changes and age-associated diseases such as hypertension, atherosclerosis, varicose veins, pulmonary emphysema and osteoporosis.

The problem of reversibility of the disturbance induced by cortisone after withdrawal of the hormone is important because it is concerned with the influence exerted by past events such as illness, surgery, and therapeutic cortisone administration upon rate of aging. Obviously information obtained on growing rats may not be applicable to human physiology but preliminary studies reveal that a decrease in H/C ratio may be observed in patients receiving therapeutic doses of cortisone for several weeks (Wright and Sobel, to be reported).

Lung, sternum, aorta and vena cava exhibit complete or nearly complete recovery of the H/C ratio following withdrawal of cortisone. Whether the original composition of the ground substance is reestablished remains to be determined.

Skin fails to reestablish the initial H/C ratio before growth continues. Apparently, the greater the depression of the H/C ratio induced by cortisone, the slower the recovery. When growth continues the changes in H/C ratio follow a complicated course. It seems however, that when the weights of the experimentally treated animals approach those of their untreated litter mates, differences in the H/C ratio no longer exist.

Summary. The effect of cortisone administration upon the hexosamine-collagen ratio (H/C) of several tissues was investigated. The H/C of skin, sternum, trachea, lung, aorta and vena cava was significantly reduced when a 20% loss in weight was induced by cortisone. When injections were discontinued and the original weight was regained, H/C of sternum and vena cava returned to starting level. This was nearly true of lung and aorta. Skin lagged behind considerably in this respect. Multiple biopsies were taken from skin following cortisone administration and withdrawal. When depression of H/C was greatest, recovery lagged the most. The findings are pertinent to the question of the general nature of cortisone effect upon connective tissue and the reversibility of this effect.

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Additional Evidence for the Existence of a New Factor Essential for Mevalonic Acid Utilization.* (24330)

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Preincubation of rat liver homogenates with crystalline ribonuclease (RNAse) was found to abolish the capacity of such homogenates to utilize mevalonic acid (MVA) for biosynthesis of nonsaponifiable material (NSF) or cholesterol (CHL)(1,2,3). Control homogenates preincubated without added RNAse ordinarily retained this biosynthetic capacity.

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The RNAse effect was observed only with homogenates that had been subjected to a brief centrifugation to remove connective tissue, etc.(3). Inactivation with RNAse could not be obtained, for example, with homogenates that had been subjected to centrifugation at 500 or 9000 xg. The synthetic capacity of homogenates that had been inactivated with RNAse could be restored by addition of an autoclaved extract of fresh liver. The existence in autoclaved liver extract of a previously unrecognized factor essential for biosynthesis of NSF or CHL directly or indirectly from MVA was postulated(3). This paper presents additional evidence for the existence of such a factor and reports preliminary information concerning its distribution and properties. Some consideration is given to possible mechanisms involved in inactivation of MVA utilization by RNAse.

Materials and methods. The experimental methods involved in NSF biosynthetic studies have been described(4). In brief, liver from young rats was homogenized with buffer (0.067 M K_2HPO_4, 0.042 M KH_2PO_4, 0.006 M $MgCl_2$, 0.03 M nicotinamide, pH 6.90) in the proportions of 1:2 with a "loose" homogenizer and centrifuged at 200 xg for 3 minutes. The supernatant layers were decanted and 5 ml aliquots were pipetted into flasks containing 1 mg ATP and 1 mg DPN in a volume of 1 ml. Crystalline RNAse (Worthington), where used (2-5 mg), was present in 1 ml of water or buffer. Control flasks without RNAse contained a corresponding volume of water or buffer. The flasks were individually aerated for 20 seconds with a stream of oxygen, stoppered, and preincubated for 30-45 min at 37°. The flasks were then opened and 1 ml of supplement or 1 ml of water or buffer plus 1 ml of 2-C^{14}-MVA solution were added to each flask. The flasks were then reaerated, stoppered, and incubated for 4 hours. 10 ml of 2N KOH in 80% ethanol was added to each flask and saponification carried out overnight on a steam bath. The alkaline solution was then extracted 4X with 50 ml amounts of petroleum ether. The combined ether extracts were dried with anhydrous sodium sulfate, filtered, and evaporated to a small volume. The concentrated NSF were transferred to tared planchets, dried briefly under an infra-red lamp and counted with a gas-flow counter. The MVA contained in 1 ml 0.5 mg of MVA calculated as the dibenzylethylene diammonium (DBED) salt from which the DBED had been removed with ether at alkaline pH. Various preparations of MVA contained 15-20,000 cpm/ml. In certain experiments material to be studied in the liver incubation system was added at the start of the experiment rather than after RNAse inactivation. Autoclaved liver extract was prepared by heating liver homogenate (whole homogenate, 200 or 9000 xg supernatant) in the autoclave at 105-110° usually for 15 min after which the mixture was centrifuged and the coagulum discarded.

Results. Assay system. As pointed out previously, the assay system depends upon the capacity of natural material to restore synthetic activity of RNAse-treated homogenates. In certain experiments material to be examined was added to the homogenates prior to inactivation with RNAse. The results obtained were essentially the same. Since evidence for the existence of a new factor seemed clearer when natural material *restored* the synthetic activity of an enzyme system after inactivation rather than *prevented* inactivation in the first place, supplements to be tested ordinarily were added after inactivation. In many assays, for example Exp. 5 and 6, synthetic capacity was restored to that of control homogenates that had not been inactivated with RNAse. On the other hand, in some assays, for example Exp. 1 and 2, restoration has been less than complete, sometimes even as low as 10-20%. Whether or not these low results are a reflection of an uncontrolled variable in the assay system or to inability consistently to prepare autoclaved liver extracts of high activity is not known.

Distribution. The factor is present in autoclaved liver extract prepared from rats immediately after killing. In one experiment, for example, an aliquot of a liver homogenate that was autoclaved immediately after preparation had an arbitrary activity of 100; another aliquot that stood at 5° for 30 min before autoclaving had an activity of 75, while a third aliquot incubated at 37° for 30 min

TABLE I. Influence of Various Supplements on Incorporation of Mevalonic Acid into Non-Saponifiable Material by Rat Liver Homogenates.

Exp.	RNAse	Supplement	Activity of NSF, cpm/mg
1*	–	None	410
	+	"	1
	+	3 ml autoclaved liver extract	67
	+	5 ml *Idem*	244
2	–	None	2020
	+	"	4
	+	1 ml autoclaved liver extract	15
	+	3 ml *Idem*	239
	+	5 ml "	585
3*	–	None	572
	+	"	82
	+	5 ml autoclaved liver extract	363
	+	20 mg distillers' dried solubles, yeast extract, bacto peptone or dried whey	≦ 104
4	–	None	2130
	+	"	34
	+	5 ml autoclaved liver extract (200 xg)	1230
	+	5 ml autoclaved liver extract (9000 xg)	1540
	+	5 ml autoclaved liver extract (9000 xg) dialyzed	33
5	–	None	1335
	+	"	16
	+	5 ml liver extract (9000 xg) autoclaved 5 min.	1445
	+	*Idem*, but autoclaved 30 min.	1195
6*	–	None	564
	+	"	1
	+	5 ml autoclaved liver extract	616
	+	*Idem*, dialyzed	6
	+	5 mg coenzyme A	1
7	–	None	1030
	+	"	9
	+	1 ml autoclaved liver extract	45
	+	3 ml *Idem*	312
	+	5 ml "	453
	+	10 mg DPN	40
8	–	None	1145
	+	"	27
	+	1 ml autoclaved liver extract	137
	+	3 ml *Idem*	236
	+	5 ml "	337
	+	5 ml autoclaved liver extract incubated 30 min.	292
	+	*Idem*, with 2.5 mg RNAse	296
	+	" + 2.5 mg RNAse	321
9*	–	None	1040
	+	"	0
	+	10 mg cysteine, glutathione, lipoic acid, thioglycollic acid, BAL or coenzyme A	≦ 2
10*	–	None	909
	+	"	3
	+	10 mg yeast RNA	7
11	–	None	790
	+	"	2
	+	15 mg acid-treated yeast RNA	0
12*	–	None	514
	+	"	168
	–	DPN replaced with TPN	761
	+	*Idem*	240
	–	2 mg coenzyme A	625
	+	*Idem*	7
	–	DPN replaced with TPN + 2 mg coenzyme A	904
	+	*Idem*	5
	–	DPN one-half replaced with TPN	647
	+	*Idem*	198
	–	DPN one-half replaced with TPN + 2 mg coenzyme A	676
	+	*Idem*	6
13*	–	None	1560
	+	"	3
	–	15 mg ascorbic acid	983
	+	*Idem*	12
	–	15 mg glutathione	1300
	+	*Idem*	10
	–	15 mg cysteine	1510
	+	*Idem*	5

* Indicates experiments in which supplements tested were added prior to RNAse inactivation.

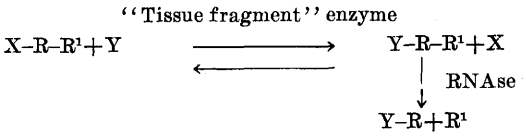
had an activity of 25. The factor is also present in autoclaved liver extracts prepared from beef or sheep immediately after killing. Autoclaved extracts prepared from beef liver purchased on the open market have been inactive. The factor appears to be absent from distillers' dried solubles, Bacto yeast extract, Bacto peptone, and commercial dried whey (see Exp. 3). In view of these results, it may be predicted that other natural material that has undergone autolysis or self-digestion will be inactive.

Properties. Active preparations of the factor usually turn out to be inactive or at least less active after dialysis especially when dialyzed against buffer (see Exp. 4 and 6). In several experiments it has been possible to show a substantial amount of the original activity remaining after dialysis when thoroughly soaked and washed tubing is used and the dialysis is carried out against water. Attempts at recovery of activity in the dialysate have been unsuccessful. Presumably the fac-

tor is essentially nondialyzable but is inactivated by some unknown mechanism during the process. When autoclaved liver extract in buffer solution is brought to pH 0.5 and held there for 18 hours at 0°C followed by neutralization the resulting solution is inactive. Inactivation is considerably less in samples exposed to pH 11. Tentatively it would appear that the factor is more labile to acid than to alkali. The factor appears to be quite stable to heat at neutral pH. Preparations of liver extract autoclaved for 30 min were as active as preparations that had been autoclaved for only 5 min (see Exp. 5). The factor is not retained by a Dowex-1-Cl column. Studies with a weak cation exchange resin (IRC-50) have given equivocal results but there may be some retention under defined conditions. The factor as encountered in autoclaved liver extract is not labile to RNAse digestion (Exp. 8) despite the fact that the factor restores the synthetic capacity of liver homogenates that have been inactivated with RNAse. An explanation for this phenomenon is considered later.

Inactivity of known compounds. Over 30 known compounds of some biological significance have been examined for activity. None has shown activity with the possible exception of DPN. In certain experiments large amounts of DPN (10 mg per flask) have sometimes shown low activity. In other experiments autoclaved liver extract has shown activity while DPN has been essentially inactive (Exp. 7). It may be concluded that the factor is not DPN, but DPN may be related somehow to the phenomenon under study. Large amounts of TPN (10 mg per flask) are quite inhibitory. When DPN routinely used as a supplement in the incubations was one-half or completely replaced with TPN, incorporation of MVA into NSF was usually slightly better than with DPN alone (see Exp. 12). Some stimulation with coenzyme A has been obtained with DPN or TPN in flasks that did not contain added RNAse. With RNAse in the presence of DPN or TPN inactivation appears to be increased in the presence of CoA. The factor under consideration is not, therefore, a mixture of DPN or TPN plus coenzyme A. Intact yeast ribonucleic acid has no activity in the system (see Exp. 10). Similarly, yeast ribonucleic acid partially hydrolyzed at random with hydrochloric acid after the technic of Merrifield and Wooley(5) also is inactive (see Exp. 11).

Discussion. Our data substantiate the hypothesis that there exists in natural material an unknown factor that is involved in and essential for mevalonic acid utilization. Some consideration is in order concerning the mechanism of RNAse inactivation of NSF or CHL biosynthesis. A previous paper reported (3) that inactivation with RNAse was not obtained with homogenates that had been subjected to moderate centrifugation (500-9000 xg) before use. These preparations did, however, retain their capacity for NSF and CHL biosynthesis. Homogenates that were treated with RNAse and then centrifuged at 9000 xg were inactivated. Treatment of the pellet obtained by centrifuging homogenates at 9000 xg separately with RNAse did not yield an inactivated preparation when this digest was added back to the 9000 xg supernatant fraction. These experiments indicate a definite role for material sedimentable below 9000 xg, loosely characterized as "cell fragments," in the mechanism of RNAse inhibition and demonstrate that the following 3 components must be present together for inactivation: (a) RNAse, (b) cell fragments, and (c) a component of 9000 xg supernatant fraction. The most reasonable mechanism of inactivation is that there exists an unknown component of the 9000 xg supernatant fraction that is essential for NSF and CHL biosynthesis which in itself is not susceptible to RNAse attack but becomes susceptible after alteration by a component of the cell fragment. The hypothesis is presented diagrammatically as follows:



X–R–R¹ = essential factor (RNAse insensitive)
Y–R–R¹ = altered factor (RNAse sensitive)
Y–R+R¹ = products of RNAse action

A number of other explanations have been considered and rejected. For example, it may

be suggested that the factor of liver extract is only an inhibitor of RNAse and is unrelated to the synthetic capacity of the system. Since the liver extract will restore the synthetic capacity of a system *after* it has been inactivated with RNAse it is apparent that this explanation is incorrect. Similarly, it may be suggested that RNAse inactivates by destroying the structure of tissue particles. Since preparations that have been autoclaved and subjected to further chemical manipulation have shown activity it is apparent that such an explanation also must be rejected.

It is premature to speculate concerning the chemical nature of the factor. Present information would suggest, however, that it is some sort of a polynucleotide of high molecular weight. All the data thus far obtained point up the extreme lability of the substance.

The hypothesis presented explains the interesting enigma that to obtain homogenates that are highly active in NSF or CHL biosynthesis a "loose" homogenizer must be used (6), yet such homogenates can then be fractionated by high speed centrifugation and still retain high synthetic capacity(7,8). It is suggested that homogenization of liver with a "tight" homogenizer releases large amounts of tissue fragments which in combination with "endogenous" RNAse destroy the essential factor under discussion here. On the other hand, a "loose" homogenizer releases enough soluble enzyme, small tissue particles as well as the present factor to yield preparations with high activity even after high speed centrifugation.

A recent paper by Popjak *et al.*(9) reports that a microsomal component of liver homogenate which on the basis of some indirect evidence may be presumed to be an SH-compound is essential for cholesterol biosynthesis by a dialyzed liver system. Coenzyme A was stated to have some activity and the authors concluded that "the evidence suggests, but does not prove, that the microsomal SH-compound is coenzyme A." Possibly the factor of Popjak is the same as the one described here. If so, the factor is not coenzyme A in view of the negative results obtained in this study (Exp. 6, 9, 12 and 13).

With respect to the probable locus of action of the present factor it may be essential directly in biosynthetic conversions of MVA or it may be required in some "supporting" reaction, for example, in reduction of DPN or TPN. A previous paper(2) reported that added MVA or material with microbiological activity equal to it accumulates in RNAse-treated homogenates. Studies of Rilling *et al.* (10) indicate that in biosynthetic reactions of MVA a monophosphate and subsequently a diphosphate are formed prior to isoprene(?), farnesene(?), and eventually squalene. The microbiological activity of these intermediates has not been reported but it is suggested that the block in MVA utilization accomplished with RNAse must be between MVA and the first compound in the sequence without significant microbiological activity. The present factor may well be a cofactor or phosphate donor for MVA phosphorylation.

Summary. Additional evidence is presented for the existence of a new factor essential for utilization of mevalonic acid. This factor is present in autoclaved extracts of fresh liver from several species but absent from autolyzed liver. It is heat stable but is lost on dialysis or exposure to pH 0.5 and to a lesser extent by exposure to pH 11.0. A variety of biological compounds has been examined for activity. No compound thus far studied, including coenzyme A, will replace natural material.

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