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THE ENERGY-YIELDING OXIDATION OF NADH BY FUMARATE IN SUBMITOCHONDRIAL PARTICLES OF RAT TISSUES

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SUMMARY

1. Oxidation of NADH by fumarate coupled to synthesis of ATP was found to occur in cyanide-poisoned rat heart submitochondrial particles. The reaction was inhibited by amytal, thenoyltrifluoroacetone and malonate, indicating the involvement of a portion of the electron transfer chain.

2. Cytochrome *b* became oxidized (while the other cytochromes remained reduced) during the oxidation of NADH by fumarate, suggesting that cytochrome *b* is part of the reaction pathway.

3. Succinate was recovered as the reaction product and accounted for the NADH oxidized.

4. The P/*2e* ratios indicate that one ATP was produced for each pair of electrons transferred to fumarate.

5. The reaction was also demonstrated to be present in liver and gastrocnemius muscle of rat. The reaction rate in heart was 2.0 times that of gastrocnemius and 3.3 times that of liver. These differences are not related to the activities of NADH or succinate dehydrogenase.

6. The ubiquitous nature of this reaction suggests that it could serve as an important physiological mechanism for generating extra glycolytic energy during periods of anoxia.

INTRODUCTION

The oxidation of NADH by fumarate demonstrated in anaerobic beef heart submitochondrial particles is of special interest for it includes a phosphorylation at the first site of the electron transfer chain[1,2]. The production of such ATP might add substantially to that generated by anaerobic glycolysis and thereby play an important role in maintaining the viability of a tissue exposed to anoxia. This is suggested by the results of CASCARANO *et al.*[3] who found that addition of fumarate to the glucose-perfused anoxic rat heart elevated the rate of beating above that obtained in the presence of glucose alone. Comparable physiological protection has been obtained in rabbits subjected to severe tissue ischemia by hemorrhagic shock. Survival was significantly increased when animals were infused with fumarate instead of glucose or NaCl[4].

In the light of the demonstrated physiological influence of fumarate under anaerobiosis, it appeared to be important to determine if anaerobic mitochondrial ATP production could indeed account for the effects obtained. Although the oxidation of NADH by fumarate is suggested by the experimental results in the perfused rat heart, this reaction has not been directly demonstrated in the rat. Experiments were therefore undertaken to determine if this reaction did occur in rat heart and if it had the same characteristics as the reaction obtained in beef heart particles[1]. This would be essential for further exploration of the physiological and metabolic implications of this reaction in the isolated perfused rat heart. Since SANADI AND FLUHARTY[1] demonstrated involvement of only succinate and NADH dehydrogenases in the reaction pathway, an attempt was made in the present investigation to determine if additional components of the electron transfer chain were also involved. Lastly, it would appear that if this reaction was of physiological importance it would be extensively distributed in different animal tissues. For this reason the reaction was explored in liver and gastrocnemius in addition to heart.

MATERIALS AND METHODS

Male Sprague–Dawley rats, 300–350 g, were decapitated and the heart, median lobe of the liver and the gastrocnemius rapidly removed and placed in saline at 0°. The homogenization procedure and isolation medium varied with each tissue.

The liver was sliced, washed and blotted, and a 5 % (v/v) homogenate prepared in 0.25 M sucrose–0.01 M Tris–HCl buffer (pH 7.6), using a Potter–Elvehjem glass homogenizer and a motor-driven Teflon pestle.

The heart was cut open, washed in cold saline, minced and homogenized in 20 ml of 0.25 M sucrose, 1 mM EDTA, 0.02 M Tris–HCl buffer (pH 7.6). Preliminary homogenization in a Potter–Elvehjem homogenizer was accomplished with a loosely fitting serrated stainless-steel pestle. Homogenization was then completed using a Teflon pestle with standard clearance.

The gastrocnemius was minced and a 10 % (v/v) homogenate prepared in 0.175 M KCl, 0.01 M Tris–HCl buffer (pH 7.6). The tissue mince was first homogenized in a Waring blender for 30 sec. The homogenate was then transferred to a Potter–Elvehjem glass homogenizer and homogenization completed with the serrated stainless-steel pestle and finally the Teflon pestle.

The procedure for isolating mitochondria and preparing submitochondrial particles was identical for all tissues. The homogenate was centrifuged for 5 min at 500 × *g* and decanted. The supernatant was recentrifuged for 5 min at 500 × *g*, decanted and centrifuged at 14000 × *g* for 15 min. The mitochondrial pellet was washed once by resuspending in 0.25 M sucrose, 0.01 M Tris buffer (pH 7.6) and recentrifuged for 15 min at 14000 × *g*. The mitochondria from six animals were combined and resuspended in a total volume of 25 ml of 0.01 M Tris–HCl buffer (pH 7.6), 1 mM EDTA (protein concentration of 2–5 mg/ml). The suspension was subjected to sonic oscillations for 30 sec at 0°, using a Branson sonifier, Model LS-75 at a power output of 3–4 A. Large particles and unbroken mitochondria were removed by centrifuging at 25000 × *g* for 10 min. The supernatant was centrifuged for 45 min at 78500 × *g* and the resulting pellet resuspended in 0.25 M sucrose, 1 mM EDTA, 0.02 M Tris–HCl buffer (pH 7.6) and recentrifuged at 78500 × *g*. The submitochondrial particles

were finally suspended in 0.25 M sucrose, 0.01 M Tris–HCl buffer (pH 7.6) (protein concentration 4–6 mg/ml), frozen at −20° and used within a week. These submitochondrial particles will be referred to as electron transport particles.

The assay for the fumarate-dependent NADH oxidation was essentially that of SANADI AND FLUHARTY[1]. The reaction mixture contained 0.05 M Tris–HCl buffer (pH 7.5), 0.1 mM EDTA, 1.6 mM KCN, 0.16 mM NADH, and 200–300 μg electron transport particles protein in 350 μl. The reaction was begun by the addition of 10 μl of 0.175 M fumarate and NADH oxidation at 27° was followed at 340 nm using a Cary-15 split-beam recording spectrophotometer. Since some NADH oxidation occurred in the absence of fumarate, a control containing 10 μl buffer instead of fumarate was always used.

Succinate dehydrogenase activity was determined by the method of BONNER[5] and NADH dehydrogenase activity by the method of MINAKAMI *et al.*[6]. For both assays, the reduction of $Fe(CN)_6^{3-}$ at different concentrations was followed at 420 nm and the results extrapolated to v_{max} with respect to oxidant. 2 moles of $Fe(CN)_6^{3-}$ were reduced by 1 mole of succinate or NADH[7]. The medium contained 0.04 M potassium phosphate buffer (pH 7.4), 1 mM KCN, and 0.5, 0.9, 1.3 or 1.7 mM $K_3Fe(CN)_6$. The reaction mixture for NADH dehydrogenase also contained 0.32 mM NADH and 5–15 μg electron transport particles and that for succinate dehydrogenase 5 mM succinate and 75–150 μg electron transport particles.

Identification of succinate was carried out by anion-exchange chromatography as described by BUSCH *et al.*[8]. The protein-free sample and standards (in 6 % (v/v) $HClO_4$) were neutralized with 90 % (w/v) KOH and centrifuged to remove the precipitated $KClO_4$. The residue was washed, the supernatant fractions combined and filtered onto a 1 cm × 12 cm column of Dowex 1 X8 (formate), 200–400 mesh. Formic acid of gradually increasing concentration passed through the column and the eluate was collected in 2-ml fractions. The fractions were evaporated to dryness in heated vacuum desiccators and the residue dissolved in 1 ml of 10 % (v/v) ethanol containing 0.01 % phenolphthalein and titrated with 0.05 M NaOH. Standards containing 6 μmoles of succinate, malate and fumarate were run immediately before and after each sample.

Protein was determined by the method of LOWRY *et al.*[9].

All experiments were repeated on different electron transport particles preparations and representative data are presented in figures and tables.

RESULTS

The first objective of this investigation was to determine if fumarate could induce the oxidation of NADH in cyanide-poisoned rat heart electron transport particles. As seen in Fig. 1, NADH was oxidized by fumarate. This oxidation of NADH occurring in the absence of fumarate was 20–30 % of that obtained in the complete reaction system. Further increases in cyanide concentration had no effect on this background oxidation. Reaction rate corrected for background oxidation was approximately one half that reported for beef heart submitochondrial particles prepared under somewhat different conditions[1,2]. The anoxic fumarate-dependent oxidation is only 2–3 % of the rate obtained when NADH is oxidized by O_2.

In order to determine the pathway of the reaction and to establish whether

a portion of the electron transfer chain was involved, the effect of various inhibitors on the reaction was examined. As shown in Figs. 2, 3 and 4, the reaction was inhibited by amytal, thenoyltrifluoroacetone, and malonate. This not only indicates the involvement of both NADH dehydrogenase and succinate dehydrogenase in the reaction pathway but also implicates the transport of electrons over a portion of the electron transfer chain.

The use of the inhibitors, however, gave no indication of whether or not cytochrome *b* was a connecting link between these two branches of the electron transfer chain. In order to investigate the possible involvement of cytochrome *b*, absorption spectra of the cytochromes were obtained in the presence and absence of fumarate.

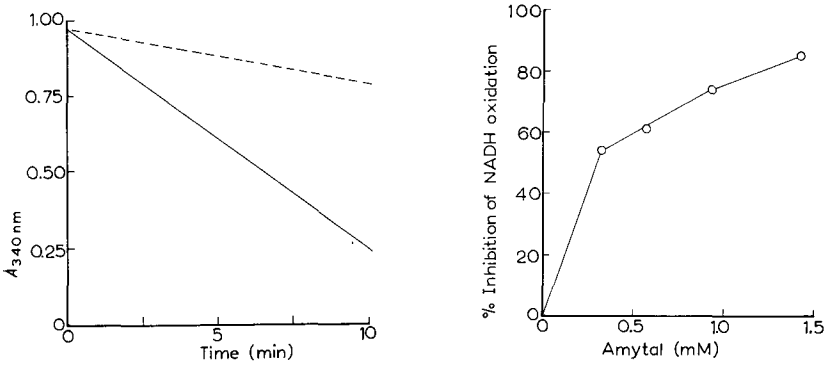

Fig. 1. Fumarate-dependent oxidation of NADH. The assay was carried out as described under MATERIALS AND METHODS. The control (– – – –) received 10 μl of buffer instead of fumarate (——).

Fig. 2. Effect of amytal on the oxidation of NADH by fumarate in heart electron transport particles. The reaction mixture was the same as that described in MATERIALS AND METHODS with the exception that the concentration of fumarate was 10 mM.

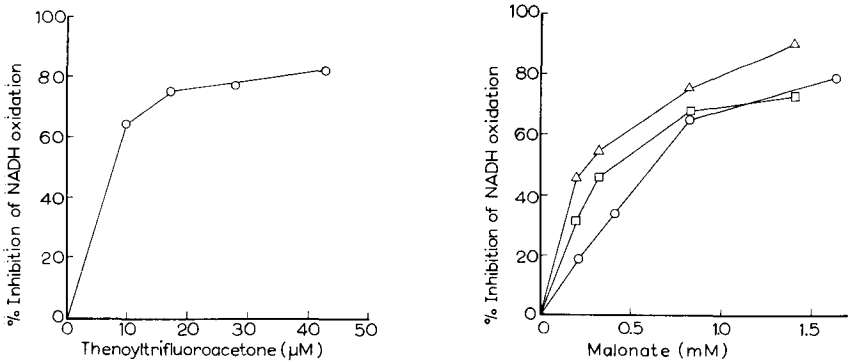

Fig. 3. Effect of thenoyltrifluoroacetone on the oxidation of NADH by fumarate in heart electron transport particles. The reaction mixture was the same as that described in MATERIALS AND METHODS with the exception that the concentration of fumarate was 10 mM. Thenoyltrifluoroacetone was added in a 10% (v/v) ethanol solution and the control received a similar amount of 10% ethanol.

Fig. 4. Effect of malonate on the oxidation of NADH by fumarate in heart, liver and gastrocnemius electron transport particles. The reaction mixture was the same as that described in MATERIALS AND METHODS. ○, heart; △, liver; □, gastrocnemius.

Addition of fumarate to a reaction mixture reduced by NADH resulted in the disappearance of cytochrome *b* peaks at 432 and 562 nm (Fig. 5). Since the reference cuvette was in the fully oxidized state, this indicated a shift in cytochrome *b* to a more oxidized state and suggested a flow of electrons from NADH through cytochrome *b* to the electron acceptor fumarate. An upward shift in the trough at 465 nm indicated oxidation of flavoprotein as might be expected from the involvement of NADH dehydrogenase and succinate dehydrogenase in the reaction pathway.

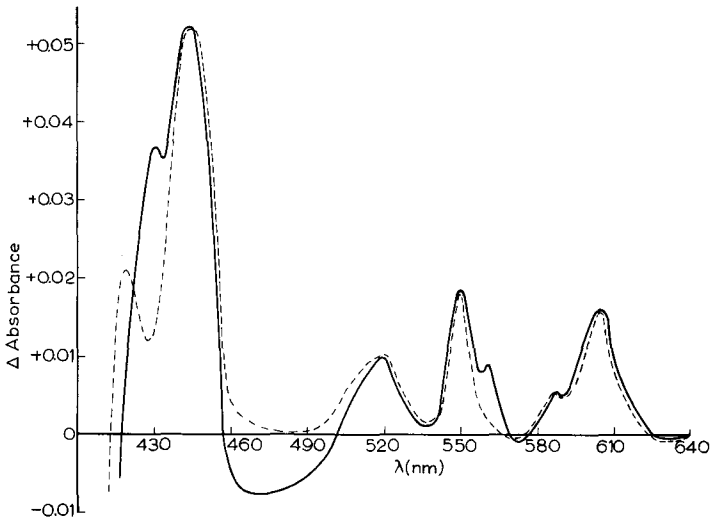

Fig. 5. Difference spectrum of cytochromes of heart electron transport particles as recorded by a Cary-15 split-beam spectrophotometer. The reference cuvette contained oxidized electron transport particles and the sample cuvette electron transport particles reduced by KCN and substrate. ———, spectrum of the cytochromes fully reduced by the addition of NADH; – – –, shift to a more oxidized state that occurred in cytochrome *b* after the addition of fumarate (cytochrome b_γ at 432 nm and b_α at 562 nm). Oxidation of flavoprotein is also indicated by an upward shift in the trough at 465 nm.

The fact that fumarate-dependent oxidation of NADH was inhibited by malonate suggested that succinate dehydrogenase was involved in the reaction. Therefore production of succinate should be stoichiometrically related to the NADH oxidized. Table I shows that succinate was recovered as a reaction product and accounted for 93 % of the NADH oxidized by fumarate. Malate was also produced, indicating fumarase activity.

When NADH is oxidized by O_2, a phosphorylation occurs between NADH and cytochrome *b*. The oxidation of NADH by fumarate, in beef heart submitochondrial particles, includes this phosphorylation site[1]. As seen in Table II, a comparable phosphorylation, although loosely coupled was obtained in rat heart mitochondria. Although the P/2*e* values are low, they are similar to the values of 0.43 reported for beef heart electron transport particles[1].

It was considered important to determine if this reaction was peculiar to heart or was present in other cells as well. Oxidation of NADH by fumarate was also found in electron transport particles prepared from gastrocnemius and liver. The sensitivity of the reaction to malonate in these two tissues was similar to that occur-

ring in heart electron transport particles (Fig. 4). As seen in Table III, the rate of NADH oxidation by fumarate varied considerably among the three tissues; the rate in gastrocnemius was 1.7 times that of liver and the rate in heart was 2.0 times that of gastrocnemius and 3.3 times that of liver.

On the basis of the differences observed in rate in the three tissues, the activities of NADH dehydrogenase and succinate dehydrogenase were investigated to determine

TABLE I

STOICHIOMETRIC RELATIONSHIP BETWEEN SUBSTRATES USED AND PRODUCTS FORMED

The reaction mixture was similar to that described in the text with the exception that the total volume (24.5 ml) was 70 times that regularly used and the concentration of NADH was 0.32 mM. A 0.35-ml sample was taken and NADH oxidation followed at 340 nm on a Cary-15 recording spectrophotometer. At the completion of the reaction (25 min), protein was precipitated by the addition of 12.1 ml 18% (v/v) $HClO_4$ and removed by centrifugation. The reaction products present in the supernatant were identified by column chromatography.

NADH used (μmoles)	*Succinate formed (μmoles)*		
5.09	4.71		
	Fumarate (μmoles)	*Malate (μmoles)*	*Succinate (μmoles)*	*Total (μmoles)*
Initial	120.75	0.0	0.0	120.75
Final	110.99	7.42	4.71	123.12

TABLE II

PHOSPHORYLATION COUPLED TO THE OXIDATION OF NADH BY FUMARATE

ATP formation was determined by following the fate of labeled inorganic phosphate. Hexokinase (EC 2.7.1.1) and glucose were added to serve as a trap for the labile phosphate that was formed. The reaction mixture contained 2 mM $MgCl_2$, 0.48 mM EDTA, 5 mM Tris–H_2SO_4 buffer (pH 7.4), 1.6 mM KCN, 32 mM glucose, 0.2 mg bovine serum albumin per 100 ml, 1 mM ATP, 2 units hexokinase, 4.3 mM potassium phosphate buffer (pH 7.4) (containing $1.1 \cdot 10^6$–$1.2 \cdot 10^6$ counts/min per μmole phosphate), whole heart mitochondria (1.6–1.7 mg protein), and 0.25 M sucrose in a final volume of 1.5 ml (after the addition of NADH and fumarate). NADH (0.5 or 0.8 μmole) was added and the reaction initiated by the addition of 40 μl of 0.175 M fumarate. A second test tube, representing the control, received the same components with the exception of fumarate. Exactly 500 μl was removed from both the sample and control mixtures and placed in a Cary-15 split-beam recording spectrophotometer to follow the oxidation of NADH at 340 nm. When the NADH was completely oxidized, 100 μl of 50% trichloroacetic acid was added to the 1 ml remaining in the test tubes. Following centrifugation, the supernatants were extracted with molybdic acid and isobutyl alcohol–benzene as described by LINDBERG AND ERNSTER[10]. The difference in counts between the sample and control was used to determine the phosphorylation (counted as glucose 6-[^{32}P]phosphate) resulting from the oxidation of NADH by fumarate. The difference in total counts/min between sample and control in the three experiments was 19626, 49126 and 28126, respectively.

Expt. No.	*NADH oxidized (μmole)*	*Glucose 6-[^{32}P]phosphate formed (μmole)*	*Ratio P/2e*
1	0.071	0.017	0.24
2	0.096	0.041	0.43
3	0.083	0.026	0.31

TABLE III

COMPARISON OF THE RATE OF NADH OXIDATION BY FUMARATE IN SUBMITOCHONDRIAL PARTICLES OF LIVER, GASTROCNEMIUS, AND HEART

The assay procedure is described in MATERIALS AND METHODS. Values in the table represent averages of six determinations ± S. E.

	Liver	*Gastrocnemius*	*Heart*
nmoles NADH oxidized per mg protein per min	4.1 ± 0.22	6.9 ± 0.18	13.6 ± 0.64

TABLE IV

SUCCINATE DEHYDROGENASE AND NADH DEHYDROGENASE ACTIVITIES AS DETERMINED BY THE REDUCTION OF $Fe(CN)_6^{3-}$

The assay procedures are described in MATERIALS AND METHODS.

	Liver	*Gastrocnemius*	*Heart*
Succinate dehydrogenase (μmole succinate oxidized per mg protein per min)	0.19	0.19	0.25
	0.19	0.17	0.29
	0.18	0.14	0.20
	$\bar{X}$ = 0.19	$\bar{X}$ = 0.17	$\bar{X}$ = 0.25
NADH dehydrogenase (μmoles NADH oxidized per mg protein per min)	2.8	3.9	8.8
	3.2	3.6	12.1
	3.3	3.5	13.7
	$\bar{X}$ = 3.1	$\bar{X}$ = 3.7	$\bar{X}$ = 11.5

if they could be rate-limiting factors in the reaction. Using $Fe(CN)_6^{3-}$ as an electron acceptor, it was found that succinate dehydrogenase and NADH dehydrogenase activities were, respectively, 20–45 and 500–850 times greater than the fumarate-dependent oxidation of NADH (Table IV). This suggested that neither dehydrogenase was exerting a rate-limiting effect on the reaction and that the rate-limiting step might be electron flow through some site located between the two dehydrogenases.

DISCUSSION

The oxidation of NADH by fumarate was first observed in Keilin–Hartree particles of beef heart mitochondria[11]. SANADI AND FLUHARTY[1] investigated the reaction in phosphorylating particles of beef heart mitochondria and found the reaction to be an ATP-producing one. A comparable reaction was found in this study in rat heart submitochondrial particles. In both rat and beef heart, the oxidation of NADH by fumarate has the same response to inhibitors, accumulates succinate as an end product and produces ATP. However, SANADI AND FLUHARTY[1] only demonstrated the involvement of NADH dehydrogenase and succinate dehydrogenase. Identifica-

tion of the components involved in the reaction pathway has been extended by this investigation; the oxidation of cytochrome *b* by fumarate suggests that cytochrome *b* is also included. It is postulated that electrons from NADH flow through NADH dehydrogenase *via* cytochrome *b* to succinate dehydrogenase, resulting in the reduction of fumarate to succinate.

This pathway is consistent with the one proposed by CHANCE AND HOLLUNGER[12] for the energy-linked reduction of NAD^+ by succinate, a reaction believed to be a reversal of the oxidation of NADH by fumarate[1]. CHANCE AND HOLLUNGER observed that addition of ATP and succinate to nonrespiring mitochondria resulted in a reduction of NAD^+ and cytochrome *b*, while cytochrome *c* and cytochrome *a* were oxidized. The concept that electron flow proceeded from succinate to NAD^+ *via* cytochrome *b* was further strengthened by the studies of HINKLE *et al.*[13]. An ATP-dependent oxidation of succinate-reduced cytochrome *b* by NAD^+ occurred when electron reversal from the other cytochromes was blocked by antimycin *a*. Cytochrome *b* therefore appears to be directly involved as an electron carrier in both the oxidation of NADH by fumarate and the reduction of NAD^+ by succinate, another piece of evidence that one reaction is the reverse of the other.

Of physiological interest is the demonstration of this reaction in cytochrome oxidase-deficient sarcosomes of *Ascaris lumbricoides*[14]. Formation of ATP was coupled to a dismutation of malate. Furthermore, succinate accumulates in the enteric fluid of the intact organism. It appears as if this mitochondrial production of ATP is an adaptive mechanism since the larval form is an aerobe and has cytochrome oxidase[15].

Succinate has also been found to concentrate in tissues of other invertebrates living in essentially anaerobic environments, apparently as a result of glucose degradation and the subsequent fixation of CO_2 into phosphoenolpyruvate or pyruvate. This has been demonstrated in the cestode *Moniezia expansa*[16] and in the pelecypods *Rangia cuneata*[17] and *Crassostrea virginica*[18].

Experiments on isolated rat heart suggest that this reaction could be of importance in producing energy during periods of anoxia in mammals as well. Addition of fumarate, malate and glutamate to the glucose perfused anoxic rat heart increased the rate of beating 5-fold[3]. It was hypothesized that the stimulation in anoxic beating was the result of a reaction identical to that found in beef heart submitochondrial particles[1]. The present investigation demonstrates that this reaction does indeed occur in rat heart.

It was also found in this study that this reaction occurs in striated muscle and liver in addition to heart. Appreciable differences were found in the activity of these three tissues which appear unrelated to the activities of either succinate or NADH dehydrogenase. The presence of this reaction in liver might explain in whole or part the accumulation of succinate obtained in the hypoxic perfused rat liver[19]. In addition the ubiquity of this reaction probably serves as the basis for the protection afforded rabbits infused with fumarate during hemorrhagic shock[4]. It would appear in terms of the general tissue distribution of the fumarate-dependent oxidation of NADH and its activity that it does have the potential for supplementing the ATP generated by anaerobic glycolysis. This could be of physiological importance in the ability of a variety of animals to cope with anoxia.

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REFERENCES

1 D. R. SANADI AND A. L. FLUHARTY, *Biochemistry*, 2 (1963) 523.
2 D. W. HAAS, *Biochim. Biophys. Acta*, 92 (1964) 433.
3 J. CASCARANO, W. L. CHICK AND I. SEIDMAN, *Proc. Soc. Exptl. Biol. Med.*, 127 (1968) 25.
4 W. L. CHICK, R. WEINER, J. CASCARANO AND B. W. ZWEIFACH, *Am. J. Physiol.*, 215 (1968) 1107.
5 W. D. BONNER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 1, Academic Press, New York, 1955, p. 722.
6 S. MINAKAMI, R. L. RINGLER AND T. P. SINGER, *J. Biol. Chem.*, 237 (1962) 569.
7 C. VEEGER, D. V. DERVARTANIAN AND W. P. ZEYLEMAKER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 13, Academic Press, New York, 1969, p. 81.
8 H. BUSCH, R. B. HURLBERT AND V. R. POTTER, *J. Biol. Chem.*, 196 (1952) 717.
9 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
10 O. LINDBERG AND L. ERNSTER, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 3, Interscience Publishers, New York, 1956, p. 1.
11 E. C. SLATER, *Biochem. J.*, 46 (1950) 484.
12 B. CHANCE AND G. HOLLUNGER, *J. Biol. Chem.*, 236 (1961) 1562.
13 P. C. HINKLE, R. A. BUTOW, E. RACKER AND B. CHANCE, *J. Biol. Chem.*, 242 (1967) 5169.
14 I. SEIDMAN AND N. ENTNER, *J. Biol. Chem.*, 236 (1961) 915.
15 H. OYA, L. C. COSTELLO AND W. N. SMITH, *J. Cellular Comp. Physiol.*, 62 (1963) 287.
16 K. S. CHEAH AND C. BRYANT, *Comp. Biochem. Physiol.*, 19 (1966) 197.
17 T. M. STOKES AND J. AWAPARA, *Comp. Biochem. Physiol.*, 25 (1968) 883.
18 C. S. HAMMEN, *Comp. Biochem. Physiol.*, 17 (1966) 289.
19 H. D. HOBERMAN AND L. PROSKY, *Biochim. Biophys. Acta*, 148 (1967) 392.