ject to further analysis by enzymatic and metabolic studies. It is hoped that the histological changes described will clarify the point of action of chemotherapeutic agents.

Conclusions. The changes produced in Ehrlich ascites tumor cells by Columbia SK virus are described, using various staining technics including fluorescent antibody staining.

1. Cutting, W., Furst, A., Read, D., Grant, D., Cords, H., Megna, J., Butterworth, E., *Antibiot. & Chemother.*, 1960, in press.

2. Furusawa, E., Cutting, W., PROC. SOC. EXP. BIOL. AND MED., 1960, in press.

3. Armstrong, J. A., *Exp. Cell. Res.*, 1956, v11, 640.

4. Weller, T. H., Coons, A. H., PROC. SOC. EXP. BIOL. AND MED., 1955, v102, 49.

5. Niven, J. S. F., *Ann. N. Y. Acad. Sci.*, 1959, v81, 84.

Received February 15, 1960. P.S.E.B.M., 1960, v103.

Protamine and Conversion of Mevalonic Acid to Cholesterol by Liver Homogenates.* (25695)

LEMUEL D. WRIGHT AND ROSEMARIE HANSON
Graduate School of Nutrition, Cornell University, Ithaca, N. Y.

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)(1). Evidence has been presented that RNAse acting in association with easily sedimentable constituent of homogenate (2,3) destroys a system concerned with maintenance of ATP(4,5,6). This system appears to require a polynucleotide, presumably "free" or "combined" RNA as an essential component since inactivation may be prevented or reversed by a "nucleoprotein" of autoclaved liver extract, or by relatively large amounts of RNA, or by DNA, or by a number of "unnatural" anionic polymers including polyethylene sulfonate (PES) or heparin(7,8). It has now been found that capacity of the system for maintaining adequate ATP levels as judged indirectly by utilization of MVA for biosynthesis of NSF is lost by preincubation of the homogenate with protamine. This protamine inhibition is associated also with absence of ATP in the homogenates and is, furthermore, preventable with RNA, DNA, or PES and, using PES as an example, is associated with a level of ATP in the homogenate equal to that of control. Reasons are presented here for believing that preincubation of liver homogenates with protamine results in formation of a protamine-RNA complex that renders RNA unavailable for its role in maintenance of ATP levels adequate for phosphorylation of MVA.

Methods and materials. The biosynthetic experiments involved preincubation of 5 ml aliquots of a 200 x g supernatant fraction of rat liver homogenate prepared as previously described(1-9) with 5-8 ml amounts of solution containing 1 mg ATP, 10 mg DPN, 100 mg sodium succinate, various amounts of protamine (neutralized), and other supplements to be studied. Each flask was aerated with a stream of oxygen and preincubated with agitation for 30 min at 37°. Following preincubation each flask was opened and 1 ml MVA-2-C^{14} solution added, the contents were aerated and the flasks then reincubated for additional 3 hrs. After final incubation, homogenates were saponified, extracted with petroleum ether, the ether extracts dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture and counted. Previous studies have shown(1,9) that under experimental conditions employed the counts found in the NSF fraction are essentially CHL or other digitonin-precipitable material. In experiments involving determination of ATP

* Supported by research grants from Nat. Science Fn. and N.I.H. and funds available through State Univ. of N. Y. We are indebted to Dr. J. H. Flokstra, Upjohn Co., for samples of polyethylene sulfonate.

TABLE I. Biosynthesis of NSF by Liver Homogenates as Influenced by Preincubation with Protamine or Protamine and Various Anionic Polymers.

Exp. No.	Protamine, mg	Additional supplement, mg	NSF, cpm
1	0	None	8254
	1	"	8148
	2	"	8011
	5	"	40
2	0	"	5982
	1	"	5862
	2	"	5889
	5	"	161
	10	"	75
3	0	"	7652
	5	"	6709
	10	"	89
	0	PES, 5 mg	6474
	5	*Idem*	6693
	10	"	7089
	0	DNA, 5 mg	7480
	5	*Idem*	7863
	10	"	162
	0	RNA, 5 mg	8078
	5	*Idem*	9202
	10	"	97
4	0	None	7962
	10	"	489
	"	PES, 5 mg	7500
	"	10	7384
	"	20	7394
	"	DNA, 5	1337
	"	10	8597
	"	20	8795
	"	RNA, 5	521
	"	10	855
	"	20	7154
5*	0	None	9332
	10	"	64
	10	PES, 5 mg	8545

* In this experiment supplementation of homogenate with additional ATP was intentionally omitted.

content of homogenates at time of MVA addition following preincubation, the contents of paired flasks were treated with equal volume of cold 4% perchloric acid. The precipitate that formed was removed by centrifugation and the supernatant solutions were freed of excess perchloric acid by careful neutralization with cold 10% potassium hydroxide. The solutions were centrifuged to remove potassium perchlorate and supernatant solutions were subjected to anion exchange chromatography and gradient elution on Dowex-1 as described by Allfrey and Mirsky(10) and used previously(4). ATP was determined spectrophotometrically in the eluates. In biosynthetic experiments involving effect of added ATP the ATP was added to homogenates following preincubation at time of MVA addition(5). The influence of protamine on activity of ATP *in vitro* was determined by coupling hexokinase and glucose-6-phosphate dehydrogenase reactions under conditions where an increase in optical density at 340 mμ from TPNH is a measure of ATP availability(11). Protamine sulfate used was a commercial preparation (Mann Research Labs). The polyethylene sulfonate (Upjohn) had a mean molecular weight of 12,900. ATP was used as crystalline disodium salt (Pabst Labs).

Results. Biosynthesis of NSF from MVA is completely inhibited by preincubation with about 5 mg of protamine/flask (Table I). Inhibition produced by as much as 10 mg of protamine is preventable with 5 mg of PES. DNA and RNA are less active. In one experiment 5 mg of DNA showed detectable activity and 10 or 20 mg showed complete activity against 10 mg of protamine. In the same experiment 10 mg of RNA showed detectable activity and 20 mg showed complete activity against this level of protamine.

Fig. 1 shows that a control homogenate of liver preincubated without protamine had an adequate level of ATP (calculated to be 2 mg) as determined by ion exchange chromatography while a paired flask supplemented with MVA following preincubation was highly active in conversion of MVA to NSF. On the other hand, a homogenate preincubated with protamine was devoid of ATP and a paired flask supplemented with MVA following preincubation was inactive in conversion of MVA to NSF. Homogenate preincubated with protamine plus PES contained a level of ATP equal to the preincubated control homogenate (2 mg) and conversion of MVA to NSF in a paired flask was equal to that in control.

Additional evidence that preincubation of a homogenate with protamine results in a system that does not maintain a level of ATP is presented in Table II. In this experiment paired flasks were preincubated with 0, 5 and 10 mg of protamine. Following preincubation 10 mg of ATP was added to one flask of each pair at time of MVA addition. As noted in

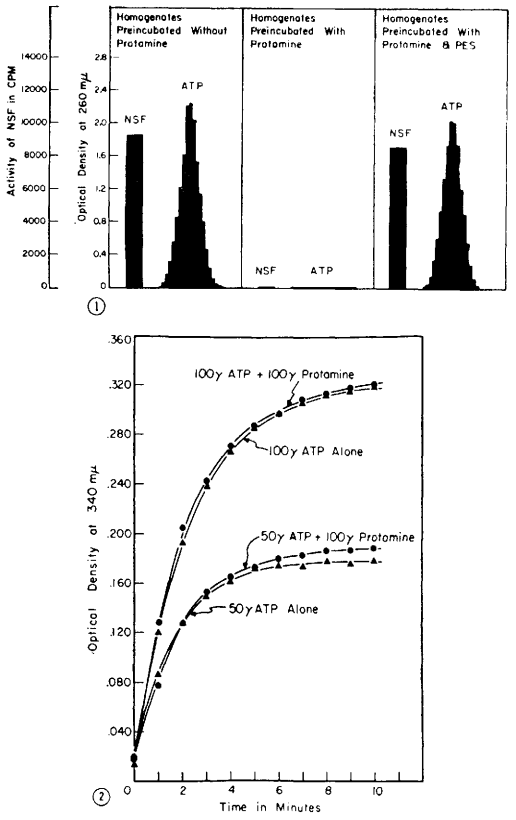

FIG. 1. ATP content of rat liver homogenates preincubated without protamine, preincubated with protamine, or preincubated with protamine and PES. Activity of NSF isolated from paired flasks to which MVA-2-C^{14} was added following preincubation also is indicated.

FIG. 2. Absence of an effect of protamine on availability of ATP for phosphorylation of glucose to glucose-6-phosphate by hexokinase as determined by TPNH formation in coupled oxidation of glucose-6-phosphate to 6-phosphogluconolactone by zwischenferment.

earlier experiments, preincubation of homogenates with protamine is associated with an almost complete absence of MVA incorporation into NSF (8651 cpm in control *vs.* 39 and 49 cpm with 5 and 10 mg protamine). In agreement with previous studies involving ATP(5), addition of ATP in this way to control homogenates is associated with some reduction in capacity to convert MVA to NSF. (5932 cpm with ATP *vs.* 8651 cpm without ATP). However, conversion of MVA to NSF in the flask containing 5 mg of protamine to which ATP was added was essentially equal to that in the control homogenate with ATP (4921 cpm *vs.* 5932 cpm or 4921 cpm with ATP *vs.* 39 cpm without ATP) while in the flask containing 10 mg of protamine subsequently supplemented with ATP there was marked conversion of MVA to NSF (1931 cpm with ATP *vs.* 49 cpm without ATP). The active conversion of MVA to NSF in flasks preincubated with protamine and then subsequently supplemented with ATP as compared with the unsupplemented flasks is evidence that the first inhibitory effect of protamine in homogenates is against a system concerned with maintenance of an ATP level.

Since the inhibitory effect of protamine is preventable with RNA, DNA or PES and is reversible with ATP the possibility existed that protamine functions by combining with either or both types of compounds *i. e.*, the relatively large anionic polymers or the relatively small coenzyme. Evidence that protamine has no effect on at least one biochemical function of ATP is furnished in Fig. 2. In this experiment ATP was made the limiting factor in phosphorylation of glucose by hexokinase and this reaction was coupled to oxidation of glucose-6-phosphate by zwischenferment where TPN reduction as measured by absorption at 340 mμ could be used to measure rate and extent of phosphorylation. At levels employed (protamine: ATP ratio of 1 or 2) protamine is without effect on rate or extent of ATP activity. These results indicate that the inhibitory effect of protamine in the biosynthetic experiments is against compounds concerned in maintaining a level of ATP rather than against ATP itself.

Discussion. Our data show that preincubation of liver homogenates with protamine yields a system that does not convert MVA to NSF (largely CHL). Inactivation may be prevented by carrying out preincubation with

TABLE II. Influence of ATP on Biosynthesis of NSF from MVA in Homogenates Preincubated with Various Levels of Protamine.

Protamine, mg	Activity of NSF, cpm	
	Unsupplemented homogenates	ATP supplemented homogenates*
0	8651	5932
5	39	4921
10	49	1931

* 10 mg ATP added/flask at time of MVA addition following preincubation with indicated level of protamine.

protamine in the presence of added RNA, DNA or PES. Control homogenate preincubated without protamine maintained a level of ATP while homogenate preincubated with protamine was devoid of ATP. Homogenate preincubated with protamine plus PES maintained a level of ATP equal to the control. Protamine inhibition also could be reversed with large amounts of ATP. Evidence that protamine combines with factors, presumably "free" or "combined" RNA, concerned with maintenance of ATP levels rather than with ATP *per se*, was found by activity of ATP in an *in vitro* enzyme system that requires ATP was not influenced by protamine to ATP levels comparable to those involved in the biosynthetic experiments. Our findings lead to the interesting question, implied or discussed by a number of authors, of the extent to which control of biochemical activity within the cell is regulated *via* basic proteins. It appears that if a strongly basic protein such as protamine were elaborated intracellularly activity requiring ATP might be depressed as a consequence of unavailability of "free" or "combined" RNA. On the other hand, some degree of RNA-protein combination appears essential to protect RNA from intracellular RNAse. Our studies have shown that "nucleoprotein" of autoclaved liver extract is resistant to RNAse but after separation of the moieties the RNA is then susceptible to RNAse. Thus a number of factors that have been loosely characterized in the literature dealing with "tightness" with which phosphorylation is coupled (or uncoupled) to oxidation may represent proteins of varying affinity for polyanions of the cell that seem to be concerned with maintenance of ATP levels.

Summary. Protamine is a potent inhibitor of a system essential for conversion of mevalonic acid to cholesterol. Evidence was presented that protamine combines with a polyanion, presumably "free" or "combined" RNA, that is essential for maintenance of an ATP level required in utilization of mevalonic acid.

1. Wright, L. D., Cleland, M., Dutta, B. N., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 425.

2. Wright, L. D., Cleland, M., Norton, J. S., *J. Am. Chem. Soc.*, 1958, v80, 3485.

3. Wright, L. D., Cleland, M., Norton, J. S., Loeb, M., PROC. SOC. EXP. BIOL. AND MED., 1958, v99, 299.

4. Wright, L. D., Loeb, M., Norton, J. S., *ibid.*, 1959, v101, 866.

5. Wright, L. D., Loeb, M., *ibid.*, 1959, v102, 540.

6. ———, *ibid.*, 1960, v103, 183.

7. Wright, L. D., Rakowska, M., Hanson, R., *Fed. Proc.*, 1960, v19,

8. Wright, L. D., Loeb, M., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 546.

9. Wright, L. D., Cleland, M., *ibid.*, 1957, v96, 219.

10. Allfrey, V. G., Mirsky, A. E., *Proc. Nat. Acad. Sci.*, 1957, v43, 589.

11. Cowgill, R. W., Pardee, A. B., *Experiments in Biochemical Research Techniques*, John Wiley & Sons, N. Y., 1957, 106.

Received February 15, 1960. P.S.E.B.M., 1960, v103.

Effect of Neomycin on Cholesterol Atherosclerosis in the Rabbit. (25696)

EDWIN R. FISHER*

Depts. of Pathology, University of Pittsburgh and Vet. Admin. Hospital, Pittsburgh, Pa.

A significant decrease in serum cholesterol has been recently observed in 10 patients following oral administration of the antibiotic Mycifradin which contains 70% neomycin sulfate(1). However, Broitman *et al.*(2) observed a marked hypercholesterolemia and valvular sudanophilia in neomycin-treated rats fed a cholesterol and cholate diet. In both studies the effect of neomycin was attributed to alterations of intestinal bacterial flora or certain enzyme systems of the gastrointestinal tract. The purpose of this report is to note the effect of oral and parenteral administration of this agent on serum lipids of normal and cholesterol-fed rabbits and the

* Senior Research Fellow U.S.P.H.S.