Biochemical Pharmacology, 1967, Vol. 16, pp. 229–232. Pergamon Press Ltd., Printed in Great Britain.

Stimulation of sulfate activation by nicotinamide–adenine dinucleotide in presence of mastocytoma fractions free of mitochondria

(*Received* 31 *May* 1966; *accepted* 23 *August* 1966)

EARLIER communications from this laboratory have reported preliminary observations on the stimulation by NAD of the incorporation of radioactive sulfate into the heparin fraction in the presence of Dunn–Potter mouse mastocytoma homogenates.[1, 2] A similar effect occurred in high-speed supernatants obtained from the Furth mast cell tumor; in addition, NAD caused a marked stimulation (up to sixfold) of the ^{35}S-sulfate incorporation[3] into a "paper-fixable" fraction (PFF).* More recently it has been shown that under certain conditions the radioactivity incorporated into the PFF may be employed as a simple and rapid means of estimating the amount of ^{35}S-3′-phosphoadenylylsulfate (PAPS) present.[4] With the help of this tool, and other methods for separation and measurement of ^{35}S-PAPS, the influence of NAD on sulfate incorporation by subfractions of the Furth tumor has been examined. It was observed that, under certain conditions, NAD stimulated the formation of PAPS.

MATERIALS AND METHODS

The preparation of Furth mouse mastocytoma[5] supernatant (105,000 *g*, 1 hr) and its separation into two subfractions, an upper clear zone designated Clear S_{105}, and a lower fluffy layer called FL-l, was described elsewhere.[4] Clear S_{105} was relatively rich in enzymes catalyzing the formation of PAPS; the FL-l contained relatively more of the enzymes required for transfer of sulfate from PAPS to the heparin fraction. These tumor fractions were incubated with carrier-free ^{35}S-sulfate (Oak Ridge National Laboratory) under conditions described in the tables. NAD was obtained from Pabst or Boehringer. Buffer salts and other chemicals were reagent grade or the best grade obtainable. The incubation was terminated by addition of an equal volume of saturated ammonium sulfate.

Sulfate incorporation into two endogenous fractions, designated heparin fraction and PFF, was measured as described earlier.[3] The heparin fraction was obtained by prolonged dialysis of the incubation mixture against tap water. PFF was prepared by applying an aliquot of the incubation sample to filter paper, fixing by treatment with heat, ethanol–formalin, and Alcian blue, and washing with glacial acetic acid and water to remove inorganic ^{35}S-sulfate. After relatively brief incubations, the radioactivity of the PFF represents a constant fraction of the total counts per minute of the PAPS in solution and provides a measure of sulfate activation.[4] For quantitative determination, radioactive PAPS was separated from inorganic ^{35}S-sulfate and ^{35}S-APS by column chromatography on Ecteola.[6] The identity of ^{35}S-PAPS and ^{35}S-APS was further established by paper chromatography and electrophoresis.[4]

Radioactivity was determined by a micromil end-window gas-flow counter. Corrections were made for self-absorption. Luciferase and a Farrand photoelectric fluorometer were used to measure ATP, essentially as described by Strehler and Bergmeyer.[7]

RESULTS AND DISCUSSION

The effects of NAD on sulfate uptake in the presence of mastocytoma high-speed supernatant have been reported briefly.[3] Sulfation of the heparin fraction was found to be approximately doubled upon addition of the nucleotide. Sulfate incorporation into the PFF was much more rapid, and it was stimulated to a much greater degree by NAD, during the first 30 min of the incubation. During later stages, a decrease in radioactivity of the PFF was observed, believed to result from the action of a PAPS-hydrolase.[4]

* Abbreviations used: APS, adenylylsulfate; PAPS, 3′-phosphoadenylylsulfate; Clear S_{105} and FL-l, ultracentrifugal tumor fractions described in text; PFF, paper-fixable fraction described in text.

When the tumor homogenate was fractionated further, the action of NAD was re-examined. In the FL-l, the nucleotide was found to stimulate sulfate incorporation into both the heparin and paper-fixable fractions. Considerable variability was found, however, in the response of various Clear S_{105} preparations to NAD. Freshly prepared batches were generally very active in forming PAPS but were unaffected, or even inhibited, by addition of NAD. Preparations which had been stored in an ice bath for several hours or lyophilized and kept at $-18°$, frequently had a greatly reduced capacity to activate sulfate, as evidenced by ^{35}S-sulfate incorporation into the PFF; however, addition of NAD restored activity. Since an NAD sample practically free of impurities (obtained by further purification of a commercial preparation according to Goldberg *et al.*[8]) produced the same degree of stimulation of sulfate incorporation into PFF as did the untreated commercial product, it was assumed that the observed phenomenon was caused by NAD itself, and not by a contamination.

In order to confirm the stimulation of sulfate activation by NAD, lyophilized Clear S_{105} was incubated with ^{35}S-sulfate in the presence and absence of NAD, the labeled PAPS and APS formed were isolated by Ecteola column chromatography, and the radioactivity incorporated into these nucleotides was determined. Table 1 shows that approximately six times as much ^{35}S-PAPS was recovered when

TABLE 1. INFLUENCE OF NAD ON THE FORMATION OF PAPS AND APS IN THE PRESENCE OF CLEAR S_{105}

NAD added (M)	^{35}S-Sulfate added (counts/min $\times 10^{-6}$)	Radioactivity recovered		^{35}S-PAPS/^{35}S-APS
		^{35}S-PAPS (counts/min $\times 10^{-6}$)	^{35}S-APS (counts/min $\times 10^{-6}$)	
0	15·1	0·395	0·137	2·89
2×10^{-5}	15·4	2·40	0·161	14·90

Composition of incubation medium: 0·4 ml enzyme preparation (lyophilized Clear S_{105}) derived from 0·2 g tumor (wet wt.); 0·25 ml Tyrode's solution; and the following in μmoles: Tris buffer (pH 7·4), 52; phosphate buffer (pH 7·4), 6·0; ATP, 2·0; $MgCl_2$, 1·2; cysteine, 0·54. NAD concentrations and carrier-free ^{35}S-sulfate added as indicated. Total volume, 1·0 ml. Bath temperature, 37°. Incubation period 30 min. PAPS and APS were isolated by column chromatography on Ecteola,[6] and identified by paper electrophoresis and chromatography.[4]

NAD was added. Furthermore, the ratio of labeled PAPS to APS was greatly increased in the presence of NAD. When FL-l was incubated with ^{35}S-sulfate, a similar phenomenon was observed; in the absence of NAD much less ^{35}S-PAPS was formed, and the ratio of PAPS to APS was relatively low (2·3). Suzuki and Strominger reported that the relative amounts of PAPS and APS formed in an enzyme system derived from hen oviduct were greatly influenced by the concentration of ATP.[9] As the ATP concentration was elevated, the proportion of PAPS to APS increased. In view of these findings, results shown in Table 1 were interpreted to suggest that the ATP concentration was relatively low in these experiments and that the stimulation of PAPS formation in the presence of NAD might be based on a mechanism that provided additional ATP.

The effect of NAD was then studied at a higher ATP concentration, as well as in a medium in which ATP was continuously regenerated from ADP with creatine phosphate and creatine kinase (ATP-creatine phosphotransferase, E.C. 2.7.3.2). In both the lyophilized Clear S_{105} and FL-l (Table 2), supplemental ATP produced an elevation in sulfate incorporation into the PFF and heparin fraction, but no further increase occurred upon addition of NAD. These results seemed to indicate that, under those conditions, the stimulatory effect of NAD was based on a regeneration of ATP resulting in an increase in PAPS synthesis. This interpretation was confirmed by measuring the ATP concentration during an incubation of lyophilized Clear S_{105} with and without NAD (and a starting concentration of 2×10^{-3} M ATP). The results showed that, in the presence of NAD, ATP levels were maintained or even increased somewhat during an initial period of 15–30 min, while in the control samples, the ATP concentration fell very rapidly.

Since no mitochondria were present in the enzyme preparations, the possibility was considered that NAD enhanced ATP synthesis via the glycolytic pathway. Such a mechanism would stimulate PAPS formation both by providing additional ATP and by removal of ADP. In order to test this hypothesis, the influence of NAD on sulfate incorporation into the PFF was examined in the presence and absence of exogenous glucose (in all previous experiments glucose had been present as a component of Tyrode's solution). The results indicate that glucose was indeed required for a demonstration of the stimulating action of NAD (Table 3).

The results reported here indicate that the acceleration by NAD of sulfate incorporation into the heparin fraction was largely an indirect effect resulting from an increase in PAPS formation. The possibility cannot be excluded, however, that NAD also enhanced the formation of precursors of the carbohydrate moiety of the heparin fraction. NAD-dependent reactions, such as the conversion

TABLE 2. EFFECT OF NAD ON SULFATE INCORPORATION IN THE FL-1 AS INFLUENCED BY ATP AND CREATINE PHOSPHATE AND CREATINE KINASE

Fraction analyzed	ATP conc. (mM)	Creatine phosphate + creatine kinase	^{35}S-Sulfate incorporation		
			−NAD	+NAD (counts/min)	% change
Paper-fixable	2	−	26,300	70,000	+166·0
	6	−	199,000	189,000	−5·03
	2	+	230,000	231,000	+0·43
Heparin fraction	2	−	21,000	33,600	+56·3
	6	−	39,700	37,400	−5·79
	2	+	39,700	42,600	+7·30

Incubation conditions as described in Table 1, except for quantities of components indicated and total volume halved; freshly prepared enzyme (FL-1) used; $6{\cdot}5 \times 10^6$ counts/min carrier-free ^{35}S-sulfate; and 0·5 μmole each of L-glutamine and niacinamide added; 2×10^{-5} M NAD added, where indicated; ATP concentrations as shown; 3·8 m-moles creatine phosphate and 22 μg creatine kinase added where indicated.

TABLE 3. EFFECTS OF NAD ON SULFATE INCORPORATION INTO PAPER-FIXABLE FRACTION IN PRESENCE OF CLEAR S_{105} AS INFLUENCED BY GLUCOSE CONCENTRATION

Exogenous glucose concn. ($M \times 10^{-3}$)	^{35}S-Sulfate incorporated		
	− NAD	+NAD (counts per min)	% Change
0	149,000	156,000	+4·70
1·6	157,000	303,000	+92·8

Experimental conditions as described in Table 1, except for quantities of all components and total volume halved; $2{\cdot}4 \times 10^6$ counts/min carrier-free ^{35}S-sulfate; and 0·5 μmole each of L-glutamine and niacinamide added; Tyrode's solution prepared without glucose; exogenous glucose and 2×10^{-5} M NAD added where indicated.

of a UDP-hexose into the corresponding UDP-hexuronic acid, or the epimerization of a hexose into one of its isomers, would fall into this category.

Acknowledgements—The authors are much obliged to Mr. R. H. Green and Mr. Z. Tokes for technical assistance, to Dr. W. G. Clark and the late Dr. W. J. Hartman,V.A. Hospital, Sepulveda, Calif., for some tumor-bearing mice, and to Dr. D. W. Visser and Dr. P. Roy-Burman for a sample of highly purified NAD. This work was supported in part by Research Grant HEO-1596 from the

National Heart Institute, by Institutional Grant 1 N-21 from the American Cancer Society, and by a grant from Riker Laboratories.

Department of Biochemistry,
School of Medicine,
University of Southern California,
Los Angeles 90033,
and Veterans Administration Hospital,
Sepulveda, Calif., U.S.A.

L. SPOLTER
L. I. RICE
R. YAMADA
W. MARX

REFERENCES

1. L. SPOLTER and W. MARX, *Fedn Proc.* **20**, 162 (1961).
2. W. MARX and L. SPOLTER, *Helv. physiol. Acta* **19**, C85 (1961).
3. L. SPOLTER, L. I. RICE and W. MARX, *Biochim. biophys. Acta* **69**, 583 (1963).
4. L. I. RICE, L. SPOLTER, Z. TOKES, R. EISENMAN and W. MARX, *Archs Biochem. Biophys.* In press.
5. J. FURTH, P. HAGEN and E. W. HIRSCH, *Proc. Soc. exp. Biol. Med.* **95**, 824 (1957).
6. W. MARX, L. I. RICE, J. SHARON, A. S. BALASUBRAMANIAN and L. SPOLTER. In preparation.
7. B. L. STREHLER, in *Methods of Enzymatic Analysis* (Ed. H. U. BERGMEYER), p. 563 (1963).
8. N. D. GOLDBERG, J. L. DAHL and R. E. PARKS, *J. biol. Chem.* **238**, 3109 (1963).
9. S. SUZUKI and J. L. STROMINGER, *J. biol. Chem.* **235**, 257 (1960).

Biochemical Pharmacology, 1967, Vol. 16, pp. 232–235. Pergamon Press Ltd., Printed in Great Britain.

The effect of the dietary lipids upon the ability of chlorpromazine to inhibit oxidative phosphorylation in liver mitochondria

(*Received* 28 *November* 1965; *accepted* 30 *August* 1966)

IT HAS been previously reported that fatty acid compositions of tissue lipids in animals[1–3] and in man[4] can be made to differ by feeding nutritionally adequate diets containing lipids of different fatty acid composition. These changes in the lipoprotein structure of the cellular membranes and the tissue enzymes can be quite substantial, and it appeared reasonable to anticipate that cellular and subcellular functions and the response of enzyme systems to drugs and chemical reagents could be influenced by feeding different fats to growing animals.

The marked effect of dietary lipids on the appearance of various signs of vitamin E deficiency in different animal species has been documented.[5–9] The swelling of liver mitochondria in response to various toxic substances has been shown to be affected by the lipid fed to experimental animals.[10] Chlorpromazine was also shown to depress the uptake of ^{32}P into rat brain phospholipids *in vivo* at different rates in rats fed diets containing cod liver oil, corn oil, or beef fat.[11] Walker and Kummerow[12] reported that erythrocytes from rats fed coconut oil were more readily hemolyzed by glycerol and thiourea than if the animals were fed corn oil; and Tepperman and Pownall[13] found that feeding a saturated fat to rats resulted in increased activities of liver hexose monophosphate and malic dehydrogenases. The present investigation describes the inhibition of chlorpromazine on the uptake of inorganic phosphate in an oxidative phosphorylating system as a function of the lipid fed to the rat.

EXPERIMENTAL

Weanling male Sprague–Dawley rats were fed semisynthetic diets containing 15 per cent beef fat, 15 per cent corn oil, or 7 per cent cod liver oil for 7–10 weeks, as previously described,[14] except that