the presence of calcium phosphate in these crystals which appear in the vicinity of trabeculae unable to mineralize in rickets as well as in osteolathyrism of the chick(1), BAPN poisoning of young rats and human osteogenesis imperfecta.

Calcium and norethandrolone help bone growth but do not favor maturation(6). Calcium and norethandrolone combined have apparently produced maturation of the organic matrix(6); the present experiments reveal however that extratrabecular crystallization is not reduced by that treatment as it is by Vit. D (Fig. 2). The present experiments indicate also that even in rickets, the local mineral deposit is fairly abundant. The numerous osteoclasts which inhabit the vicinity of the growth sites might be instrumental in local mobilization of salt in order to "make the interstitial fluid rich in calcium," a condition essential for mineralization as postulated by Leriche and Policard(7). The primary lesion of bone in rickets which is cured by Vit. D would then be related to the organic matrix. On the other hand, the increased $Ca^{45}$ uptake by bone under high Ca deposit(8) may be intimately responsible to the presence of extratrabecular crystals and not to an activation of bone growth.

*Summary.* Large extratrabecular crystals are present in rachitic bones of chicks. They disappear after treatment with Vit. D.

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## Additional Evidence for Involvement of RNA in Maintenance of ATP Levels in Homogenates.* (26224)

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Rat liver homogenates preincubated with ribonuclease (RNAse) lose the capacity to convert mevalonic acid (MVA) to non-saponifiable material (NSF) and cholesterol(1). This loss of synthetic capacity is associated with absence of ATP in these homogenates (2,3). ATP is present and conversion of MVA to NSF occurs if the homogenates are supplemented prior to or after preincubation with autoclaved liver extract, or with relatively large amounts of RNA, or with DNA, or with various anionic polymers such as polyethylene sulfonate or heparin (4,5,6). It has been concluded that a polyanion, which in the case of whole liver is RNA or a ribonucleoprotein, is essential for maintenance of ATP levels in tissues(7,8). Inactivation of biosynthetic capacity in liver homogenates with respect to MVA conversion to NSF also is observed on preincubation with various polycations such as protamine or polylysine(9,10). The effect of these agents similarly is preventable or reversible with the same group of polyanions. These findings with polycations raise the question as to whether inactivation with RNAse is attributable to its enzymatic activity or to its capacity as a basic protein to combine with or precipitate polyanions unrelated in structure to RNA. The experiments described here demonstrate that when RNAse is denatured by acid and heat under condi-

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tions where enzymatic activity is largely lost without significant alteration of acid-base properties the capacity to inactivate homogenates disappears. These findings provide additional evidence that RNA is, indeed, a component of an aerobic system concerned with the maintenance of ATP levels in liver homogenates.

*Materials and methods.* The biosynthetic experiments involved a preliminary preincubation period where essentially whole homogenates of rat liver (1 part liver, 2 parts phosphate-magnesium-nicotinamide buffer, loose homogenizer, 200 xg supernatant fraction, aeration) were treated (30 min, 37°, agitation) with various amounts of native or denatured RNAse followed by a biosynthetic period where the homogenates were supplemented with MVA-2-$C^{14}$ (0.5 mg DL MVA, 60,000 cpm), aerated, and reincubated (3 hr). All flasks contained supplements of 1 mg ATP, 10 mg DPN, and 100 mg sodium succinate. Following the biosynthetic period the contents of each flask were saponified with alcoholic KOH, extracted with petroleum ether, the extracts dried with sodium sulfate, filtered, evaporated to dryness, taken up in scintillation mixture, and counted. The results given are in terms of total cpm per incubation flask. Further details of the biosynthetic experiments have been published(1-10). RNAse (Schwarz, 5 x crystallized) was denatured by heating 10 ml amounts of a solution (5 mg/ml) at pH 1.0 in a vigorously boiling water bath for the time intervals indicated. Following denaturation the solutions were neutralized and used immediately in the biosynthetic experiments. Determinations of RNAse activity as a measure of the extent of inactivation were carried out by the method of Kunitz(11) within 3-4 hrs of heat treatment.

*Results.* As indicated by Exp. 1, Table I, preincubation of liver homogenate with essentially inactivated RNAse (90 min. 84% inactivation), in contrast to native RNAse, is associated with no reduction in capacity to convert MVA to NSF. Similarly RNAse heated for a shorter time (Exp. 2, 60 min, 72% inactivation) is not deleterious and, in fact, at intermediate levels some stimulation of MVA utilization is evident. With RNAse preparations less extensively inactivated (Exp. 3 and 4, 51 and 38% inactivated according to the Kunitz assay) the phenomenon that began to be apparent with the 72% inactivated preparation *i.e.* stimulation of MVA utilization at intermediate levels is evident. The data from Exp. 3 and 4 (51 and 38% inactivation) are summarized in Fig. 1. Clearly, preincubation of liver homogenates with only partially de-

TABLE I. Biosynthesis of NSF by Liver Homogenate as Influenced by Preincubation with Various Native and Heat-Acid Denatured RNAse Preparations.

| | RNAse preparation | | |
|---|---|---|---|
| Exp. | Level, mg | Treatment | NSF, cpm |
| 1 | 0 | Native RNAse | 4976 |
| | 1 | | 1436 |
| | 2 | | 426 |
| | 5 | | 199 |
| | 10 | | 132 |
| | 20 | | 155 |
| | 1 | 90 min., pH 1.0, 100°, | 5226 |
| | 2 | 84% inactivation | 5180 |
| | 5 | | 5982 |
| | 10 | | 6228 |
| | 20 | | 6630 |
| 2 | 0 | Native RNAse | 5132 |
| | 1 | | 790 |
| | 2 | | 65 |
| | 5 | | 37 |
| | 10 | | 25 |
| | 20 | | 21 |
| | 1 | 60 min., pH 1.0, 100°, | 7409 |
| | 2 | 72% inactivation | 7769 |
| | 5 | | 8166 |
| | 10 | | 7285 |
| | 20 | | 7065 |
| 3 | 0 | Native RNAse | 5743 |
| | 1 | | 1186 |
| | 2 | | 504 |
| | 5 | | 213 |
| | 10 | | 176 |
| | 20 | | 280 |
| | 1 | 30 min., pH 1.0, 100°, | 12002 |
| | 2 | 51% inactivation | 9773 |
| | 5 | | 3793 |
| | 10 | | 1617 |
| | 20 | | 1424 |
| 4 | 0 | Native RNAse | 5557 |
| | 1 | | 424 |
| | 2 | | 124 |
| | 5 | | 77 |
| | 10 | | 32 |
| | 20 | | 96 |
| | 1 | 30 min., pH 1.0, 100°, | 8208 |
| | 2 | 38% inactivation | 4094 |
| | 5 | | 427 |
| | 10 | | 169 |
| | 20 | | 79 |

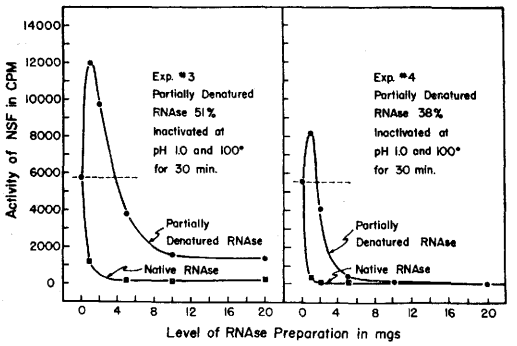


FIG. 1. Biosynthesis of NSF by liver homogenate as influenced by preincubation with native or partially denatured RNAse.

natured RNAse is associated with 2 superimposed effects: (1) stimulation of MVA utilization at low levels and (2) inhibitiion of MVA utilization at higher levels. In all experiments preincubation of liver homogenates with native RNAse was associated with at least 95% reduction in capacity to convert MVA to NSF.

*Discussion.* The data presented demonstrate that the capacity of RNAse to inactivate a system essential for conversion of MVA to NSF is associated with the enzymatic activity of RNAse rather than with its basic properties. Thus it would appear that a substrate for RNAse is involved in the entire liver homogenate system. Previous experiments have provided evidence that the natural factor, at least as far as liver is concerned, is an RNA or ribonucleoprotein and the locus of activity is somewhere in maintenance of ATP levels presumably either as a factor essential in respiration or oxidative phosphorylation or as an inhibitor of ATPase activity.

Person and Fine recently have reported(12) that the activity of isolated preparations of cytochrome oxidase is inhibited by a number of basic componds including RNAse and protamine. This inhibition is reversed by the strongly anionic compound polyglucose sulfate. Person and Fine conclude that basic polymers are direct inhibitors of cytochrome oxidase. If there is any analogy between the experiments of Person and Fine and those involving RNAse and protamine as inactivators of a system concerned with maintenance of ATP levels of liver homogenates, it is suggested that RNAse and protamine may not inhibit cytochrome oxidase directly but do so indirectly by hydrolyzing an associated RNA in the case of RNAse or by combining irreversibly with the RNA in the case of protamine. It should be noted that at least some cytochrome preparations that have been obtained in a state of relative purity are strongly basic and it is reasonable to postulate that they function in association with RNA. Possibly the integrated function of the cytochromes in electron transport is a consequence of a close and orderly arrangement on a polyanionic framework.

Some comment is in order concerning the phenomenon observed with partially denatured RNAse where preincubation of liver homogenates with small amounts of this material is associated with a greater conversion of MVA to NSF, and presumably, a higher level of ATP, than encountered in controls. It is known from published and unpublished experiments in this laboratory that frequently stimulation of MVA conversion to NSF is obtained with anionic polymers in homogenates that have been preincubated without added RNAse or high molecular weight basic compounds. Apparently in the preincubation of liver homogenates various "endogenous" diesterases and other enzymes sometimes reduce the level of RNA to a limiting extent. It is suggested that partially denatured RNAse shows stimulation because part of it has been altered to the extent that it combines with substrate to yield a ribonucleoprotein where RNA moiety is "protected" against other enzymes but is not bound to the extent of interference with function. Obviously at higher levels of the altered material the unaltered or native portion must somehow outstrip that portion affording protection and the resultant effect is hydrolysis of RNA, loss of ATP level, and resulting failure of MVA conversion to NSF. Other interpretations of the data may be formulated.

*Summary.* Preincubation of liver homogenates with RNAse preparations nearly completely denaturated with respect to enzymatic activity by heat and acid is associated with no reduction in capacity to convert mevalonic acid to non-saponifiable material. Mevalonic acid utilization is completely eliminated by

preincubation with native RNAse. The preincubation of liver homogenates with less completely inactivated RNAse involves more complicated phenomena. The results are interpreted to mean that the inactivation of MVA utilization observed with native RNAse is due to enzymatic activity of RNAse rather than to the basic properties of the protein that are not significantly altered on mild denaturation. Additional evidence is thus provided for the involvement of RNA (or a ribonucleoprotein) in the complete system essential for conversion of mevalonic acid to nonsaponifiable material by liver homogenates. Evidence has been presented previously that the RNA is essential for maintenance of ATP levels required in a number of steps involved in utilization of mevalonic acid.

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## Production of Circulating Antibody against Defined Antigen (BGG) by Neonatal Guinea Pigs. (26225)

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The immunologic capacity of young guinea pigs is generally acknowledged as being considerably less than that of adults(1-5). Investigations leading to this conclusion employed chemically undefined antigens, such as sheep serum(1), diphtheria toxin(2), tubercle bacillus(3), typhus vaccine(4), and thyroid tissue(5), and the demonstrations principally concerned sensitization rather than antibody detection. On the other hand, studies of acquired tolerance, naturally induced *in utero,* toward postnatally implanted maternal skin homografts(6,7), and demonstration of plasma cells in lymph nodes of newborn guinea pigs(8) constitute indirect evidence that guinea pigs may already possess the capacity to form antibodies in neonatal stages.

The present study was undertaken to ascertain whether guinea pigs can form humoral antibody against defined antigen, bovine gamma globulin (BGG), immediately after birth.

*Methods.* Guinea pigs of outbred stock were inoculated 6 to 30 hours after delivery. Sensitization was accomplished by single intramuscular injections of BGG* (25 mg/100 g body weight) combined with Freund adjuvant.† Animals were bled by intracardiac puncture or challenged for anaphylaxis by intracardiac or intravenous injection. Sera were stored at −19°C.

The passive cutaneous anaphylactic (PCA) procedure, as developed by Ovary (10), was employed for detection of circulating antibody; the method is capable of demonstrating precipitable and nonprecipitable antibody and is sensitive down to the order of 0.003 μg antibody nitrogen(9,10). Intracutaneous injection of 0.1 ml undiluted antiserum was followed, 5 to 6 hours later, by

* Bovine gamma globulin, fraction II, from Nutritional Biochemicals Corp., Cleveland, O.

† Freund adjuvant, complete, from Difco Laboratories, Detroit, Mich.