washed sarcotubules retained 60–80% of the original activity; treatment with freezing and thawing yielded comparable results.

The association of phosphofructokinase with the sarcotubules appears of interest because relaxation-factor preparations, which other studies have shown to derive from fragmentation of the sarcotubular system[5], are able to form adenosine 3′,5′-phosphate[8], a strong activator of phosphofructokinase[9]. Since phosphofructokinase is the rate-limiting enzyme of glycolysis[10], and phosphofructokinase activity is increased during muscular activity[11], it is conceivable that the sarcotubular system may be the basis for the integration of metabolic and physiological control mechanisms.

A detailed account of this work will be presented in a forthcoming paper[12].

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The flavin components of the NADH dehydrogenase of the respiratory chain

The relationship between the flavin prosthetic group of the NADH dehydrogenase of the respiratory chain[1–3] and that of the mitochondrial NADH:cytochrome *c* oxidoreductase (EC 1.6.2.1)[4–8] is obscure. First, the properties of the FAD in lipoflavoprotein[1] and in NADH dehydrogenase[2,3] are different. The FAD of lipoflavoprotein[1] is stable under the usual conditions of deproteinization preparatory to the flavin assay, while acid or thermal denaturation of NADH dehydrogenase yields FMN, AMP and riboflavin as well as FAD. SINGER *et al.*[2,3] suggest that the prosthetic group is FAD attached by multiple bonds to the protein and that unfolding of the

protein during denaturation results in partial cleavage of the FAD. Secondly, both ZIEGLER *et al.*[1] and SINGER *et al.*[2,3] suggest that acid–ethanol treatment of mitochondrial preparations is responsible both for splitting of the FAD and for liberation of NADH:cytochrome *c* oxidoreductase activity. Thus, if their interpretations of this unusual property of the dehydrogenase flavin were true, then all mitochondrial FMN-containing[6,7,9,10] NADH:cytochrome *c* oxidoreductases[4–8] should be regarded as artefacts.

The transformation of NADH dehydrogenase into NADH:cytochrome *c* oxidoreductase appears to be the result of denaturation of the former enzyme[11]. However, recently it has been reported that not only acid–ethanol or heat treatment but also proteolytic digestion of NADH dehydrogenase results in the emergence of NADH:cytochrome *c* oxidoreductase activity[11,12]. Moreover, as was first shown by HUENNEKENS[9] for MACKLER'S enzyme[8] all mitochondrial NADH:cytochrome *c* oxidoreductases are reactivated only by FMN[10]. Thus, if the NADH:cytochrome *c* oxidoreductase is liberated from the parent molecule without any configurational alteration[11], FMN would be the natural prosthetic group; on the other hand, the splitting off of the NADH:cytochrome *c* oxidoreductase might involve simultaneous cleavage of FAD[2,3] (see ref. 13 for a fuller discussion). We now wish to report that the flavin liberated from NADH dehydrogenase after proteolytic digestion at room temperature shows the same pattern as after heat denaturation of the enzyme[2,3].

The enzyme was isolated from the Keilin and Hartree heart-muscle preparation by snake-venom digestion at 30° as previously described[11]. After partial purification up to the step of differential centrifugation[3,14] the enzyme contained no lipoamide

TABLE I

THE FAD AND FMN CONTENT OF NADH DEHYDROGENASE

The same methods for flavin release were used as in Fig. 1. The deproteinized extracts were concentrated by lyophilization for spectrophotometric assay of total flavin by measuring light absorption at 450 mμ. For quantitative determination of FAD and FMN the method of CERLETTI AND IPATA[19] was used.

Flavin	*Method of flavin liberation*	
	Heat denaturation	*Proteolytic digestion*
	% of total flavin	
FAD	11–32	<2
FMN	30–53	33–60

dehydrogenase (EC 1.6.4.3) when tested with lipoic acid as acceptor at pH 5.65 under the conditions of VEEGER AND MASSEY[15] and the ratio of ferricytochrome *c* activity[4] to ferricyanide activity[16], both calculated at infinite concentration of acceptor, was 0.004, indicating that these FAD- and FMN-containing contaminants were practically absent.

The total amount of flavin liberated from NADH dehydrogenase by heat deproteinization or under conditions where neither heat nor acid treatment was used were about the same and both procedures yielded FAD, FMN and riboflavin

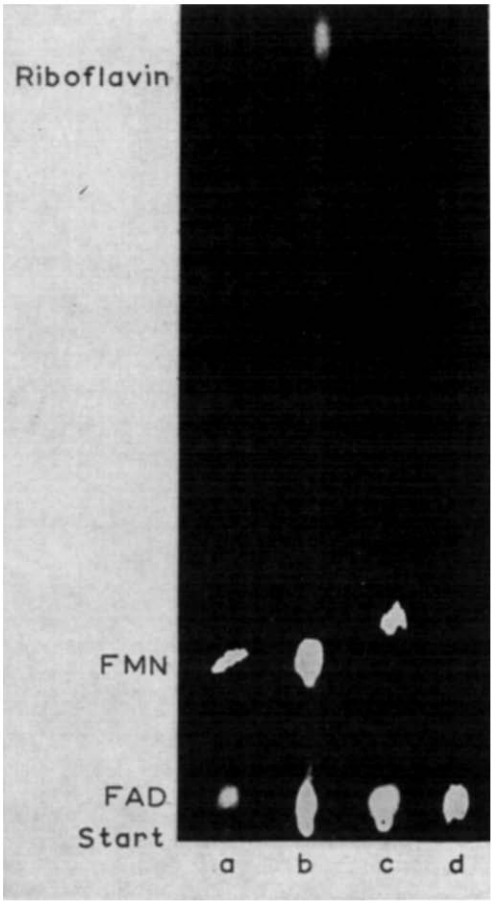

Fig. 1. Identification of the flavin components of NADH dehydrogenase. Descending chromatography on Whatman No. 1 paper with *n*-butanol–acetic acid–water (4:1:5) as solvent system. For the liberation of flavin two different methods were used: (1) The enzyme was denatured by heat according to MINAKAMI *et al.*[2]. (2) The enzyme was first digested with trypsin and chymotrypsin (0.2 mg of each per mg of protein) at pH 7.6 for 18h at 16°; then the flavins were adsorbed in the cold onto and eluted from a Florisil column essentially according to DIMANT *et al.*[17]. Lipoamide dehydrogenase[18] was treated in the same way except that *Naja naja* snake venom was added before the proteolytic digestion. For paper chromatography the aqueous solutions of the flavins were concentrated first by lyophilization, followed by phenol extraction. (a) The flavins of NADH dehydrogenase after proteolytic digestion; (b) standards of FAD, FMN, and riboflavin; (c) the flavins of heat-denatured NADH dehydrogenase; (d) the flavin of lipoamide dehydrogenase.

(Fig. 1 and Table I). The main difference is that proteolytic digestion yielded a much smaller amount of FAD and a larger amount of riboflavin. On the other hand, only one spot, corresponding to FAD, was obtained with lipoamide dehydrogenase (Fig. 1), when proteolytic digestion was carried out in the presence of snake venom. This shows that the presence of FMN in digests of NADH dehydrogenase is not a result of FAD degradation by contamination with nucleotide pyrophosphatase present in the snake venom.

These results cannot distinguish between the two possibilities: (1) The flavin

prosthetic group of NADH dehydrogenase is FAD which is partially degraded[2,3,14] during the deproteinization, almost completely in the case of proteolytic digestion. (2) The NADH dehydrogenase contains besides FMN also FAD, which easily undergoes degradation yielding FMN and riboflavin.

The existence of a labile flavin compound as a source of the FMN must be considered in connection with a recent report of MASSEY* that the FMN content of heart-muscle preparation increases upon aging. Thus, the conclusion of ZIEGLER *et al.*[1] concerning the splitting of FAD would be true not for the FAD of lipoflavoprotein but for the flavin present in the "electron-transfer particle", which, in fact, was used for the acid–ethanol treatment. The same type of unstable flavin appears to be present also in NADH dehydrogenase[2,3,14]. Therefore, in our opinion, it is impossible to decide yet what is the origin of the FMN in NADH:ubiquinone reductase[20], in NADH:cytochrome *c* oxidoreductases[4–8] and mitochondrial preparations[21,22].

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