BBA 3961

BIOCHEMISTRY OF THE DEVELOPING RAT BRAIN

II. NEONATAL MITOCHONDRIAL OXIDATIONS

M. R. V. MURTHY AND D. A. RAPPOPORT

Department of Pediatrics, The University of Texas Medical Branch, Galveston, Texas (U.S.A.)

(Received October 2nd, 1962)

SUMMARY

Intracellular distribution of tricarboxylic acid-cycle enzymes in neonatal rat brain revealed a preponderance of NADP-isocitric dehydrogenase, malic dehydrogenase and aconitase (pH 7.4) activities in the soluble fraction while NAD-isocitric dehydrogenase, aconitase (pH 5.8), succinicoxidase and cytochrome oxidase were localized in mitochondria.

Comparison of brain and liver mitochondria from the same neonatal rats showed differences in coenzyme requirements for oxidation of substrates. Addition of NAD inhibited the oxidation of succinate in both tissues; addition of cytochrome *c* greatly increased succinate oxidation in liver mitochondria but was comparatively less effective in brain.

Of the 3 cofactors tested for α-ketoglutarate oxidation, CoA, thiamine pyrophosphate and NAD, neonatal brain mitochondria required only NAD for maximal oxidation and oxidative phosphorylation. In liver mitochondria, neither oxidation of this substrate nor oxidative phosphorylation was affected when NAD was omitted. Under optimal conditions, neonatal-rat-brain mitochondria gave P:O ratios of 1.0 for succinate and 3.0 for α-ketoglutarate.

INTRODUCTION

Histological and biochemical investigations of the brain during development have shown changes not only in chemical composition, but also in enzyme content. Thus BURTON[1] reported that the neonatal rat brain exhibited very low activities of NADase and the activity rose progressively as the rat matured, reaching adult levels in three weeks. POTTER *et al.*[2], and DUBOIS *et al.*[3], using rat brain slices and homogenates, showed that among the respiratory enzymes, succinic dehydrogenase and cytochrome oxidase increased in concentration during growth. SAMSON *et al.*[4] found that during the first three weeks of development rat-brain-mitochondrial protein per cell increased three fold with a concomitant rise in number, size and weight of mitochondria. Since these observations indicated changes in oxidative metabolism in the developing brain, investigations were undertaken to examine with some detail,

Abbreviation: TPP, thiamine pyrophosphate.

the enzymology of neonatal rat-brain mitochondria for eventual comparison with mature rat brain.

This report summarizes the results from studies on the (a) fractionation and characterization of subcellular fractions in neonatal rat brain, and (b) mitochondrial enzyme activities from brain as compared to liver, and influence of added cofactors on these activities.

METHODS

Preparation of subcellular fractions

Brains and livers, from one day old Sprague–Dawley rats, were removed immediately after decapitation and homogenized in a Potter–Elvehjem homogenizer. For each gram of fresh tissue, 5 ml of a 0.25 M sucrose solution containing 0.02 M Tris buffer (pH 7.4) was used for homogenization unless otherwise specified. This was then further diluted with an equal volume of the above medium. The homogenates were first centrifuged at 1000 × *g* for 10 min, and after removing the cell debris, the supernatant was recentrifuged at 10000 × *g* for 15 min. The mitochondrial pellet was separated and washed twice by resuspending in the extraction medium and sedimenting at 10000 × *g* for 15 min. Subsequently the pellet was suspended in isotonic sucrose to give approx. 10 mg of mitochondrial protein/ml. Other subcellular fractions were collected according to the procedure outlined in Fig. 1.

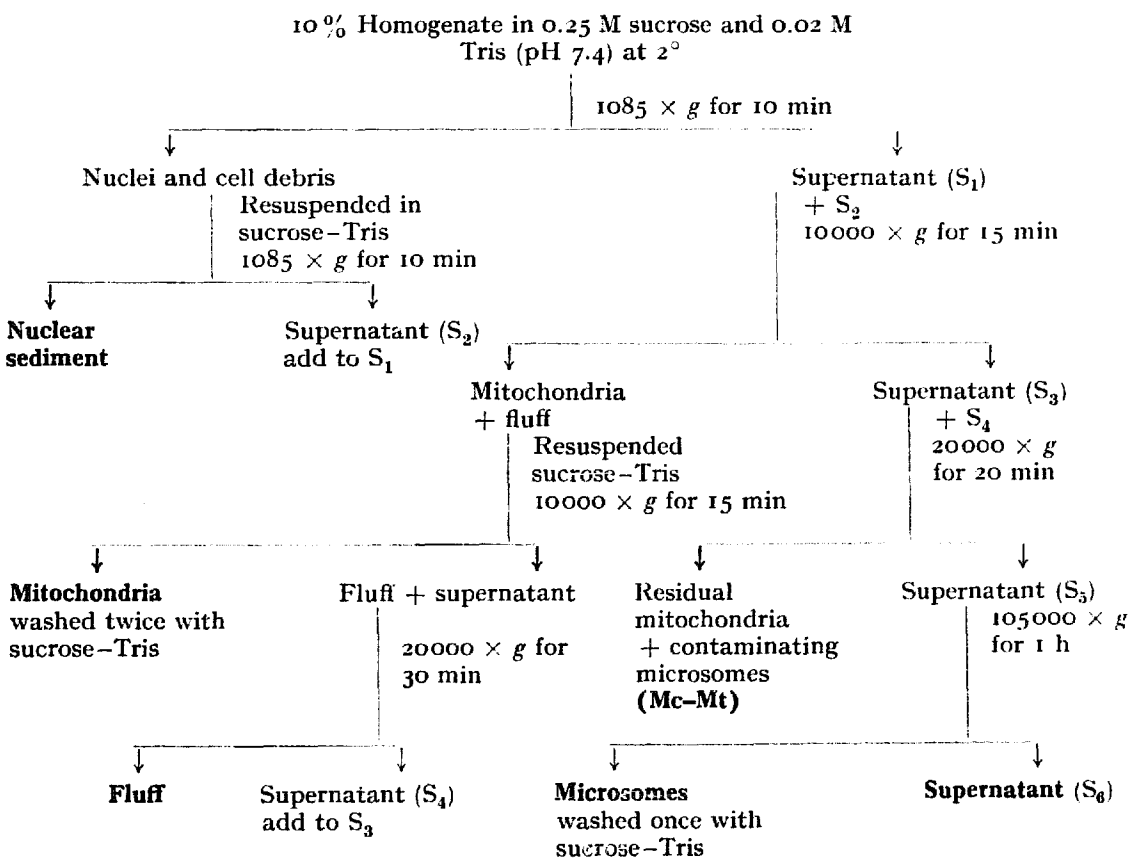

Fig. 1. Scheme for the preparation of subcellular fractions from neonatal rat brain

Determination of enzyme activities

Succinicoxidase and cytochrome oxidase were determined by the manometric method described by SCHNEIDER AND POTTER[5] using a Warburg apparatus at 37°. NADP-isocitric dehydrogenase was determined by the spectrophotometric method of GRAFFLIN AND OCHOA[6]. NAD-isocitric dehydrogenase activity was measured by the procedure described by PLAUT AND SUNG[7] except KCN and nicotinamide were omitted from the reaction mixture. In order to prevent NADH reoxidation by atmospheric oxygen, all reagents were prepared in water previously boiled to remove dissolved oxygen and then gassed with nitrogen just before use. Malic dehydrogenase was estimated using the procedure described by MEHLER *et al.*[8]. Aconitase was determined by measuring the reduction of NADP in the presence of citrate with an excess of added NADP-isocitric dehydrogenase[9]. All spectrophotometric determinations were made using a double-beam Beckman spectrophotometer with the sample chamber maintained at 30° by circulating water. RNA was determined by the SCHNEIDER method[10]. Proteins were estimated either by the WARBURG AND CHRISTIAN spectrophotometric method[11] or by the biuret reaction[12].

Oxidative phosphorylation was measured by the method of HUNTER[13] at 30°. Inorganic phosphate was estimated by the FISKE AND SUBBAROW method[14].

RESULTS AND DISCUSSIONS

Preparation of mitochondria: effect of the homogenizing medium

When mitochondria were isolated in isotonic sucrose in the presence of 10^{-4} M EDTA and 0.02 M Tris buffer (pH 7.4), respectively, it was found that EDTA inhibited oxidation of α-ketoglutarate, succinate, and isocitrate, but Tris yielded mitochondria which oxidized these substrates at a high rate (Table I).

TABLE I

EFFECT OF HOMOGENIZING MEDIA ON OXYGEN UPTAKE BY BRAIN MITOCHONDRIA

The reaction mixture (3.0 ml) contained 40 μmoles phosphate buffer (pH 7.4), 40 μmoles substrate, 15 μmoles $MgCl_2$, 2 μmoles $MnSO_4$, 30 μmoles potassium fluoride, 5 μmoles ATP, 60 μmoles glucose, 140 K.M. units hexokinase, 0.04 μmole cytochrome *c* and mitochondria equivalent to 0.5 g fresh brain. In addition, NAD (3 μmoles) was added to the reaction mixture of Expt. I, NAD (3 μmoles) and potassium malonate (30.0 μmoles) to reaction mixture of Expt. II and $CaCl_2$ (1.3 μmoles) and $AlCl_3$ (1.3 μmoles) to reaction mixture of Expt. III.

		μl O_2/mg protein/h		
	Substrate	*0.25 M sucrose*	*0.25 M sucrose 10^{-4} M EDTA*	*0.25 M sucrose 0.02 M Tris (pH 7.4)*
Expt. I	Isocitrate	8.9	7.2	11.5
Expt. II	α-Ketoglutarate	8.2	7.2	12.3
Expt. III	Succinate	22.3	18.8	32.4

Of the three substrates tested, succinate was oxidized at a much higher rate than either α-ketoglutarate or isocitrate. This observation conforms with the polarographic determination by SACKTOR *et al.*[15] and the manometric data reported by BERNSOHN *et al.*[16] and BERGER *et al.*[17] using adult-rat-brain mitochondria. However, in neonatal-

rat-brain mitochondria the actual rate of oxidation of each of these substrates was lower than in the adult.

During centrifugation, a "fluff" was obtained which remained loosely packed above the mitochondrial sediment. It required fewer washings to remove the "fluff" from mitochondria in sucrose–Tris medium than in sucrose medium.

Effect of sedimentation rate

The activities of succinic oxidase and cytochrome oxidase were measured in mitochondrial preparations isolated from centrifugations at 10000 × *g* and 20000 × *g*, respectively, for 15 min. The resulting mitochondrial pellets exhibited the same levels of enzyme activities with the same relative ratio of these activities, as shown in Table II.

TABLE II

SUCCINICOXIDASE AND CYTOCHROME *c* OXIDASE ACTIVITY IN MITOCHONDRIAL FRACTIONS FROM NEONATAL BRAIN

Activity in μl O_2/mg protein/h

	Succinicoxidase (A)	*Cytochrome c oxidase (B)*	*Ratio B/A*
Mitochondia (10000 × *g*)	26.7	78.7	2.9
Mitochondria (20000 × *g*)	29.7	84.0	2.9

Effect of repeated washings

Although the sedimentation characteristics of mitochondria and microsomes are widely separated, it was impossible to obtain mitochondria free from microsomes at 10000 × *g* for 15 min unless the mitochondrial pellet was repeatedly washed. Such repeated washings with sucrose–Tris medium effectively decreased the microsomal contamination from mitochondria, as can be seen from the RNA analysis shown in Table III. The succinicoxidase activity increased during the washing process and reached a maximum value after two washings (Table III), indicating that this increase was probably due to the removal of the inactive "fluff". However, oxidative phosphorylation, with succinate as the substrate, decreased drastically by one-third of the original value after four washings and by one-half the original value after six washings (Table III).

On the basis of the above results, the protocol described under METHODS was

TABLE III

EFFECT OF WASHING ON SUCCINICOXIDASE ACTIVITY AND OXIDATIVE PHOSPHORYLATION

	Succinicoxidase (μl O_2/mg protein/h)	*P:O*	*RNA (μg P/mg protein)*
Mitochondria washed once	26.9	1.21	1.63
Mitochondria washed 2 times	33.1	1.12	1.48
Mitochondria washed 4 times	33.6	0.84	1.30
Mitochondria washed 6 times	34.8	0.69	1.29

adopted for the isolation of neonatal-rat-brain mitochondria for all subsequent studies. This procedure yielded a mitochondrial preparation almost free of "fluff" and one which exhibited a maximal rate of substrate oxidation with a high degree of oxidative phosphorylation (Table III). A slight contamination of microsomes was noted, as indicated by RNA analysis, but this was not considered critical in this investigation.

Intracellular distribution of enzymes

In order to characterize biochemically the discrete cell fractions obtained by differential centrifugation of the neonatal rat brain, a complete fractionation was undertaken by the procedure illustrated in Fig. 1. In these preparations, some of the tricarboxylic acid cycle enzymes in each fraction were measured and the results are shown in Table IV.

TABLE IV

DISTRIBUTION OF TRICARBOXYLIC ACID CYCLE ENZYMES IN SUBCELLULAR FRACTIONS OF THE NEONATAL BRAIN

Fraction	*Succinicoxidase*	*Cytochrome oxidase*	*Isocitric dehydrogenase (NADP)*	*Malic dehydrogenase*	*Aconitase (pH 7.4)*	*Aconitase (pH 5.8)*	*RNA*
	(μl O_2/h/mg protein)		*($\Delta A \times 10^3$/min/mg protein)*				*(μg P/mg protein)*
Mitochondria	25.8	75.0	204	326	25	48	1.34
Fluff	7.0	15.0	56	1.36	—	—	2.30
Mc–Mt	7.5	14.8	103	1.78	—	—	1.85
Microsomes	0.0	0.0	64	64	—	—	6.69
Supernatant	0.0	0.0	412	924	198	0.0	2.35

Succinicoxidase and cytochrome oxidase

These two enzymes were found exclusively in the mitochondrial fraction and the cytochrome oxidase was three times as active as the succinicoxidase (Table IV). Although these enzymes were absent in the supernatant and microsomal fractions, the "fluff" and the "Mc–Mt" fractions showed both of these enzyme activities, presumably due to entrapped mitochondria.

Isocitric dehydrogenases

The soluble fraction contained twice as much of the NADP-isocitric dehydrogenase as did the mitochondria (Table IV). This stands in sharp contrast to the distribution of this enzyme in rat liver[10], where the ratio of enzyme activities in the soluble and particulate fractions was 10:1. SHEPHERD AND KALNITSKY[19], have shown, however, that in rabbit cerebral cortex more than half of the NADP-isocitric dehydrogenase was present in the mitochondrial fraction. While mitochondrial NADP-isocitric dehydrogenase has an acknowledged role in the tricarboxylic acid cycle oxidations[20,21], the presence of appreciable amounts of this enzyme in the soluble fraction, in such organs as the liver and brain, is difficult to interpret in terms of its role in cell metabolism[22].

When the present enzyme distribution studies were initiated, the NAD-isocitric dehydrogenase could not be demonstrated in any of the subcellular fractions by the

spectrophotometric method[7]. It was later found that addition of cyanide (used to inhibit cytochrome oxidase) and nicotinamide (used to protect pyridine nucleotides from hydrolysis) interfered with determinations of NAD-isocitric dehydrogenase. When these two ingredients were omitted and the determinations were carried out in an atmosphere of nitrogen, NAD-isocitric dehydrogenase was found exclusively in mitochondria. This enzyme was only one-sixth as active as the corresponding mitochondrial NADP enzyme. VIGNAIS *et al.*[9] reported a higher ratio for these enzymes in adult-rat-brain mitochondria. These differences may be explained as variations during brain development or due to the different methods of pretreatment of mitochondria.

Malic dehydrogenase

The soluble fraction of the neonatal rat brain contained three times as much malic dehydrogenase activity as was found in mitochondria (Table IV). JOHNSON[23] found 45 % of the malic dehydrogenase in the soluble fraction of the adult rat brain and 40 % in the mitochondrial fraction. He concluded that this mitochondrial enzyme from adult rat brain was truly particulate and not due to the presence of axons incompletely fragmented during homogenization. In neonatal rat brain, the high concentration of malic dehydrogenase in mitochondria, even after two washings, is indicative of its particulate localization.

There was a small measurable activity of malic dehydrogenase in the microsomal fraction, but this is presumed to be due to contamination from the soluble fraction.

Aconitase

Aconitase in the soluble fraction had an optimum at pH 7.4, but mitochondria exhibited very little activity at this pH (Table IV). In mitochondria the aconitase had an optimum at pH 5.8 and its activity was only one-fifth that of the soluble aconitase. DICKMAN AND SPEYER[24] showed that aconitase in rat-liver mitochondria had an optimum at pH 5.8 and that in the soluble fraction at pH 7.4. It was noted, however, that in the subcellular fractions from the neonatal brain, the activity of the mitochondrial aconitase increased two-fold when the pH was decreased to 5.8, whereas the soluble aconitase was completely inactivated under the same treatment (Table IV).

Ribonucleic acid

RNA determination of each subcellular fraction was made mainly to test for the contamination of mitochondria by microsomal or supernatant fractions. A comparison of the RNA content of mitochondria from Table IV with those shown in Table III indicates that the RNA content reached a minimum value and that mitochondria were relatively free of contaminants when prepared by the procedure described above (Fig. 1). The amounts of microsomal RNA and of soluble RNA were five- and two-fold greater, respectively, than the RNA content of mitochondria. The "fluff" and "Mc–Mt" fractions contained more RNA than mitochondria but less than the microsome and soluble fractions, thus indicating the heterogeneous composition of "fluff" and "Mc–Mt" fractions.

The actual RNA content (μg P/mg protein) of mitochondrial, microsomal, and soluble fractions of neonatal rat brain (Table IV) were approximately double the values reported by ALDRIDGE AND JOHNSON[25] in corresponding fractions from adult

rat brain. However, the distribution of RNA in the subcellular fractions of both neonatal and adult brain were similar.

Mixed cell fractions: Mc–Mt fraction

This was the fraction obtained between 10000 × *g* and 20000 × *g* after the "fluff" and mitochondrial pellet were removed. This fraction had very little succinate oxidase, cytochrome oxidase, or malic dehydrogenase activities, but there was a fairly high activity of NADP-isocitric dehydrogenase (Table IV). The RNA content in this fraction was higher than in the mitochondria. It appeared to have the characteristics of "light" mitochondria contaminated with microsomes and adsorbed components from the soluble fraction. This fraction was not further characterized since the objectives were to locate the proper centrifugal range for the isolation of intact mitochondria.

"Fluff"

This fraction was a light, almost white, layer which sedimented at the same speed as the mitochondria. Both the suspending medium and the centrifugal speed were the determinant factors in separating the "fluff" from mitochondria. Thus, in isotonic sucrose most of the "fluff" remained as a loose layer above the mitochondria and could be decanted off, while in sucrose–Tris medium and at 20000 × *g* it became a tightly packed layer above the mitochondria and required repeated washing to remove completely. Other investigators[26–28] have described a similar "fluffy" layer in adult rat brain homogenates. DAHL *et al.*[28] were of the opinion that their "light" layer did not originate from mitochondria but that some kind of association between this fraction and mitochondria occurred *in vivo*. Enzymic characterization of the "fluffy" layer showed low activities of succinate oxidase and NADP-isocitric dehydrogenase and almost no malic dehydrogenase (Table IV). It did have, however, a high RNA content, comparable to the RNA in the soluble fraction. Based on these determinations, it appears that this fraction is composed of fragmented mitochondria heavily contaminated with microsomes.

Effect of cofactors on mitochondrial oxidations: oxidation of succinate

Addition of NAD markedly inhibited oxidation of succinate by both liver and brain mitochondria from the same neonatal rats (Table V). This observation conforms with those of PARDEE AND POTTER[29] who showed that oxalacetate, formed from succinate in the presence of NAD, was inhibitory to succinic dehydrogenase. Inhibition by added NAD in the neonatal brain mitochondria, occurred even in the presence of Ca^{2+} in the reaction medium. This effect can be explained by the fact that neonatal brain has a very low NADase activity[1], hence the absence of any effect by Ca^{2+} on the hydrolysis of NAD. Oxidative phosphorylation, with succinate as the substrate, was also inhibited by added NAD (Table V).

On addition of cytochrome *c*, oxidation of succinate was increased two-fold in liver mitochondria but only by a third in brain mitochondria (Table V). This indicates that cytochrome *c* is more firmly bound in brain mitochondria than in the liver and is therefore less readily removed during the preparation of this fraction. Addition of cytochrome *c* does not affect mitochondrial oxidative phosphorylation in either of these organs.

Oxidation of α-ketoglutarate

In the oxidation of α-ketoglutarate by neonatal brain mitochondria, the effect of added CoA, thiamine pyrophosphate and NAD were tested separately. Only NAD was necessary for this oxidation. In the absence of added NAD, oxidation of this keto acid was reduced by 50 % and oxidative phosphorylation was reduced by 15 %. In liver, oxidation of this substrate was influenced slightly when NAD was omitted and oxidative phosphorylation was not affected at all (Table VI). These findings indicate that either the bound NAD is in limiting concentration in neonatal brain mitochondria, or, in contrast to liver, this cofactor is readily lost from brain mitochondria during preparation.

SACKTOR[15] showed by polarographic measurements that added NAD increased the oxidation of substrates mediated by NAD-linked dehydrogenases in adult-rat-brain mitochondria. CHANCE[30] has reported that the ratio of pyridine nucleotides to cytochrome *c* in rat-brain mitochondria was considerably lower than that in liver or heart mitochondria. This suggests that the different requirements for cofactors by

TABLE V

OXIDATION OF SUCCINATE IN THE MITOCHONDRIA OF NEONATAL BRAIN AND LIVER

The reaction mixture contained 100 μmoles of phosphate buffer (pH 7.4), 125 μmoles sodium succinate, 1.3 μmoles $CaCl_2$, 1.3 μmoles $AlCl_3$, 8 μmoles ATP, 8 μmoles $MgCl_2$, 2 μmoles $MnSO_4$ and mitochondria equivalent to 100 mg (wet wt.) of brain, in 3.0 ml total volume of reaction mixture. NAD and cytochrome *c* were added, when necessary, at a concentration of 0.8 μmole and 0.017 μmole per ml, respectively; 100 μmoles of glucose and 100 K.M. units of hexokinase were transferred from the side arm after equilibration for 7 min at 30°.

	μl O_2/mg protein/h	*P : O*
Brain mitochondria	26.2	1.10
+ NAD	12.5	0.81
+ cytochrome *c*	34.5	1.12
Liver mitochondria	30.5	1.30
+ NAD	15.2	0.84
+ cytochrome *c*	60.4	1.23

TABLE VI

OXIDATION OF α-KETOGLUTARATE IN THE MITOCHONDRIA OF NEONATAL BRAIN AND LIVER

The reaction mixture was the same as described under Table V except Ca^{2+} and Al^{3+} were omitted and CoA, NAD and TPP were added at a concentration of 0.7 μmole/ml.

	μl O_2/mg protein/h	*P : O*
Exp. 1		
Brain mitochondria	29.2	2.85
minus CoA	26.5	2.60
minus NAD	19.3	2.40
minus TPP	30.3	2.65
Expt. 2		
Brain mitochondria	28.3	2.75
minus NAD	14.9	1.94
Liver mitochondria	38.2	2.91
minus NAD	30.4	2.92

liver and brain mitochondria found in the present studies reflect differences in cofactor composition of the mitochondria from these organs, and not merely variations in permeability properties.

In this study, the maximal ratio of oxidative phosphorylation, observed with neonatal-rat-brain mitochondria, was 1.1 for succinate and 2.9 for α-ketoglutarate (Table VI). Data on the P:O ratios of neonatal brain mitochondria are lacking in the literature, but in adult brain there is a considerable degree of variation in the published results with succinate as substrate. With α-ketoglutarate, fairly consistent data have been reported. Thus, Voss *et al.*[31] found a P:O ratio of 1.98 for succinate and 2.8 for α-ketoglutarate, whereas ABOOD *et al.*[32], using Triton–Alconox-treated mitochondria, obtained P:O values of 1.5 for succinate and 2.7 for α-ketoglutarate.

ACKNOWLEDGEMENTS

This work was supported in part by grant RG-7926 from the National Institutes of Health, U.S. Public Health Service, and by the Medical Research and Development Command, Department of the Army under a contract No. DA-49-193-MD-2139.

REFERENCES

1 R. M. BURTON, *J. Neurochem.*, 2 (1957) 15.
2 V. R. POTTER, W. C. SCHNEIDER AND G. J. LIEBL, *Cancer Res.*, 5 (1945) 21.
3 K. P. DUBOIS AND V. R. POTTER, *J. Biol. Chem.*, 150 (1943) 185.
4 F. E. SAMSON, Jr., W. M. BALFOUR AND R. J. JACOBS, *Am. J. Physiol.*, 199 (1960) 693.
5 W. C. SCHNEIDER AND V. R. POTTER, *J. Biol. Chem.*, 149 (1943) 217.
6 A. L. GRAFFLIN AND S. OCHOA, *Biochim. Biophys. Acta*, 4 (1950) 295.
7 G. W. E. PLAUT AND S. C. SUNG, *Methods in Enzymol.*, Vol. 1, Academic Press, New York, 1955, p. 710.
8 A. H. MEHLER, A. KORNBERG, S. GRISOLIA AND S. OCHOA, *J. Biol. Chem.*, 174 (1948) 961.
9 P. V. VIGNAIS AND P. M. VIGNAIS, *Biochim. Biophys. Acta*, 47 (1961) 515.
10 W. C. SCHNEIDER, *J. Biol. Chem.*, 161 (1945) 293.
11 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
12 J. GOA, *Scand. J. Clin. Lab. Invest.*, 5 (1953) 218.
13 F. E. HUNTER, *Methods in Enzymol.*, Vol. 2, Academic Press, New York, 1955, p. 610.
14 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
15 B. SACKTOR, L. PACKER AND R. W. ESTABROOK, *Arch. Biochem. Biophys.*, 80 (1959) 68.
16 J. BERNSOHN, I. NAMAJUSKA AND L. S. G. COCHRANE, *Arch. Biochem. Biophys.*, 62 (1956) 274.
17 M. BERGER, H. J. STRECKER AND H. WAELSCH, *Nature*, 177 (1956) 1234.
18 G. H. HOGEBOOM AND W. C. SCHNEIDER, *J. Biol. Chem.*, 186 (1950) 417.
19 J. A. SHEPHERD AND G. KALNITSKY, *J. Biol. Chem.*, 207 (1954) 605.
20 N. O. KAPLAN, M. N. SWARTZ, M. E. FRECH AND M. M. CIOTTI, *Proc. Natl. Acad. Sci. U.S.*, 42 (1956) 481.
21 J. L. PURVIS, *Biochim. Biophys. Acta*, 52 (1961) 148.
22 L. ERNSTER, *Biochem. Soc. Symp. (Cambridge, Engl.)*, 16 (1959) 54.
23 M. K. JOHNSON, *Biochem. J.*, 84 (1962) 25P.
24 S. R. DICKMAN AND J. F. SPEYER, *J. Biol. Chem.*, 206 (1954) 67.
25 W. N. ALDRIDGE AND M. K. JOHNSON, *Biochem. J.*, 73 (1959) 270.
26 M. K. JOHNSON, *Biochem. J.*, 77 (1960) 610.
27 R. BALAZS AND J. R. LAGNADO, *J. Neurochem.*, 5 (1959) 1.
28 D. R. DAHL, R. J. JACOBS AND F. E. SAMSON, *Am. J. Physiol.*, 198 (1960) 467.
29 A. B. PARDEE AND V. R. POTTER, *J. Biol. Chem.*, 176 (1948) 1085.
30 B. CHANCE, *Science*, 120 (1954) 745.
31 D. O. VOSS, A. P. CAMPELLO AND M. BACILA, *Biochem. Biophys. Res. Commun.*, 4 (1961) 48.
32 L. G. ABOOD, E. BRUNNGRABER AND M. TAYLOR, *J. Biol. Chem.*, 234 (1959) 1307.