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BIOCHEMISTRY OF THE DEVELOPING RAT BRAIN

III. MITOCHONDRIAL OXIDATION OF CITRATE AND ISOCITRATE AND ASSOCIATED PHOSPHORYLATION

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SUMMARY

Oxygen uptake, reduction of added pyridine nucleotides and enzyme activities concerned with isocitrate oxidation show that aged mitochondria from both neonatal and adult rat brain oxidize isocitrate by two separate and independent pathways, one mediated by NAD and $NADH_2$-oxidase system and the other by NADP and $NADPH_2$-oxidase system.

The neonatal and adult brain mitochondria contain equal concentrations of NAD-isocitrate dehydrogenase (Ls-isocitrate: NAD oxidoreductase (decarboxylating), EC 1.1.1.41) and $NADH_2$-cytochrome *c* reductase ($NADH_2$: cytochrome *c* oxidoreductase, EC 1.6.2.1) activities. However, the neonatal mitochondria have a much greater NADP-isocitrate dehydrogenase (Ls-isocitrate: NADP oxidoreductase (decarboxylating), EC 1.1.1.42) activity and much lower $NADPH_2$-cytochrome *c* reductase ($NADPH_2$: cytochrome *c* oxidoreductase, EC 1.6.2.3) activity than the adult.

Oxidation of citrate occurred at a lower rate than isocitrate oxidation in intact mitochondria from both neonate and adult rat brains. But in aged mitochondria from the adult, citrate was oxidized at a higher rate than isocitrate indicating an alternate pathway for citrate oxidation in this preparation in addition to the tricarboxylic acid cycle.

Intact mitochondria from adult rat brain oxidized citrate and isocitrate with a P:O ratio of 2. This ratio was increased by added NAD and decreased by added NADP. Mitochondria aged in phosphate buffer failed to oxidize either citrate or isocitrate unless pyridine nucleotides were supplemented. Addition of either NAD or NADP restored oxidation completely, but the oxidative phosphorylation did not exceed a ratio of 1.

INTRODUCTION

The pyridine nucleotide requirements for isocitrate oxidation have been studied by ERNSTER[1], KAPLAN *et al.*[2], VIGNAIS AND VIGNAIS[3], and others[4,5], in adult rat liver, kidney and brain mitochondria aged in phosphate buffer. The higher concentration in liver mitochondria of the NADP-isocitrate dehydrogenase (EC 1.1.1.42) and a $NADH_2$-oxidase system compared to NAD-isocitrate dehydrogenase (EC 1.1.1.41) and a

$NADPH_2$-oxidase system, and the presence of an active NAD(P) transhydrogenase ($NADPH_2$: NAD oxidoreductase, EC 1.6.1.1) suggested to KAPLAN and others[2,4], that the oxidation of isocitrate is accomplished by the pathway: NADP-isocitrate dehydrogenase → NAD(P) transhydrogenase → $NADH_2$-oxidase system. However, ERNSTER *et al.*[1,23], concluded from their oxygen uptake studies that isocitrate was oxidized primarily by the NAD-linked isocitrate dehydrogenase. VIGNAIS *et al.*[3] suggested that this discrepancy could be explained on the basis of their findings that aged liver mitochondria were able to synthesize necessary amounts of NADP from NAD by the action of NAD-kinase (ATP: NAD 2'-phosphotransferase, EC 2.7.1.23). They were of the opinion, however, that the pathway of isocitrate oxidation in brain mitochondria may differ from liver since the brain contains negligible amounts of NAD(P) transhydrogenase and a high concentration of NAD-isocitrate dehydrogenase.

However, analytical data to be presented in this report showed that the concentration of enzymes in the rat brain concerned with the oxidation of isocitrate varied with the age of the organ. Thus neonatal rat brain mitochondria had a much higher NADP-isocitrate dehydrogenase and a much lower NADP-cytochrome *c* reductase (EC 1.6.2.3) activity than the adult rat brain mitochondria. In addition, it was observed that the relative rates of oxidation of citrate and isocitrate in aged mitochondria differed in the neonate and the adult. The following report summarizes the results relating to these oxidations in intact and aged mitochondria from the neonatal and adult rat brains. Data are also presented indicating the role of NAD and NADP in phosphorylations associated with the oxidation of isocitrate in the adult rat brain mitochondria.

METHODS

Preparation of mitochondria

Male Sprague-Dawley rats were used in these experiments. The designation "neonate" denotes one-day-old rats and the "adult" rats of approx. 12 weeks of age. After decapitation, the brains were removed, rinsed free of blood in ice-cold homogenizing medium, then rough-dried on cheese-cloth and weighed. 2 g at a time were homogenized in 10 ml of a medium containing 0.25 M sucrose, 0.06 M nicotinamide, 0.004 M ATP, and 0.02 M Tris buffer (pH 7.4). This medium was used for all experiments except for those recorded in Table I. The combined homogenates were diluted with the same medium to 10 vol. per gram of tissue and then centrifuged at 1000 × *g* for 10 min (0°) to remove nuclei and cell debris. Mitochondria were sedimented by centrifugation of the supernatant at 10000 × *g* for 15 min and the sediment was washed twice with the homogenizing medium. The inside walls of the centrifuge tube were carefully wiped to remove any adhering solution and the pellet was washed a third time with an excess of cold 0.25 M sucrose in order to remove all traces of the additives contained in the homogenizing medium. The mitochondria were finally suspended in 0.25 M sucrose to represent 1 g of fresh brain per ml of solution. Mitochondria obtained by the above procedure and exhibiting P:O ratios approaching theoretical values are referred to in this report as the "intact" mitochondria.

Since isolation of mitochondria required approx. 2 h, the homogenizing medium was formulated with nicotinamide in order to preserve pyridine nucleotides[6], and with ATP in order to maintain oxidative and phosphorylating activities in the mitochondria[7].

Aging

Mitochondria intended for aging were prepared as described above except that the final washing was made with 0.25 M sucrose buffered with 0.05 M potassium phosphate (pH 7.4). Mitochondria were then suspended in this phosphate–sucrose solution to a concentration representing 1 g of fresh brain per ml. For aging, this suspension was incubated at 30° for 1 h with occasional shaking[8].

Analyses

Oxygen uptake was determined in a Warburg apparatus at 30° using 15-ml flasks. Determinations of NAD- and NADP-isocitrate dehydrogenases were made according to the procedure described previously[9]. $NADH_2$- and $NADPH_2$-cytochrome *c* reductases were assayed according to the methods of MAHLER *et al.*[10], and HASS *et al.*[11], respectively, as modified by VIGNAIS AND VIGNAIS[3]. NAD(P) transhydrogenase was determined by a modification of the method of COLOWICK *et al.*[12]. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and NADP were used as the $NADPH_2$-generating system for the transhydrogenase reaction instead of the usual reaction mixture containing isocitrate, isocitrate dehydrogenase, and NADP. This change was necessary because the presence of an NAD-α-ketoglutarate dehydrogenase (α-ketoglutarate: NAD oxidoreductase (decarboxylating)) and NAD-glutamate dehydrogenase (L-glutamate: NAD oxidoreductase) in rat brain mitochondria render the system producing α-ketoglutarate unsuitable for accurate determination of NAD(P) transhydrogenase.

Aconitate hydratase (citrate (isocitrate) hydro-lyase, EC 4.2.1.3, formerly known as aconitase) activity was determined spectrophotometrically by the formation of $NADPH_2$ from NADP in the presence of citrate and an excess of purified NADP-isocitrate dehydrogenase[3]. NAD-kinase was measured by the method of WANG[13] and the citrate oxalacetate lyase (CoA acetylation, ATP hydrolysis) by the procedure described by SREERE[14]. Oxidative phosphorylation was measured by the method of HUNTER[15] at 30°. Inorganic phosphate was estimated by the FISKE AND SUBBAROW procedure[16].

NAD and $NADH_2$ were obtained from the Pabst Laboratories; NADP, $NADPH_2$ and disodium ATP from the Sigma Chemical Company; trisodium DL-isocitrate was purchased from Mann Research Laboratories and cytochrome *c* from Calbiochem.

RESULTS

Homogenization and reaction media

Maximal oxidation of citrate and isocitrate was obtained when mitochondria were prepared in a homogenizing medium containing 0.25 M sucrose, 0.06 M nicotinamide, 0.004 M ATP, and 0.02 M Tris buffer (pH 7.4). (Table I, Expt. 3). When the Tris buffer was omitted during homogenization, oxidation of citrate in the presence of either NAD or NADP decreased by 30 %, while the oxidation of isocitrate was decreased by 30 % with NADP but was not affected in the presence of NAD (Table I, Expts. 1 and 2). Maintenance of an adequate buffer capacity in the reaction mixture was also found to be necessary, since a change in buffer concentration from 1.3 mM to 0.65 mM phosphate completely abolished citrate oxidation and decreased the oxidation of isocitrate by one-third (Table I, Expts. 1 and 2).

TABLE I

EFFECT OF MEDIA USED IN HOMOGENIZATION AND INCUBATION ON CITRATE AND ISOCITRATE OXIDATIONS BY NEONATAL BRAIN MITOCHONDRIA (AGED)

The reaction medium (3.0 ml) contained 15 μmoles $MgCl_2$, 2 μmoles $MnSO_4$, 30 μmoles KF, 5 μmoles ATP, 0.04 μmole cytochrome *c*, 60 μmoles glucose, 140 K. M. units hexokinase, 40 μmoles citrate or DL-isocitrate, 3 μmoles each of the pyridine nucleotides, and "aged" mitochondria equivalent to 0.5 g fresh brain. In addition, 20 μmoles phosphate buffer (pH 7.4) was added for Expt. 1 and 40 μmoles phosphate buffer for Expts. 2 and 3. Incubation, 60 min, 30°. In Expts. 1 and 2 mitochondria were isolated from homogenates prepared in 0.25 M sucrose, 0.08 M nicotinamide and 0.004 M ATP, in Expt. 3 the same homogenate was used but with 0.02 M Tris buffer (pH 7.4) added.

Experiment No.	*1*		*2*		*3*	
Additions	*Citrate*	*Isocitrate*	*Citrate*	*Isocitrate*	*Citrate*	*Isocitrate*
	(μl O_2 uptake per mg protein per hour)					
None	0	0	0.5	0.6	0.5	0.6
NAD	0	4.8	2.8	7.6	4.1	7.3
NADP	0	1.2	1.4	1.7	2.1	2.7
NAD + NADP	0	5.8	4.1	8.0	5.5	10.2
NAD + NADP (calculated)	(0)	(6.0)	(4.2)	(9.3)	(6.2)	(10.0)

Inactivation of pyridine nucleotides by aging

Aging of mitochondria destroys endogenous pyridine nucleotides[17]. It can be seen from Fig. 1 that the oxidation of isocitrate was negligible in the absence of added coenzymes and was restored on addition of either NAD or NADP.

Oxidation of isocitrate in aged mitochondria, from both neonatal and adult brains, increased with an increase in the concentration of added NAD up to a level of 2.0 μmoles per flask (3 ml of reaction mixture containing mitochondria equivalent to 500 mg

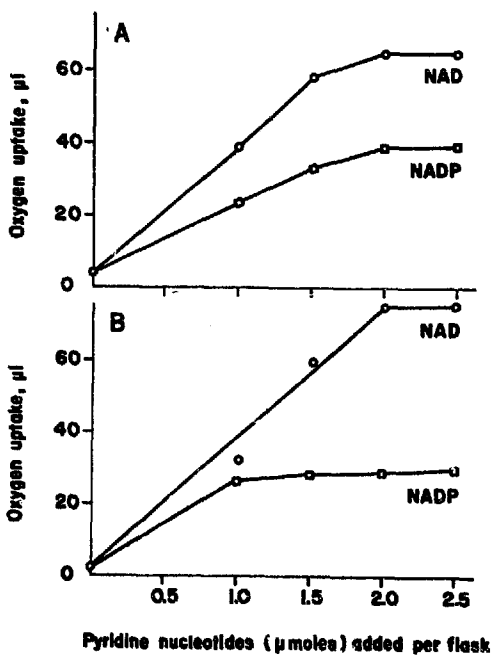

Fig. 1. Effect of pyridine nucleotide concentrations on oxidation of isocitrate by aged mitochondria; (A) adult, incubation 50 min, 30°; (B) neonate incubation 40 min 30°. Composition of reaction mixture was the same as that described in Table I.

fresh brain). Further additions of the coenzyme were ineffective. On the other hand, in the presence of NADP as the coenzyme, the maximum oxygen uptake was reached at a level of 1.0 μmole in the neonate (Fig. 1, B) and 2.0 μmoles (Fig. 1, A) in the adult. The maximum oxygen uptake with NAD was higher than that obtained with NADP, in both the adult and the neonate. All oxygen uptake experiments in the present studies were made with 3 μmoles of added pyridine nucleotides per flask to insure that NAD and NADP were not at limiting concentrations during the experimental period.

TABLE II

OXIDATION OF CITRATE AND ISOCITRATE BY INTACT BRAIN MITOCHONDRIA

Composition of the reaction mixture the same as that described in Table I, Expt. 3. Incubation, 40 min, 30°.

Additions	*Neonate*		*Adult*	
	Citrate	*Isocitrate*	*Citrate*	*Isocitrate*
	(μl O_2/flask/40 min)			
None	11.8	13.5	11.3	11.7
NAD	30.1	41.8	30.6	40.0
NADP	18.5	22.3	30.0	36.6
NAD + NADP	45.5	70.1	48.8	53.6
NAD + NADP (calculated)	(48.6)	(64.1)	(66.6)	(76.6)

TABLE III

OXIDATION OF CITRATE AND ISOCITRATE BY AGED BRAIN MITOCHONDRIA

Composition of the reaction mixture the same as in Table I, Expt. 3. Incubation, 60 min, 30°.

Additions	*Neonate*			*Adult*		
	None	*Citrate*	*Isocitrate*	*None*	*Citrate*	*Isocitrate*
	(μl O_2 uptake per flask per hour)					
None	0	4.8	5.8	0	9.4	7.0
NAD	1.5	40.5	72.6	2.2	59.2	45.6
NADP	0	20.5	27.0	1.1	44.7	32.0
NAD + NADP	2.8	55.2	101.5	4.1	76.3	52.0
NAD + NADP (calculated)	(1.5)	(61.0)	(99.6)	(3.3)	(103.9)	(77.6)

Oxidation by intact mitochondria

Freshly prepared mitochondria from neonatal or adult rat brain oxidized both citrate and isocitrate even in the absence of added pyridine nucleotides (Table II). Additions of either NAD or NADP to the reaction mixture enhanced these oxidations. The effect of added NAD was more pronounced than that of NADP in the neonate than in the adult. When the two nucleotides were added together, the oxygen uptake by adult mitochondria was less than the calculated sum of the values obtained in the presence of either nucleotide alone. In the neonate, however, the effects of NAD and NADP were additive. In both the neonate and the adult, the oxidation of citrate was equal to or lower than that of isocitrate in the presence or absence of added pyridine nucleotides, indicating that in intact mitochondria the major pathway for citrate oxidation is through the tricarboxylic acid cycle.

Oxidation by aged mitochondria

No oxygen uptake was observed by aged mitochondria (neonatal or adult) in the absence of exogenous substrate even when the mitochondria were supplemented with NAD and NADP individually or in combination (Table III). With citrate or isocitrate as the substrate, oxygen uptake was negligible unless pyridine nucleotides were added. Addition of either NAD or NADP restored oxidation of these substrates. This shows that these preparations of aged mitochondria did not contain any endogenous substrates.

Addition of NAD was more effective than NADP in promoting the oxidation of citrate and isocitrate in the aged mitochondria of the neonate, but this difference was much less pronounced in the adult (Table III). Also, in the presence of both nucleotides, oxygen uptake was equal to the sum of the indiviadul values with NAD and NADP in the neonate mitochondria but the uptake was much less than additive in the adult preparations.

Aged mitochondria from neonatal brain oxidized citrate at a rate much lower than isocitrate in the presence of the added coenzymes (Table III). In contrast to this, oxidation of citrate by adult brain mitochondria was higher than that of isocitrate in the presence of either NAD or NADP. This suggests that aging of adult brain mitochondria activated a new reaction for citrate dismutation in addition to the Krebs' cycle pathway.

Isocitrate dehydrogenase and cytochrome c reductase activities

When NAD or NADP was used as the final electron acceptor, the rate of reduction of NADP was six times greater than the reduction of NAD in neonatal mitochondria and approximately equal in the adult (Table IV). When both coenzymes were added

TABLE IV

ISOCITRATE DEHYDROGENASE AND CYTOCHROME *c* REDUCTASE ACTIVITIES IN AGED BRAIN MITOCHONDRIA

The reaction mixture (1.0 ml) for isocitrate dehydrogenase determinations contained 15 μmoles DL-isocitrate, 0.8 μmole oxidized pyridine nucleotides, 0.7 μmole $MnCl_2$, 50 μmoles Tris (pH 7.3) and 10–100 μg mitochondrial protein. Increase in absorbancy was recorded at 340 mμ against a blank containing all reagents except the pyridine nucleotides. The reaction mixture (1.0 ml) for cytochrome *c* reductase determinations contained 0.5 μmole reduced pyridine nucleotides, 0.1 μmole oxidized cytochrome *c*, 1.0 μmole KCN, 75 μmoles Tris (pH 7.3) and 50–200 μg mitochondrial protein. Reduction of cytochrome *c* was recorded at 550 mμ against a blank containing all reagents except the pyridine nucleotides.

	Isocitrate (mμmoles of pyridine nucleotides reduced/mg protein/min)		*Cytochrome c (mμmoles of cytochrome c reduced/mg protein/min)*	
	Neonate	*Adult*	*Neonate*	*Adult*
NAD	14.0	13.4	—	—
$NADH_2$	—	—	16.5	17.2
NADP	86.8	16.6	—	—
$NADPH_2$	—	—	0	0.65
NAD + NADP	97.5	28.8	—	—
NAD + NADP (calculated)	(100.8)	(30.0)	—	—
$NADH_2$ + $NADPH_2$	—		16.5	7.1
$NADH_2$ + $NADPH_2$ (calculated)	—		(16.5)	(17.9)

TABLE V

OXIDATION OF ISOCITRATE BY AGED BRAIN MITOCHONDRIA (ADULT) WITH NAD AND NADP AS FINAL ELECTRON ACCEPTORS

Composition of the reaction mixture and methods of determination of enzyme activities were the same as in Table III. When added, arsenite and KCN concentrations were 10 μmoles/ml and 0.1 μmole/ml, respectively.

	NAD	*NADP*	*NAD+NADP*	*NAD + NADP (calculated)*
	(mμmoles of pyridine nucleotides reduced/mg protein/min)			
Isocitrate	8.1	16.2	23.8	24.2
Isocitrate + arsenite	8.4	16.8	26.4	25.2
Isocitrate + KCN	6.3	16.1	23.2	22.4

to either neonatal or adult preparations, the total amounts of reduced pyridine nucleotides formed was equal to the calculated sum of $NADH_2$ and $NADPH_2$ produced when each coenzyme was added individually. These reactions were carried out in the absence of KCN, since this is a known inhibitor of NAD-isocitrate dehydrogenase[3]. In order to avoid interference due to the presence of a $NADH_2$-oxidase system activity, the reactions were carried out anaerobically under an atmosphere of nitrogen, as described previously[9]. Thus, a higher rate of NAD reduction was observed in the absence of KCN than in its presence (Table V). It was also noted that a mitochondrial NAD-α-ketoglutarate dehydrogenase did not interfere in the assay since addition of arsenite, an inhibitor of this enzyme[18], had no effect on the rate of $NADH_2$ formation.

Aged mitochondria from adult brain reduced cytochrome *c* in the presence of either $NADH_2$ or $NADPH_2$ (Table IV). $NADPH_2$-cytochrome *c* reductase had a very low activity compared to the $NADH_2$-cytochrome *c* reductase, the ratio being approx. 1:30. When both $NADH_2$ and $NADPH_2$ were added, the amount of reduced cytochrome *c* formed was less than half of the total that would be expected if $NADH_2$ and $NADPH_2$ were oxidized separately. This suggests that one or both of the $NADH_2$- and $NADPH_2$-cytochrome *c* reductases are competitively inhibited by the coenzymes $NADPH_2$ and $NADH_2$ respectively. While the $NADPH_2$-cytochrome *c* reductase had comparable activity in the neonate and adult mitochondria, the $NADH_2$-cytochrome *c* reductase in the neonate was below the range of detection.

Enzymes in isocitrate oxidation

The NAD-isocitrate dehydrogenase and $NADH_2$-cytochrome *c* reductase had the same activities in the neonatal and adult mitochondria (Table VI). NADP-isocitrate dehydrogenase in the neonate had approx. 5 times the activity of that in the adult. NADPH-cytochrome *c* reductase activity was very low in both the neonatal and adult mitochondria, but it had a measurable activity in the adult while it could not be detected in the neonate by the assay method used. Activities of NAD(P) transhydrogenase, citrate oxalacetate lyase, NAD-kinase and aconitate hydratase could not be detected spectrophotometrically in either mitochondrial preparation.

TABLE VI

ENZYMES IN AGED BRAIN MITOCHONDRIA

Composition of these reaction mixtures described under METHODS. The activities denote an average of 6 determinations.

	Neonate	*Adult*
NAD-isocitrate dehydrogenase*	13.3	12.5
NADP-isocitrate dehydrogenase*	87.9	16.5
$NADH_2$-cytochrome *c* reductase**	16.5	18.8
$NADPH_2$-cytochrome *c* reductase**	0	0.7
Aconitate hydratase	Trace	Trace
NAD(P) transhydrogenase	0	0
NAD-kinase	0	0
Citrate oxalacetate lyase	0	0

* mμmoles of pyridine nucleotides reduced or oxidized/mg protein/min.
** mμmoles of cytochrome *c* reduced/mg protein/min.

Oxidative phosphorylation by adult intact mitochondria

In the absence of added pyridine nucleotides, the oxidative phosphorylation associated with the oxidation of either citrate or isocitrate was the same with a P:O ratio of 2 (Table VII). When NAD was added, the P:O ratio was increased slightly, but with added NADP this ratio was drasti ally reduced. When both NAD and NADP (3 μmoles each per flask) were added, the ratio was intermediate to that obtained with either of the pyridine nucleotides alone.

TABLE VII

OXIDATIVE PHOSPHORYLATION IN ADULT BRAIN MITOCHONDRIA (INTACT)

The reaction mixture was the same as in Table I, Expt. 3. Mitochondria equivalent to 0.5 g fresh brain was added in a total volume of 3 ml. Incubation, 40 min, 30°.

Additions	*Citrate*		*Isocitrate*	
	O_2 uptake (μatoms)	*P:O*	*O_2 uptake (μatoms)*	*P:O*
None	1.9	1.91	3.1	1.80
NAD	3.8	2.06	7.1	2.25
NADP	4.0	1.25	5.8	1.32
NAD + NADP	5.5	1.75	9.2	1.46
NAD + NADP (calculated)	(7.8)	—	(12.9)	—

Oxidative phosphorylation by adult aged mitochondria

Oxidative phosphorylation was only partially uncoupled by aging the mitochondria in phosphate buffer (Table VIII). In the absence of any added coenzymes, oxygen uptake was very low and oxidative phosphorylation was zero. In contrast to the effect on oxidation, oxidative phosphorylation was not completely restored by the addition of pyridine nucleotides. The highest P:O ratios from citrate or isocitrate oxidation were noted in the presence of NAD and the lowest ratios in the presence of NADP. In the presence of both NAD and NADP, the P:O ratios had values intermediate to those with NAD and NADP added individually.

TABLE VIII

OXIDATIVE PHOSPHORYLATION IN ADULT BRAIN MITOCHONDRIA (AGED)

Composition of the reaction mixture the same as that described in Table I, Expt. 3. Incubation in Expt. 1 for 40 min and in Expt. 2 for 30 min at 30°.

Expt. No.	Substrate	No additions		NAD		NADP		NAD + NADP		Protein per flask (mg)
		O_2 uptake (μatoms)	P:O	O_2 uptake (μatoms)	P:O	O_2 uptake (μatoms)	P:O	O_2 uptake (μatoms)	P:O	
1	Isocitrate	0.72	0.00	4.43	0.90	3.20	0.64	5.95	0.68	9.6
2	Citrate	1.72	0.00	5.28	1.00	4.00	0.69	6.81	0.85	12.7

DISCUSSION

Aging of adult brain mitochondria was used as a means of depriving them of endogenous pyridine nucleotides[17]. Applicability of this aging process to the neonatal brain mitochondria was somewhat in question because of the very low activity of NAD glycohydrolase (EC 3.2.2.5) in the neonatal rat brain[19]. It was found that in the absence of added NAD or NADP, oxidation of isocitrate by aged mitochondria was negligible (Fig. 1, B; Table I), but with the addition of pyridine nucleotides a high oxygen uptake occurred. Freshly prepared intact mitochondria, on the other hand, oxidized these substrates even in the absence of added coenzymes (Table II). These observations indicated that aging destroyed the pyridine nucleotides in the neonatal brain mitochondria in spite of the low NAD glycohydrolase activity.

Use of aged mitochondria in elucidating the role of pyridine nucleotides in isocitrate oxidation has the following disadvantages: (a) the observed oxidation in presence of NAD alone may be due to traces of residual NADP in aged mitochondria, which would oxidize isocitrate via the NAD(P) transhydrogenase reaction[20], (b) NADP may be formed from added NAD by NAD-kinase[3] and (c) large quantities of mitochondria used in oxygen-uptake measurements may magnify trace contents of NADP or NAD-kinase activity or both[4].

The above criticisms are valid only in such cases where the NAD(P) transhydrogenase reaction is prominent, for example in liver or kidney mitochondria[3]. No NAD (P) transhydrogenase activity was detected in aged neonatal or adult brain mitochondria by the spectrophotometric method (Table VI). It may be argued that the large amount of mitochondria employed in the present studies (equivalent to 500 mg fresh brain per flask) may contain NAD(P) transhydrogenase activity sufficient to give oxygen uptake, but not sufficient to be detected at dilutions used for spectrophotometric analysis. Any NAD(P) transhydrogenase activity, as a significant step in isocitrate oxidation, however, is precluded for the following reasons (Tables I, III and IV):

1. In the absence of added pyridine nucleotides, the oxidation of isocitrate was negligible.

2. Addition of either NAD or NADP promoted isocitrate oxidation.

3. The presence of both NAD and NADP in the aerobic or anaerobic oxidation of isocitrate had an effect which was either additive in the neonate or less than additive in the adult preparations (Tables III and IV). If the NAD(P) transhydrogenase was

active in these mitochondrial preparations, then these values would be much more than additive in the presence of both pyridine nucleotides, as reported in liver and kidney mitochondria[3].

4. Aged mitochondria from the neonate or adult had no detectable NAD-kinase activity (Table VI). Moreover, the reaction system used in these experiments contained hexokinase (EC 2.7.1.1) and glucose to trap any ATP which is necessary for the NAD-kinase reaction.

From the above results it is deduced that in aged mitochondria from rat brain, oxidation of isocitrate must occur by two separate and independent pathways, one mediated by NAD and $NADH_2$-oxidase system, and the other by NADP and $NADPH_2$- oxidase system. Since the NAD(P) transhydrogenase is absent, it can be calculated on the basis of oxygen uptake data (Table III) that in the neonatal mitochondria approx. two-thirds of isocitrate is oxidized via the NAD-pathway and the rest through the NADP-pathway. In the adult, isocitrate oxidation appears to be equally divided between the two pathways. However, whether this reflects the actual bifurcation of isocitrate oxidation in brain remains to be ascertained.

The activities of $NADH_2$- and $NADPH_2$-cytochrome *c* reductases were low in our preparations of aged mitochondria from both the neonatal and adult brains (Table IV) compared to the results reported by VIGNAIS AND VIGNAIS[3] for adult rat brain. This may be attributed to the fact that in the present studies the mitochondria were not pretreated to reveal the maximal cytochrome *c* reductase activities[21]. The activities of isocitrate dehydrogenases and cytochrome *c* reductases in neonatal rat brain mitochondria have not been previously reported, hence no comparison can be made.

Aging of mitochondria in phosphate buffer not only destroys the pyridine nucleotides but also inactivates aconitate hydratase[22]. No aconitate hydratase activity was detected in aged mitochondria from neonate or adult by the spectrophotometric method used in this study, although this method revealed measurable aconitate hydratase activity in intact mitochondria[9]. However, when citrate was incubated with high concentrations of aged mitochondria (equivalent to 500 mg fresh brain per flask) in an atmosphere of nitrogen, isocitrate was produced (2 μmoles of isocitrate after 1 h of incubation at 30°). When the reaction mixture was supplemented with NAD or NADP, 1–2 μmoles of α-ketoglutarate were formed with a simultaneous production of CO_2. This indicated that, although aconitate hydratase activity was low in aged mitochondria, sufficient enzyme remained after aging to permit a partial oxidation of citrate via the tricarboxylic acid pathway. In neonatal mitochondria aged in phosphate buffer, aconitate hydratase activity was a limiting factor in citrate oxidation, since there was a lower oxygen uptake with citrate than with isocitrate (Table III). In contrast to this, adult brain mitochondria after aging, oxidized citrate at a higher rate than isocitrate. Similar results in aged mitochondria from adult rat liver were reported by ERNSTER AND GLASKY[23]. These observations suggest that there may exist an alternate pathway of citrate oxidation in mitochondria from adult rat tissues, in addition to the usual pathway mediated by aconitate hydratase and isocitrate dehydrogenase. This aspect of citrate oxidation is being further investigated.

Neonatal mitochondria had much more NADP-isocitrate dehydrogenase activity and much less $NADPH_2$-cytochrome *c* reductase activity than the adult brain (Table VI). The significance of this difference in activities of the above enzymes in the oxidation of isocitrate in neonatal brain mitochondria is not clear. It is possible that the

generation of $NADPH_2$ by the NADP-isocitrate dehydrogenase is required for the rapid synthesis of lipids in the rat brain during postnatal development[24].

Studies with intact mitochondria from adult rat brain showed that oxidative phosphorylation had a ratio of approx. 2 with isocitrate or citrate as the substrate (Table VII). This ratio is not in agreement with the results of VIGNAIS AND VIGNAIS[25], who reported that in adult rat brain mitochondria phosphorylation associated with the oxidation of a mixture of acids (isocitrate, aconitate and citrate) did not exceed a P:O of 1. Assuming that the major pathway for isocitrate oxidation was through the sequence of NADP-isocitrate dehydrogenase → $NADPH_2$-cytochrome *c* reductase → cytochrome oxidase (EC 1.9.3.1), they suggested that the oxidation of $NADPH_2$ was associated with a single phosphorylating step occurring at the oxidation of reduced cytochrome *c*. The results reported here agree with those of VIGNAIS *et al.* in respect to the first step in the above sequence, since the oxidation of isocitrate occurred at a higher rate with NADP as the final electron acceptor than with NAD (Table IV). However, during oxidation of isocitrate under aerobic conditions, oxygen uptake was greater with NAD than with NADP (Tables II and III). This presumably, is due to the much higher activity of $NADH_2$-cytochrome *c* reductase in brain mitochondria compared to the $NADPH_2$-cytochrome *c* reductase[3] (Table IV). If in the aerobic oxidation of isocitrate the maximum P:O ratio associated with $NADH_2$oxidati on is 3, and that associated with $NADPH_2$ oxidation is 1 (see ref. 25), oxidative phosphorylation in brain mitochondria would be expected to assume a value somewhere between these figures. The observed P:O ratio of 2 conforms to this estimate (Table VII).

Intact mitochondrial preparations used in these studies were permeable to added pyridine nucleotides and were able to utilize them for the oxidation of isocitrate (Tables II and VII). BIRT AND BARTLEY[26] have shown that intact mitochondria from liver have a low permeability to NAD and the small amount of this coenzyme that does enter the mitochondria has no access to intramitochondrial oxidations and reductions. On the other hand, NADP could enter mitochondria readily and participate in mitochondrial reactions. However, the results of these authors and those of LEHNINGER[27] show that the role of added pyridine nucleotides in mitochondrial function is dependent on the conditions used for the preparation and pretreatment of mitochondria. It is presumed that the preparation and incubation conditions used in the present studies may be responsible for the permeability of added NAD and NADP into intact mitochondria.

Addition of NAD to intact mitochondria increased the phosphorylation associated with citrate or isocitrate oxidation, but the addition of NADP decreased this phosphorylation (Table VII). This can be explained in the following manner: (a) NAD and NADP are in limiting quantities in intact mitochondria and their relative concentration determines the proportion of isocitrate oxidized by the NAD- or the NADP-pathway. Added NAD increases the proportion of NAD-mediated oxidation, while added NADP increases the proportion of NADP-mediated oxidation; (b) the greater the fraction of isocitrate oxidized through NAD, the higher the P:O ratio and the greater the portion of this substrate oxidized through NADP, the lower the P:O ratio.

Addition of either NAD or NADP to aged mitochondria gave P:O ratios approaching 1 (Table VIII). This indicated that in aged mitochondria one phosphorylation step, common to both NADH and NADPH pathways, remained coupled to oxidation. Since the oxidation of NADPH leads to only one phosphorylation associated with the

oxidation of reduced cytochrome *c* by cytochrome oxidase[25], it appears that this is the site of phosphorylation that remained intact during mitochondrial aging.

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