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Relationship Between Ribonuclease and Certain Anionic Polymers in Utilization of Mevalonic Acid by Liver Homogenates.* (25591)

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Preincubation of essentially whole homogenates of rat liver with crystalline ribonuclease (RNAse) abolishes capacity of these homogenates to convert mevalonic acid (MVA) into non-saponifiable material (NSF) or cholesterol (CHL). Control homogenates similarly preincubated ordinarily retain the capacity for biosynthesis(1). Inability of RNAse-treated homogenates to utilize MVA is associated with absence of ATP in these preparations. Homogenates preincubated with RNAse and an autoclaved liver extract (ALE) previously treated with an anion exchange resin to remove residual ATP maintain a level of ATP and convert MVA to NSF and CHL(2). Evidence has been presented for existence in these heated liver extracts of a factor concerned with maintenance of a functional level of ATP in homogenates, presumably involving oxidative phosphorylation(3,4), and a tentative mechanism for explaining the experimental data has been proposed(2,5,6). Allfrey and Mirsky have previously described a possibly somewhat similar system involving isolated thymus nuclei which when pre-treated with *deoxyribonuclease* lose capacity to convert amino acids into protein or purine and pyrimidine precursors into nucleic acids. The treated nuclei are devoid of ATP but the synthetic capacity is retained on preincubation with DNA or may be established by addition of DNA after inactivation. Activity is not confined to DNA, however, and RNA as well as a number of anionic polymers substitute for DNA(7,8). It is our purpose to point out that DNA and certain anionic polymers have an effect similar to the factor of ALE in the liver homogenate system and that these anionic polymers, but not ALE, are also inhibitors of RNAse. A conclusion that all anionic polymers necessarily function in the same way in maintenance of ATP levels in tissue may not be justified.

Methods and material. The experimental procedures involved in biosynthesis of NSF and CHL from MVA by rat liver homogenates have been described(9). In brief, cooled liver from young rats is homogenized quickly in "loose" homogenizer with nicotinamide-phosphate-magnesium buffer and centrifuged at slow speed to remove connective tissue. This essentially whole homogenate is dispensed into flasks containing ATP, DPN, succinate and RNAse where indicated, as well as various materials or combinations of materials to be studied with respect to biosynthesis of NSF. The flasks are aerated with a stream of oxygen, stoppered, and preincubated with agitation. Following preincubation MVA-2-C^{14} is added to each flask, aeration is repeated and flasks reincubated. Then contents of flasks are saponified, extracted with petroleum ether, extracts dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture and counted. The results given are in terms of cpm/incubation flask. Details of procedure are essentially as described previously. Despite use of nicotinamide as inhibitor of DPNase in the homogenizing buffer, DPN some-

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times is limiting in the system employed. For this reason it is recommended that a level of 10 mg/flask rather than 1 mg previously described be used The various polymers were studied after solution in water with neutralization or solution in buffer. The appropriate controls contained by corresponding amount of water or buffer. Heparin and DNA were commercial preparations. Several samples of polyethylene sulfonate (PES) were used including material with molecular weights of 27,600, 12,900 and 5,700. ALE was prepared by treating fresh guinea pig liver in the cold with twice its weight of water in Waring Blendor for about a minute. The brei was then autoclaved at 105 - 110° for 20 min and the mixture cooled and centrifuged at 10,000 x g for 20 min. Determinations of RNAse activity were done essentially by procedure of Kunitz (10) as modified by Zöllner and Fellig(11) which depends upon the fact that when RNAse hydrolyzes RNA there is a decrease in absorption at 300 mμ. The substrate RNA solution was prepared by dissolving 25 mg commercial yeast RNA in 25 ml of 0.1M, pH 5.0, acetate buffer. The RNAse solution was prepared by dissolving 2 mg crystalline RNAse in 200 ml of water. In a typical experiment 2 cuvettes were prepared as follows: control cuvette, 1 ml RNA solution and 1.5 ml water; RNAse cuvette, 1 ml RNA solution and 0.5 ml of water. The cuvettes were placed in Beckman DU spectrophotometer and with the control cuvette in position, the instrument was arbitrarily adjusted to read an optical density of 0.175 at 300 mμ. One ml of RNAse solution was then added to the RNAse cuvette and relative optical density of this cuvette determined at intervals over 10 - 15 min. Ordinarily the instrument stayed in balance but on occasion it became necessary to make minor adjustments so that the control cuvette maintained an apparent optical density of 0.175. Where various materials were studied for effect on RNAse activity they were added in 0.5 ml to each cuvette at the expense of water. Each experiment then involved a rapid series of determinations with a pair of cuvettes both containing same amounts of RNA and water or aqueous solution of material to be examined while one contained in addition 10 γ of RNAse. Suitable controls demonstrated that the RNAse solution maintained full activity during experiments. In experiment involving ALE as substrate 1 ml of this material replaced the 1 ml of RNA solution used in the other RNAse experiments.

TABLE I. Biosynthesis of NSF as Influenced by Preincubation with Various Materials.

Exp.	RNAse*	Supplement	NSF, cpm
1	—	None	6852
	+	"	1138
	+	Liver extract, 5 ml	6811
2	—	None	5368
	+	"	1445
	+	DNA, 1 mg	3498
	+	" 10	5430
	+	RNA, 1	1636
	+	" 10	3489
3	—	None	8415
	+	"	293
	—	PES, 1 mg added prior to preincubation	8461
	—	3 *Idem*	7969
	—	5 "	7416
	—	10 "	6861
	+	1 "	8222
	+	3 "	8126
	+	5 "	7370
	+	10 "	7185
	—	10 mg following preincubation	6938
	+	10 *Idem*	7112
4	—	None	7787
	+	"	630
	—	Heparin, 1 mg added prior to preincubation	7438
	—	5 *Idem*	7516
	—	10 "	7782
	+	1 "	7814
	+	5 "	7893
	+	10 "	7330
	—	10 mg following preincubation	6979
	+	10 *Idem*	10222

* RNAse where used was present at a level of 2.5 mg/flask.

Results. The results of a number of representative biosynthetic experiments are summarized in Table I. RNA, DNA, heparin, and PES are effective in preventing inactivation of MVA utilization, accomplished by preincubation of essentially whole homogenates with RNAse. RNA is markedly less active than the other polymers, as expected, since it is the natural substrate for the enzyme RNAse used to inactivate the system. Both

heparin and PES, the 2 "unnatural" polymers studied, are as active in "reversing" RNAse inactivation, *i.e.* on addition after preincubation rather than before, as they are in preventing inactivation in the first place.

A previous paper reported that RNA is inactive in the liver homogenate system. Results of an experiment summarized in Table II afford an explanation for this apparent discrepancy. RNA and DNA were compared each at a level of 5 mg/flask against various levels of RNAse. It is apparent that at a marginal level of RNAse, RNA is active in the system while at high levels of RNAse, RNA is essentially inactive. DNA, since it is not influenced by RNAse, is equally active at all levels of the enzyme. It appears that for valid determinations of activity materials should be examined against a marginal level of RNAse.

PES, heparin, and DNA are active in that order as inhibitors of RNAse (Fig. 1). Half maximum reversal of RNAse is obtained with about 2.5 γ PES, 12.5 γ of heparin and about 50 γ per cuvette of DNA. In the latter instance complete reversal of RNAse is not obtained with any level of DNA. Since each cuvette contained 10 γ of RNAse it is apparent that inhibition ratios for one-half maximum activity vary from about 0.25 with PES to about 5 for DNA. As indicated by Exp. 4, ALE does not influence RNAse activity at a ratio of 1 ml/10 γ of RNAse. Samples of ALE examined in this assay were previously shown to be active in preventing RNAse inactivation of MVA utilization in the homogenate system. The data of Exp. 5 show that the ALE does not contain amounts of RNAse-sensitive material detectable by this procedure. RNAse is not sensitive to relatively large amounts of ATP as indicated by Exp. 6.

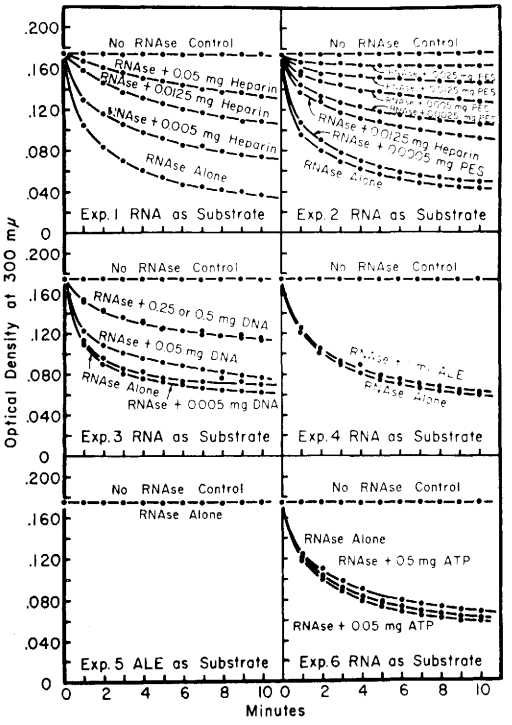

FIG. 1. Influence of various anionic polymers, ATP, or ALE on activity of RNAse.

TABLE II. Biosynthesis of NSF as Influenced by Preincubation with RNA and DNA at Various Levels of RNAse.

RNAse, mg	Supplement	NSF, cpm
0	None	5790
5.0	"	21
0	RNA, 5 mg	5482
2.5	*Idem*	465
5.0	"	31
10.0	"	24
0	DNA, 5 mg	5569
2.5	*Idem*	6634
5.0	"	6475
10.0	"	4745

Discussion. Results of biosynthetic studies demonstrate that the presence of certain polymers prevents or reverses inactivation of MVA utilization that is accomplished by preincubation with RNAse. It should be recalled that amounts of RNAse required to accomplish this inactivation are relatively large. Nevertheless in these experiments biosynthesis usually occurs when polymer to enzyme ratios of around 1 are used. In *in vitro* experiments RNAse inactivation occurred with polymer to enzyme ratios of 0.25 to 5 depending upon the polymer. As far as ALE is concerned, weight ratios are not applicable because of the crude state of the active component but 5 ml of extract ordinarily permits MVA utilization in presence of 1 - 5 mg of RNAse, a volume to weight ratio of about 1 - 5. ALE did not influence RNAse activity in the *in vitro* system at a level of 1 ml to 10 γ (0.01 mg) of RNAse, a corresponding volume to weight ratio of 100. Since anionic polymers

appear to have essentially the same activity against RNAse in the 2 systems it is quite reasonable to suggest that the effects on biosynthesis may be attributable to RNAse inhibition. It will be recalled, however, that the "unnatural" polymers reverse RNAse inactivation as well as prevent it. This observation suggests, but does not necessarily prove, that they function in a positive way by fulfilling a requirement of the system rather than by inhibiting RNAse. On the other hand, ALE is inactive against RNAse *in vitro*. It is difficult to see how its effect in biosynthesis can be explained on the basis of RNAse inhibition.

Summary. Liver homogenates preincubated with RNAse, in contrast to controls, do not incorporate mevalonic acid into nonsaponifiable material or cholesterol. This inactivation by RNAse is preventable or reversible with an autoclaved extract of liver or with certain anionic polymers including polyethylene sulfonate, heparin, DNA and even RNA. The polymers also inactivate RNAse *in vitro* at comparable ratios of inhibitor to enzyme used in the biosynthetic experiments. Inactivation of RNAse *in vitro* is not observed, however, with comparatively high levels of liver extract. It is concluded that liver extract does not function in the homogenate system merely by inactivation of RNAse but this interpretation may be applicable to the anionic polymers.

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Hemagglutination-Inhibition and Serum Neutralization Response of Horses To Eastern Equine Encephalomyelitis Virus.* (25592)

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Hemagglutinating activity for Japanese B encephalitis, one of the arthropod-borne group of viruses, was first reported in 1950(1). Subsequent work revealed that other members of this group of viruses also agglutinate erythrocytes(2-5), and methods for hemagglutination-inhibition (HI) tests were developed concurrently. Information, other than from this laboratory(6), on use of the HI test for clinical and epidemiological studies of eastern equine encephalomyelitis (EEE) has been found in only one instance. Groot *et al.*(7) testing human sera for EEE found none to be positive for either HI or neutralizing antibodies. The experiments reported here were designed to compare use of HI and neutralization tests in detecting the immunological response of horses experimentally infected with EEE virus.

Materials and methods. The EEE virus used was the V-52 strain, isolated from a naturally infected horse in Maryland in 1956. The strain had undergone 17 mouse brain passages prior to inoculation of the horses. The 4 young horses used for experimental work were obtained locally from non-enzootic areas. Three horses (#126, #127, #128) were inoculated intradermally and one (#115) was inoculated subcutaneously with a virus suspension containing approximately 10^6 mouse

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