ated through a humoral factor. This is suggested by the observation of similar results (6) in hypophysectomized animals subjected to dorsal celiotomy and subcutaneous implantation of tumor cells.

Summary. These findings reveal that the profound inhibition of metastases observed in pituitary ablated animals is overcome by hepatic damage. The effect was no longer evident in animals subjected to hepatic manipulation 7 and 14 days after injection of tumor cells.

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Elucidation of a Factor Essential in Oxidative Phosphorylation of a Mammalian System.* (27105)

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Whole homogenates of rat liver preincubated with RNAase lose the capacity to convert mevalonic acid (MVA) into non-saponifiable material (NSF) or cholesterol (CHL) (1). Such treated liver homogenates, in contrast to controls, are devoid of ATP(2). RNAase inactivation of MVA utilization may be prevented or reversed by an autoclaved extract of fresh liver previously treated with a Dowex-1 anion exchange resin to remove any residual ATP that it might contain, and conversion of MVA to NSF and CHL in this system is proportional to the ATP level maintained(3,4). The conclusion has been drawn that there exists in autoclaved liver extract a factor concerned with maintenance of ATP levels in liver homogenates incubated aerobically(3,4). Subsequent study has shown that this activity is not confined to autoclaved liver extract, however, since the effect of autoclaved liver extract may be duplicated with DNA, large amounts of RNA, or other polyanions such as polyethylene sulfonate or heparin(5). The properties of the factor from autoclaved liver extract including, for example, failure to be retained by a strong anion exchange resin, lability to both weak acid and alkali, loss of activity on dialysis, and resistance to both RNAase and DNAase (4) all suggested that the factor present in autoclaved liver extract, required by RNAase-treated homogenates to maintain a level of ATP is not simply RNA or DNA. This paper is concerned with fractionation studies that have led to some understanding of the nature of the factor of autoclaved liver extract. A preliminary report of the findings has been presented(6).

Materials and methods. The experimental procedures involved in biosynthesis of NSF and CHL from MVA by rat liver homogenates have been described(1-7). In brief, cooled liver (1 volume) from young rats was homogenized quickly in a "loose" Potter-Elvehjem homogenizer (1 mm clearance) with pH 7.0 phosphate (0.1 M)-magnesium (0.006 M)-nicotinamide (0.03 M) buffer (2 volumes) and centrifuged (200 × g, 3 min.) to remove connective tissue. This essentially whole homogenate was dispensed (5 ml aliquots) into flasks (125 ml Erlenmeyer) containing ATP (1 mg), DPN (10 mg), sodium succinate (100 mg), and RNAase where in-

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dicated as well as various materials or combinations of materials (up to 5 ml) to be studied with respect to biosynthesis of NSF. Total volume (including homogenate) was 11 ml. The flasks were aerated with a stream of oxygen (15 sec), stoppered, and preincubated with agitation (30 min., 37°, 100 oscillations per min.). Following preincubation MVA-2-C^{14} (0.5 mg DL MVA in 1 ml, 60,000 cpm) was added to each flask, aeration was repeated and the flasks then reincubated (3 hr). Following the biosynthetic period the contents of the flasks were saponified (10 ml 2 N 80% alcoholic KOH, 100°, 18 hr), extracted with petroleum ether (30-60°, 4 × with 50 ml amounts), the combined extracts dried with sodium sulphate (30 g), filtered, evaporated to dryness, taken up in scintillation mixture (10 ml, 4 g PPO (2,5-diphenyloxazole) and 30 mg POPOP (1,4-di-2(5-phenyloxazoyl)-benzene) per liter of toluene), and counted. The results given are in terms of cpm per incubation flask. Separate studies have shown that, under the experimental conditions described, these counts are essentially all CHL or other digitonin-precipitable material.

Autoclaved liver extract was prepared by treating fresh guinea pig liver in the cold with twice its weight of water in a Waring Blendor for about 1 min. The brei was then autoclaved at 105-110° for 20 min. and the mixture cooled and centrifuged at 10,000 × g for 20 min. The sedimented fraction was discarded. The supernatant fraction was then passed over a column of Dowex-1 chloride before use.

Electrophoretic separations were carried out in the cold room with a vertical apparatus having an inside column 2.5 cm in diameter and 100 cm long. The column was filled with a slurry of Geon #427 resin (B. F. Goodrich Chemical Co., Cleveland, Ohio) suspended in pH 7.0, 0.02 M phosphate buffer. The material to be fractionated, usually 10 ml, was applied to the top of the column and after being taken up, was followed by 10 ml of buffer. After the buffer was taken up, the apparatus was assembled and a potential of 700 volts (25 volts per cm at about 20 milliamps) applied for 8-10 hr. Following electrophoresis, material was eluted from the column with pH 7.0, 0.02 M phosphate buffer using a fraction collector adjusted to accumulate 3.5 ml fractions. Geon 427 has many desirable characteristics as a supporting agent in column electrophoresis and is strongly recommended for this purpose.

TABLE I. Effect of Preincubation with RNAase and Autoclaved Liver Extract in Various Combinations on Conversion of MVA to NSF by Liver Homogenates.

Exp. 1			Exp. 2		
RNAase, mg	Liver extract, ml	NSF, cpm	RNAase, mg	Liver extract, ml	NSF, cpm
None	None	7635	None	None	8507
2	"	597	2	"	19
None	5	7863	None	5	8881
2	5	7521	2	5	9054

Autoclaved liver extract where present was added prior to incubation with RNAase.

The nucleotide composition of RNA preparations was determined on KOH hydrolysates (20 mg RNA, 1 ml 0.3 N KOH, 37°, 20 hr) by spectrophotometric determinations after separation of the individual nucleotides by extended gradient elution from Dowex-1 formate with increasing concentrations of formate after the procedures of Hurlbert *et al.*(8).

Results. As pointed out previously, the preincubation of liver homogenates with RNAase is associated with the inability of these treated homogenates to convert MVA to NSF, and the so-called "factor" under consideration has been defined as the material present in autoclaved liver extract that prevents or reverses this inactivation. The data of Table I demonstrate the effect of autoclaved liver extract in the prevention of RNAase inactivation of liver homogenate. In general, smooth response curves to graded increments of autoclaved liver extract are obtained but, for reasons to be pointed out later, variations in the activity of various fractions of autoclaved liver extract and the lack of specificity of various active compounds has precluded the development of a refined biological assay for the factor.

It became apparent during the fractionation studies that the activity of a preparation

TABLE II. Influence of Phosphate Level on Activity of RNAase as an Inhibitor of a System Essential for Conversion of MVA to NSF by Liver Homogenates.

RNAase, mg	Phosphate buffer, M	NSF, cpm
0		6270
2		278 (96)
5	.05	69 (99)
10		57 (99)
0		4887
2		321 (94)
5	.06	62 (99)
10		43 (99)
0		5769
2		945 (84)
5	.075	139 (98)
10		47 (99)
0		6467
2		5370 (17)
5	.1	1582 (75)
10		332 (95)

Figures in parentheses indicate % inactivation of MVA conversion to NSF accomplished by preincubation of homogenate with various levels of RNAase and pH 7.0 phosphate buffer.

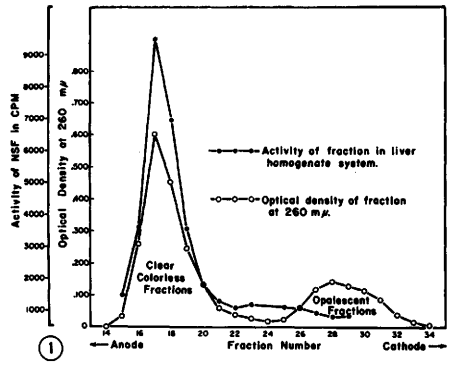
is influenced to a considerable extent by the phosphate concentration. The data of Table II demonstrate that the activity of RNAase as an inhibitor is an inverse function of the phosphate concentration of a homogenate preparation. For example, when 5 ml of liver homogenate (prepared with 0.1 M phosphate buffer) were diluted with 5 ml of 0.1 M phosphate buffer in a biological assay, approximately 10 mg of RNAase were required to produce 95% inactivation of the system. On the other hand, when the homogenate was diluted with 5 ml of water, 96% inactivation was accomplished with 2 mg of RNAase. For this reason it was found necessary that all fractions involved in a given assay contained the same phosphate concentration. Where solid preparations could be dissolved in water, this was the usual solvent for unknown materials. Where it was necessary to assay electrophoretic fractions, for example, all materials contained the same level of phosphate.

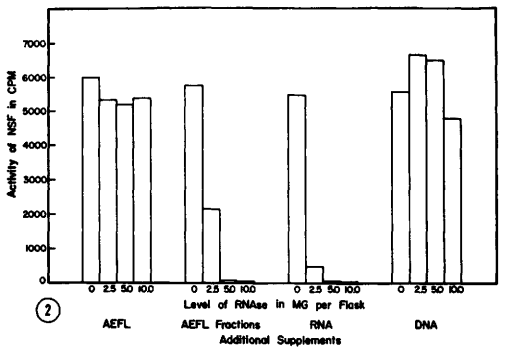
Column electrophoresis was investigated as a possible means of fractionating autoclaved liver extract, because preliminary studies of the properties of the factor(3,4) suggested that it is quite unstable, and column electrophoresis has been widely employed with success in the fractionation of labile materials. Autoclaved liver extract was subjected to column electrophoresis as described in the procedure, and the various fractions obtained were assayed. The results obtained in a typical experiment are shown in Fig. 1. Biological activity migrated towards the anode and was associated with material having strong absorption in the vicinity of 260 mμ. Such fractions, in contrast to the original autoclaved liver extract, were clear and colorless. Active fractions prepared in this way were compared in the biological assay with autoclaved liver extract on the basis of equal optical density at 260 mμ. The results obtained are summarized in Fig. 2. It is apparent that liver extract is much more active.

Preliminary investigation of active fractions migrating towards the anode on electrophoresis suggested that the factor of autoclaved liver extract might be RNA. Such active fractions were therefore combined, made 10% with respect to sodium chloride and treated with 4 volumes of ethanol. The precipitate that formed was dissolved in water, dialyzed against several changes of water, and lyophilized. Such preparations were free of DNA by the Dische reaction, were quantitatively retained by a Dowex-1 formate column as judged by UV determinations, contained about 7-8% P, and gave no color with biuret reagent. UV absorption curves were characteristic of RNA. The nucleotide composition of a typical preparation showed ratios of adenylic: uridylic: cytidylic: guanylic acid of 10:11:32:42. A typical RNA preparation was compared in the biological assay with the "homologous" autoclaved liver extract from which it was derived on the basis of equal optical density at 260 mμ, and the results obtained are summarized in Fig. 3. It is apparent that autoclaved liver extract is much more active. No activity was found in the mother liquor or dialysates obtained in various RNA preparations.

Discussion. The fractionation of autoclaved liver extract for activity in preventing the deleterious effect of RNAase against a system essential for the conversion of MVA to NSF by liver homogenates has led to the

isolation of RNA with a base composition characteristic of the guinea pig liver from which it was derived. Comparisons of activity on the basis of equal optical density at 260 mμ of this RNA with the original autoclaved liver extract from which it was derived show the autoclaved liver extract to be much more active. The most reasonable interpretation of these data is that the "factor" of autoclaved liver extract is a conjugate, presumably a nucleoprotein, with properties quite different from the RNA component. The conjugate is dissociated by mild procedures such as electrophoresis at neutral pH to yield RNA which, as such, is susceptible to attack by RNAase and as a consequence is decidedly less active in a system inactivated by RNAase. Previous papers from this laboratory have offered evidence that the "factor" of autoclaved liver extract, or other nonspecific polyanion substitute, is essential for maintenance *via* the terminal respiratory chain of a functional level of ATP required in a number of phosphorylations involved in conversion of MVA to NSF(2,3,4,5).





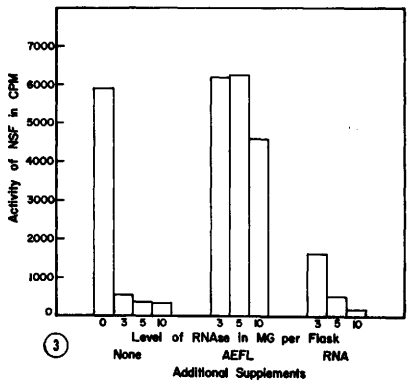


FIG. 1. Fractionation of autoclaved liver extract by column electrophoresis.

FIG. 2. Relative activity of Dowex-1-treated autoclaved liver extract (AEFL), electrophoretic fractions derived from AEFL, commercial RNA, and commercial DNA in prevention of RNAase inhibition of the conversion of MVA to NSF by liver homogenate. Comparisons made on the basis of equal optical density at 260 mμ.

FIG. 3. Relative activity of Dowex-1-treated, autoclaved liver extract (AEFL) and purified RNA derived from it in prevention of RNAase inhibition of conversion of MVA to NSF by liver homogenate. Comparison of AEFL and RNA made on the basis of equal optical density at 260 mμ.

Summary. Fractionation of autoclaved liver extract with respect to a component essential for conversion of mevalonic acid to cholesterol by RNAase-treated homogenates of liver has led to the isolation of RNA. It is concluded on the basis of comparative activity of liver extract and the RNA isolated from it that the RNA represents one moiety of a more complex component, presumably a ribonucleoprotein, less susceptible to the action of RNAase.

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