labelled compounds were obtained from the Radiochemical Centre, Amersham, Great Britain.

Rat-liver mitochondria were first loaded with either [^{14}C]succinate, L-[^{14}C]-malate or [^{14}C]malonate. This was achieved by incubating 200 mg (protein) rat-liver mitochondria with 2.5 μC of the radioactive dicarboxylic acid (specific activity 15 mC/mmole) in the presence of 2 μM rotenone and 0.2 μg/ml antimycin in a total volume of 7 ml of buffer containing 125 mM KCl and 20 mM Tris–HCl buffer (pH 7.4) at 10°. After 10 min the volume was made up to 100 ml with ice-cold 0.25 M sucrose, 5 mM Tris–HCl buffer, and 1 mM EGTA. The mitochondria were then centrifuged for 10 min at 18000 × *g*, the supernatant removed as much as possible by decantation and the pellet resuspended in 4 ml of the sucrose medium described above ready for use. When treated in this way, the mitochondria were incapable of utilising O_2 at 30° with any added substrate other than ascorbate–tetramethyl-*p*-phenylenediamine (TMPD), so that transformations of radioactive compounds by oxidation were not likely to occur.

Rat-liver mitochondria loaded in this way gave a total of 3000–4000 counts/mg of mitochondrial protein, and about 75 % of these counts appeared to be located in the intramitochondrial osmotic space. In a typical experiment mitochondria (8-mg protein aliquots) loaded with L-[^{14}C]malate as described above were added to 1 ml (incubations) of a buffer containing 125 mM KCl and 20 mM Tris–HCl buffer (pH 7.4) at 0°. After a 2-min interval the tubes were centrifuged at 18000 × *g* for 5 min, 0.9 ml of the supernatant drawn off and added to 0.5 ml 1.6 M $HClO_4$. The acidified supernatants were centrifuged at 1000 × *g* for 2 min and then neutralised with 0.5 M triethanolamine–3 M K_2CO_3 base. Excess supernatant was drawn off the mitochondrial pellets and the pellets were taken up with 0.5 ml 0.12 M KCl and acidified with 1.6 M $HClO_4$ (0.5 ml). These were centrifuged as the supernatant had been and 0.9 ml of the acid extract drawn off and neutralised. Supernatant and pellet samples were counted and assayed for L-malate by the method of HOHORST[9]. The treatment and layout of the results from such an experiment is shown in Table I.

The results show that the inclusion of 1 mM malonate in the incubation medium

TABLE I

CORRELATION OF RADIOACTIVE AND ENZYME ASSAY DATA FOR MALONATE/L-[^{14}C]MALATE EXCHANGE

See text for experimental details

Additions	*Supernatant (counts per min)*	*Pellet (counts per min)*	*Exchange** (%)	L-*Malate (nmoles)*		*Exchange** (%)
				Supernatant	*Pellet*	
None	7 950	21 400	0	9.1	25.2	0
Malonate, 1 mM	14 600	14 450	31.1	18.1	15.1	35.6
Malonate, 1 mM + butylmalonate, 15 mM	8 460	20 400	2.4	9.8	23.3	3.3

* *e.g.* percent exchange for malonate/L-[^{14}C]malate calculated from the second line of the table would be as follows:

$$\text{Exchange} = \frac{14\,600 - 7\,950}{21\,400} = 31.1\,\%.$$

caused a displacement of malate from the pellet to the supernatant fraction which was reflected in the distribution of the counts. It is interesting to note that the specific activity of the pellet and supernatant malate were very similar. The presence of 15 mM butylmalonate in addition to 1 mM malonate inhibited the exchange by about 90 % as judged both by the radioactive and the enzyme assay data.

The inhibitor stop technique used to measure the time-course of exchange reactions was similar to the technique used by QUAGLIARIELLO *et al.*[6] except that a refrigerated centrifuge instead of a microcentrifuge was used for spinning down the mitochondria. Comparison of the use of a microcentrifuge and refrigerated centrifuge showed little difference in the results obtained and the refrigerated centrifuge was used, since it gave superior separation and the trouble with heating of the head noted with the microcentrifuge was avoided. The results are expressed in terms of percentage exchange for purposes of comparison, since in the case of malonate and succinate the total amount of dicarboxylic acid intramitochondrially was much less than in the case of L-malate when the endogenous complement varied between 2 and 4 nmoles/mg of mitochondrial protein. This percentage exchange figure is an expression of the difference in extramitochondrial counts between the sample in question and the control with no additions, divided by the intramitochondrial counts in the control multiplied by 100/1. This figure was also useful since it corrected for the slow leak of labelled material that occurred in stock mitochondrial suspension from mitochondria to medium during the course of a set of incubations.

RESULTS

Rat-liver mitochondria were loaded with [^{14}C]succinate as described in METHODS AND MATERIALS and 5-mg (protein) aliquots added to 1-ml incubations at 0° as described in METHODS AND MATERIALS. The presence of L-malate in the incubation medium caused an efflux of labelled succinate from the mitochondria depending on the L-malate concentration used (Fig. 1). The exchange was reduced only slightly by the presence of 10 mM *n*-butylmalonate in the incubation medium but significantly by 10 mM *n*-pentylmalonate and 10 mM *p*-iodobenzylmalonate; 25 mM *p*-iodobenzylmalonate was found to inhibit malate/[^{14}C]succinate and malate/L-[^{14}C]malate exchange completely at concentrations of L-malate below 0.5 mM, but 25 mM butylmalonate was not as effective.

When mitochondria were loaded with L-[^{14}C]malate, efflux of the labelled compound was caused by phosphate or malonate (Fig. 2). 25 mM butylmalonate completely inhibited both of these exchanges and the inhibition appeared to be much more potent with respect to the P_i/malate exchange when 5 mM of P_i and malonate were used. It was thus possible by the use of 25 mM of butylmalonate to perform the inhibitor stop type of experiment described by other investigators[7,10,11]. Mitochondria were rapidly pipetted into 1-ml incubations containing 1 mM P_i or malonate and 25 mM butylmalonate added at time t to prevent further exchange. The mitochondria were then separated as described in METHODS AND MATERIALS and the supernatants taken for counting. A plot of percentage exchange against time is shown in Fig. 3 and as can be seen the exchange is approximately linear over the first 20 sec. When 0.1 mM L-malate was used to exchange for the L-[^{14}C]malate, it was not possible to obtain efflux kinetics since even butylmalonate (25 mM) added

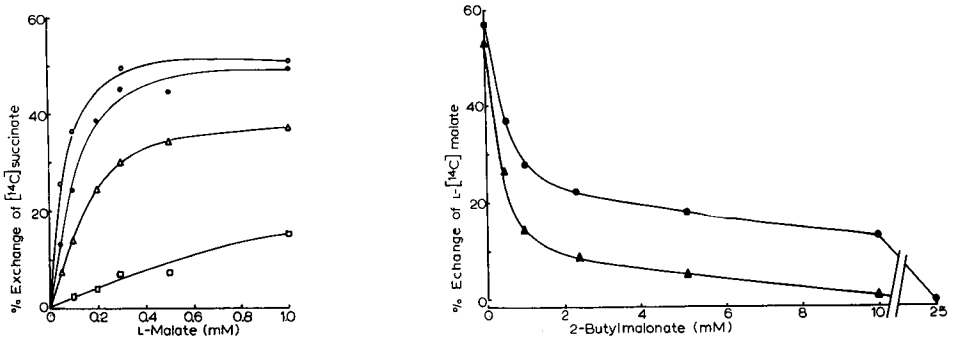

Fig. 1. Inhibition of L-malate/[^{14}C]succinate exchange by substituted malonates. Rat-liver mitochondria were loaded with [^{14}C]succinate and 8-mg (protein) aliquots added to 1-ml incubations of buffer at 0° as described in METHODS AND MATERIALS. After 2 min the mitochondria were separated as described for Table I and preparations of supernatant and pellet prepared for counting. The exchange at 0, 0.1, 0.3 and 1.0 mM extramitochondrial L-malate was investigated in the presence of 10 mM 2-butylmalonate (●—●), 10 mM 2-pentylmalonate (△—△), 10 mM *p*-iodobenzylmalonate (□—□), and with no additions other than the L-malate (○—○).

Fig. 2. Inhibition of malonate/L-[^{14}C]malate and P_i/L-[^{14}C]malate exchange as a function of 2-butylmalonate concentration. 5-mg (protein) aliquots of rat-liver mitochondria loaded with L-[^{14}C]-malate were added to 1-ml incubations of buffer at 0° as described in METHODS AND MATERIALS. After 2 min the mitochondria were separated as described for Table I and preparations of supernatant and pellet prepared for counting. The exchanges at 5 mM P_i (▲—▲) and 5 mM malonate (●—●) were investigated at concentrations of 2-butylmalonate from 0 to 25 mM. Controls with no additions were used so that calculation of percentage exchange was possible.

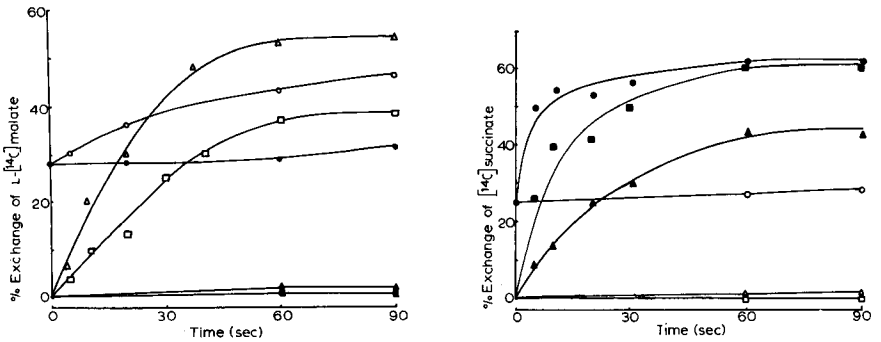

Fig. 3. Time-course of malonate/L-[^{14}C]malate and P_i/L-[^{14}C]malate exchange. Rat-liver mitochondria (5.5 mg protein) loaded with L-[^{14}C]malate were rapidly pipetted into 1-ml incubations of buffer at 0°, containing 1 mM P_i (□—□), 1 mM malonate (△—△) or 0.1 mM L-malate (○—○) and 25 mM 2-butylmalonate added at time *t* (sec) to prevent further exchange. The mitochondria were then separated as described in METHODS AND MATERIALS and the supernatant and pellet samples counted for L-[^{14}C]malate. Similar experiments were performed with 25 mM butylmalonate added at zero time in the presence of 1 mM P_i (■—■), 1 mM malonate (▲—▲) or 0.1 mM L-malate (●—●). Controls with no additions were also included so that percentage exchange could be calculated.

Fig. 4. Time-course of malonate/[^{14}C]succinate and P_i/[^{14}C]succinate exchange. Rat-liver mitochondria (6.1 mg protein) loaded with [^{14}C]succinate were rapidly pipetted into 1-ml incubations of buffer at 0° containing 1 mM P_i (▲—▲), 1 mM malonate (■—■) or 1 mM L-malate (●—●) and 25 mM 2-butylmalonate added at time *t* (sec) to prevent further exchange. The mitochondria were then separated as described in METHODS AND MATERIALS and supernatant and pellet samples counted for [^{14}C]succinate. Similar experiments were performed with 25 mM butylmalonate added at zero time in the presence of 1 mM P_i ▲—▲), 1 mM malonate (□—□) or 1 mM L-malate (○—○). Controls with no additions were also included so that percentage exchange could be calculated.

at zero time did not abolish the exchange. 25 mM butylmalonate added at zero time prevented any further exchange in samples incubated in parallel with those used for P_i/L-[^{14}C]malate and malonate/L-[^{14}C]malate exchange time-courses. The effluxes appear to follow first-order kinetics, since log $(E_{max}/E_{max}-E)$ against t plots are linear. Similar observations were made with [^{14}C]succinate-loaded mitochondria. Exchanges of [^{14}C]succinate with phosphate and malonate were monitored over time using the inhibitor stop technique (Fig. 4), while exchange with L-malate was not completely inhibited by 25 mM butylmalonate. Succinate/L-[^{14}C]malate and succinate/[^{14}C]succinate exchanges were inhibited completely by 25 mM butylmalonate.

The situation for [^{14}C]malonate-loaded mitochondria was different. In order to obtain good measurable radioactive exchange rates, the mitochondria were first incubated at 10° for 10 min with 5 mM unlabelled malonate, spun, resuspended and then incubated with [^{14}C]malonate as described in METHODS AND MATERIALS. The exchange of [^{14}C]malonate with 1 mM L-malate was, in contrast to malate exchanges with L-[^{14}C]malate and [^{14}C]succinate, completely inhibited by 25 mM butylmalonate, so that efflux kinetics could be measured (Fig. 5a). The exchange of [^{14}C]malonate with 1 mM L-malate was found to be slower than with 1 mM malonate. When the preincubation step with unlabelled malonate was omitted, the exchange was more rapid but showed the same characteristics (Fig. 5b).

The dependence for the exchange upon the concentration of the externally added anion being investigated was also monitored using the inhibitor stop technique. Fig. 6a shows the concentration dependence of the exchange of P_i, succinate and malonate, respectively, with L-[^{14}C]malate. Inhibitor stops were performed at a series

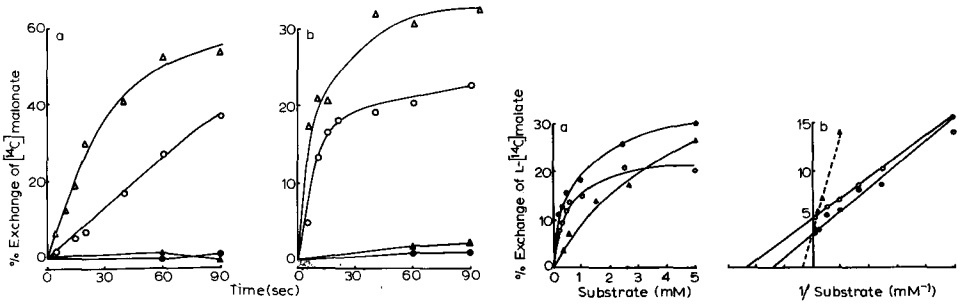

Fig. 5. Time-course of malonate/[^{14}C]malonate and L-malate/[^{14}C]malonate exchange reactions. Rat-liver mitochondria were loaded with [^{14}C]malonate as described in the text so that in (a) the specific activity of the malonate was lowered by the presence of unlabelled malonate, while in (b) it was much higher. 5.1-mg (protein) aliquots were pipetted rapidly into 1-ml incubations of buffer at 0° containing 1 mM malonate (△—△) or 1 mM L-malate (○—○) and 25 mM 2-butylmalonate added at time t (sec) to prevent further exchange. The mitochondria were then separated as described in METHODS AND MATERIALS and supernatant and pellet samples counted for [^{14}C]malonate. Similar experiments were performed with 25 mM butylmalonate added at zero time in the presence of 1 mM malonate (▲—▲) or 1 mM L-malate (●—●). Controls with no additions were also included so that percentage exchange could be calculated.

Fig. 6. The concentration dependence of the exchange of malonate, succinate and P_i for L-[^{14}C]malate. Rat-liver mitochondria (4.9 mg protein) loaded with L-[^{14}C]malate were rapidly pipetted into 1-ml incubations of buffer at 0° containing varying concentrations of malonate (●—●), P_i (○—○) or succinate (△—△, △---△). After 5 sec for malonate and succinate and 10 sec for P_i, the exchange was stopped by the addition of 25 mM butylmalonate and the mitochondria separated as described in METHODS AND MATERIALS and counted for L-[^{14}C]malate. (a) is a plot of percentage exchange against concentration, while (b) is a plot of the reciprocals of these values.

TABLE II

KINETIC CONSTANTS (K_m) OBTAINED FOR DICARBOXYLATE EXCHANGE REACTIONS

Experiments were performed as described for Fig. 6 and K_m's (mM) obtained from Lineweaver–Burk plots

	L-[^{14}C]*Malate*	[^{14}C]*Succinate*	[^{14}C]*Malonate*
L-Malate	—	—	1.20
			1.20
Succinate	1.32	0.18	—
	1.40	0.22	
Malonate	0.50	0.18	0.70
	0.35	0.15	0.91
	0.39	0.17	1.20
P_i	0.30	0.25	0.91
	0.20	0.15	1.10
		0.15	

TABLE III

RATE OF EXCHANGE OF SUBSTRATES AT 1 mM EXTRAMITOCHONDRIAL CONCENTRATION AT 0°

Experiments were performed as described in Figs. 3, 4 and 5 and values quoted are for the initial rates of exchange, with 1 mM of the unlabelled substrate added (first column). Rates expressed as nmoles/min per mg mitochondrial protein.

	L-[^{14}C]*Malate*	[^{14}C]*Succinate*	[^{14}C]*Malonate*
L-Malate (1 mM)	4.2*	—	0.09
Succinate (1 mM)	1.5	0.23	
	2.0	0.16	—
Malonate (1 mM)	4.2	0.53	0.39
		0.58	0.40
	4.1	0.36	
P_i (1 mM)	2.1	0.12	0.10
	2.5	0.08	0.11
		0.11	

* Value obtained using 25 mM *p*-iodobenzylmalonate as the inhibitor with 0.5 mM L-malate added extramitochondrially.

of concentrations at 5 sec for both malonate and succinate and at 10 sec for P_i. A Lineweaver–Burk plot (Fig. 6b) for the same data shows some scatter in the experimental points, but in a large number of experiments it was found that such plots approximated to linearity and that the K_m's obtained were reproducible. Table II shows the values obtained in a number of experiments for the various exchanges. Table III shows the rates of exchange calculated from the inhibitor stop experiments. The rates with L-[^{14}C]malate appear to be much faster, although the rate of exchange of labelled L-malate was very similar to the rates with [^{14}C]succinate and [^{14}C]malonate. This difference is due to the dilution of specific activity of the added L-[^{14}C]malate by endogenous L-malate so that for the same rate of exchange of labelled compound, the overall rate of exchange was greater. In any case, the differences in rate may reflect differing degrees of saturation of the carrier at the inside, rather than differing maximal transport capabilities.

All of the exchanges described in this paper were observed at 0°. At higher temperatures two factors became important. Firstly, the control incubations with no unlabelled dicarboxylate anion present showed that a considerable amount of labelled material was escaping to the incubation medium from the mitochondria, compared with the situation at 0°. Secondly, at 5, 10 and 20° the exchanges, which at 0° were inhibited completely by 25 mM butylmalonate, became less inhibited with increasing temperature.

It was thus not possible either to study the temperature dependence of the exchange reactions described or to estimate the rate of exchanges at physiological temperatures.

DISCUSSION

Sensitivity of dicarboxylate exchange to inhibition

The exchange reactions of the dicarboxylic acid transporting system seem in general to be sensitive to the same inhibitors, but there appears to be a difference in the degree of sensitivity depending on the exchanging species. The P_i/L-[^{14}C]malate exchange at 0° is completely inhibited by 12.5 mM butylmalonate, while the malonate/L-[^{14}C]malate exchange requires 25 mM butylmalonate for complete inhibition. L-Malate/L-[^{14}C]malate exchange requires 25 mM of the more potent inhibitor *p*-iodobenzylmalonate before inhibition is complete. The explanation for this, possibly lies in the binding of the inhibitor to the transporting system (at the extramitochondrial surface). When P_i is the exchanger, the inhibitor has no entering dicarboxylate anion to compete with, but with malonate and L-malate present the inhibitor cannot bind to the transporting system easily. Since it is likely that the transporting system carries L-malate *in vivo*, it is not surprising that L-malate/L-[^{14}C]malate exchange is the least sensitive to inhibitors. In heart mitochondria the malate/malate exchange is inhibited completely by 25 mM butylmalonate (unpublished observations). It is therefore unnecessary to invoke a separate mitochondrial transporting system which catalyses dicarboxylate/dicarboxylate exchanges in the presence of dicarboxylate transport inhibitors[4].

When the inhibitor stop technique was used to follow the time-course of exchange, it was found that in general the P_i/[^{14}C]dicarboxylate exchanges were slower than the corresponding malonate/[^{14}C]dicarboxylate exchanges when 1 mM P_i or malonate was used (see Table III). Since the K_m's for P_i- and malonate-stimulated exchanges were not very different for each category of [^{14}C]dicarboxylate anion, it is reasonable to conclude, in general, that P_i/dicarboxylate exchange is slower than dicarboxylate/dicarboxylate exchange. This could be another contributing factor to the differential sensitivity of the two exchanges to inhibitors.

The odd behaviour of L-malate when exchanging for [^{14}C]malonate is difficult to account for (Fig. 5). The slow rate of its exchange relative to that brought about by malonate and its butylmalonate sensitivity in contrast to the other L-malate-stimulated [^{14}C]dicarboxylate exchanges were two very puzzling features. Little explanation of this anomalous behaviour can be offered, unless the L-malate was preferentially exchanging with small amounts of endogenous citrate present in the mitochondria, but this state of affairs would be applicable to the other exchanges.

Variation in K_m for exchange reactions

It is obvious from Table II that the K_m found for the exchange reaction of an unlabelled species was not independent of the ^{14}C-labelled species in the exchange reaction. This indicates that the binding of the unlabelled exchanging moiety was a function of the identity of both the labelled and the unlabelled species. The K_m's for the exchanges with [^{14}C]succinate seemed to be generally lower than with L-[^{14}C]-malate and these in turn seemed to be lower than with [^{14}C]malonate. Thus the apparent K_m is influenced more perhaps by the labelled intramitochondrial species than the unlabelled extramitochondrial one. The values quoted by HARRIS AND MANGER[8] and by QUAGLIARIELLO *et al.*[6] for the K_m's for dicarboxylate permeation cannot be compared to those here, since it was not clear in these cases what the exchanging species was for the penetrating dicarboxylate anion.

Conclusions

It has been shown that the inhibitor stop method may be used to investigate the kinetics of dicarboxylate exchange reactions at low temperatures. The results show good agreement with the postulate that a single transporting system catalyses the exchange of P_i with dicarboxylate anions and the exchange of dicarboxylate anions with dicarboxylate anions. These two exchanges catalysed by the dicarboxylate anion transporting system, however, show different sensitivities to 2-butylmalonate inhibition[4], this being due probably to the relative substrate binding affinities at the extra-mitochondrial facet of the anion exchange carrier.

ACKNOWLEDGEMENTS

We thank Mrs. J. Oei for expert technical assistance and the Medical Research Council of Canada for financial support. One of us (B. H. R.) holds an M.R.C. of Canada Postdoctoral Fellowship.

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