serum cannot be regarded as quantitative because the conditions used to hydrolyze taurolithocholic acid lead to partial destruction or alteration of the lithocholic acid molecule. Evidence of this was obtained by hydrolyzing taurolithocholic acid-24-C^{14} under the same conditions and noting the appearance of several widely distributed radioactive peaks under Celite-column chromatography. Use of milder conditions normally sufficient to hydrolyze conjugates of dihydroxy and trihydroxy bile acids or use of 50 percent aqueous ethanol resulted in incomplete hydrolysis of taurolithocholic acid.

It is apparent from this study, however, that enough lithocholic acid survived hydrolysis to be detected by gas chromatography, as was demonstrated by the appearance of both the methyl ester and trifluoroacetate derivatives of lithocholic acid in the serum extracts. Using a technique employing extraction by anion-exchange resin, Sandberg *et al.* (*10*) observed a peak, believed to be lithocholic acid, in their gas-chromatographic analysis of serum bile acids from a patient with hepatitis. Sufficient data to permit direct comparison with our studies were not given; their study was primarily concerned with other bile acids in serum.

The finding of lithocholic acid in the serums of patients with liver disease raises important questions regarding the possible role of this compound in injury to the human liver. Relatively little is known, for example, about the amount of lithocholic acid absorbed from the colon; quantitative studies are needed to determine whether amounts absorbed are comparable with those required in animal-feeding experiments to produce injury to the liver. Lithocholic acid is believed to be poorly absorbed from the colon because very little or none is found in human bile (*11*) and it remains in the sediment of fecal extracts (*6*). Its presence in serum, however, raises the possibility that more lithocholic acid is absorbed from the colon than is generally recognized.

Another question concerns the toxicity of lithocholic acid to the human liver. Lithocholic acid has produced proliferation of hepatic ductular cells and other changes in the liver in birds (chicken, *12*), reptiles (iguana, *13*), and mammals (rats, *14*; rabbits, *3*; mice, *14*; and monkeys, *15*). It is likely that man also is subject to its cirrhotogenic effects, and perhaps he is more sensitive than the rat (*14*), since the human liver does not readily hydroxylate lithocholic acid to other compounds (*16*), as do the livers of Muridae (*17*).

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Template Activity of RNA from Antibody-Producing Tissues

Abstract. *An RNA fraction, which represents a small percentage of cellular RNA and which has the characteristics of nuclear messenger RNA, has been isolated from the spleen and lymph nodes of immunized rats by successive phenol extractions of these tissues at increasing temperatures. This fraction increased the amount of protein synthesized in a cell-free extract of* Escherichia coli *as much as 35 times and directed the synthesis of proteins different from those of* E. coli.

According to the "instructive" theories of antibody formation, the specificity of antibodies results from the presence, at the site of γ-globulin synthesis, of the antigen itself, which somehow determines the tertiary (*1*) or even the primary (*2*) structure characteristic of a specific antibody; the role of the genetic information is, in this model, limited to the formation of unspecific γ-globulin molecules. In contrast, the "selective" theories postulate that the specificity of all possible antibody molecules is determined by the genome itself (*3*); each of a multitude of different genes would be potentially capable of coding for that small portion of the γ-globulin molecule of which the unique amino acid sequence accounts for the specificity of a given antibody.

Since genetic information is expressed in the form of mRNA (*4*), the contribution of the genome in the control of antibody specificity could be assessed by determining whether or not mRNA of antibody-producing cells is capable, in the absence of antigen, of directing the synthesis of specific antibody molecules. We report now the first step in such a study, the isolation from antibody-synthesizing tissues of an RNA fraction with the characteristics of mRNA and, particularly, with a high template activity, defined as the ability to stimulate the synthesis of proteins.

RNA was extracted from spleen and lymph nodes of rats killed at various times after the injection, into the peritoneal cavity and the four footpads, of a mixture of *Haemophilus pertussis* and sheep red blood cells. Whole organs or subcellular fractions were homogenized in a mixture of equal volumes of buffer (0.1*M* tris, *p*H 5, with 0.5 percent naphthalene disulfonate) and 88 percent phenol; RNA was purified by repeated extractions with phenol and precipitations with ethanol (*5*). The capacity to stimulate the incorporation of amino acids into protein in a cell-free system derived from *Escherichia coli* was taken as a measure of the template activity of the RNA preparations. RNA obtained from animals which were in the primary or in the secondary phase of the immune response gave similar results and therefore will not be discussed separately.

RNA extracted from whole organs

by phenol treatment at 20°C had only negligible template activity, even at high concentration. When phenol extraction was performed at high temperature (65°C for 6 minutes followed by rapid cooling) and in the presence of detergent (SDS, 0.5 percent), the RNA obtained significantly stimulated protein synthesis (Fig. 1, "Total"). RNA extracted under similar conditions from spleen microsomes was practically devoid of template activity; nuclear RNA was somewhat active, although less so than RNA extracted from whole organs (Fig. 1). This finding could be attributed to some degree of degradation having taken place during cell fractionation, when RNA was not yet protected from the effects of nucleases by phenol and detergents.

Since RNA extracted from whole cells with both hot phenol and SDS stimulated the synthesis of protein significantly, an attempt was made to purify template RNA by fractionation of this RNA preparation on sucrose gradient (*5*). Protein synthesis was stimulated by RNA fractions that have a wide range of molecular weight; the 6 to 12*S* fraction had the highest activity per milligram of RNA (*5*). The activity of this fraction, however, was not significantly higher than the activity of the total cellular RNA; furthermore, the 6 to 12*S* RNA fraction can only be obtained in very small amounts.

Since heat and detergents are essential for the extraction of RNA with template activity, the active RNA fraction might remain in the interphase when extraction is performed at only 20°C without detergents; subsequent extraction of the interphase with hot phenol and detergents might thus result in some purification of template RNA. An analogous situation exists in the case of the rapidly synthesized RNA [which consists largely of precursors of ribosomal RNA (*6*) and of some mRNA (*7*)], since heat (*8*) or detergents (*9*) are required for the extraction of all the newly made RNA. Georgiev *et al.* (*8*) have shown that the newly synthesized RNA which remains at the interphase can be separated into two fractions: one, which is released by phenol extraction at 45° to 50°C, is of ribosomal type and the other, extractable only at 55°C and above, has a base composition close to that of DNA. Such a fractionation by successive phenol extractions at increasing temperatures has been used in our study to purify template RNA from spleen and lymph node tissues.

Table 1. Characteristics of RNA from fractions I, II, and III. The amount of RNA in each fraction was measured by absorption at 260 mμ (30 OD units = 1 mg). Radioactive labeling of RNA was determined on specimens obtained 20 minutes and 24 hours after the intravenous injection of respectively 4 and 0.5 mc of P^{32}. The relative specific radioactivities of the three fractions are expressed as a function of the values found for fraction I, arbitrarily taken as 1. Radioactivity of fraction I was 4.5 to 5 × 10^3 count min^{-1} mg^{-1} after 20 minutes of labeling and 1 × 10^6 count min^{-1} mg^{-1} after 24 hours.

Fraction	Fraction of total RNA (%)	Relative specific activity after:	
		20 min	24 hr
I	70 to 80	1	1
II	10 to 20	2–3	0.9
III	5 to 10	6–8	.8

Tissues were first homogenized at room temperature and without detergents; after centrifugation and removal of the aqueous phase, the phenol and interphase were reextracted once with buffer. The pooled aqueous phases represented fraction I. The interphase was then homogenized with fresh phenol and buffer containing 0.5 percent SDS, shaken at 45°C for 6 minutes and cooled rapidly. After separation of the aqueous phase, phenol and interphase were again extracted once at 20°C with fresh buffer; the pooled aqueous phases represented fraction II. The remaining interphase was mixed with fresh phenol and buffer containing SDS, shaken at 65°C for 6 minutes, and cooled rapidly; a second extraction was performed at 20°C as outlined for fractions I and II, and the pooled aqueous phases represented fraction III. All fractions were extracted twice more at 20°C with one-half volume of phenol and then precipitated four times in ethanol (*5*).

Extraction of tissue homogenates at 65°C in the presence of detergents yields all of the cellular RNA, uncontaminated by DNA (*10*). RNA fractions thus purified contained less than 1 percent of protein as estimated by the method of Lowry (*11*); when spleen proteins were labeled in vivo with C^{14}-valine, the radioactivity detected in the RNA fractions indicated that protein contamination represented less than 0.01 percent of the spleen proteins.

The relative yields of the three fractions and their specific radioactivities after short and long periods of labeling with P^{32} in vivo are shown in Table 1. Identical results were obtained with RNA from either spleen or lymph nodes. The observed decrease in relative specific activity of fraction III after 24 hours of labeling indicates that this fraction has a faster turnover than the two others.

These three fractions, and the rapidly labeled RNA which they contain, were further characterized by sedimentation on sucrose gradient (Fig. 2) and by analysis of their nucleotide composition (Table 2). Fraction I contains the RNA from the cytoplasm; and the sedimentation profile of rapidly labeled RNA, as well as its nucleotide composition, is in agreement with the results obtained for cytoplasmic RNA (*12*). Fraction II consists mainly of ribosomal RNA with a predominance of the 30*S* component (Fig. 2). The sedimentation profile of rapidly synthesized RNA of fraction II

Table 2. Nucleotide composition (percent) of RNA's from fractions I, II, and III. RNA was hydrolyzed in 0.3*N* KOH for 18 hours at 37°C and neutralized with perchloric acid; the nucleotides were separated by ion-exchange column chromatography (*23*). Uridylic acid was separated from possible contaminating inorganic P^{32} by adsorption on charcoal. Base composition of newly made RNA was determined in measuring the radioactivity of each nucleotide in RNA preparations obtained from rats killed 20 minutes after intravenous injection of P^{32} (4 mc). The values indicated represent the mean of several determinations. The base composition of the two components of spleen ribosomal RNA (*5*) and of rat DNA (electrophoretic separation) (*24*) are indicated for comparison.

RNA fraction	Base composition				GC/AU
	U(T)	G	C	A	
Fraction I, newly made	26.2	30.0	25.0	18.8	1.22
Fraction II, newly made	22.8	32.5	28.2	16.4	1.55
Fraction III, newly made	27.2	27.5	24.3	20.7	1.08
Fraction III, whole RNA	24.2	28.0	24.7	23.3	1.1
Ribosomal, 30*S* whole RNA	18.0	34.5	28.7	18.7	1.72
Ribosomal, 18*S* whole RNA	19.9	32.8	25.4	21.8	1.4
Rat DNA (electroph. sepn.)	28.4	21.4	21.5	28.6	0.755

shows a peak of heavy ribosomal RNA, as well as heavier components which are believed to be precursors of ribosomal RNA (*5*); the nucleotide composition of fraction II also indicates that most of the newly made RNA of this fraction is ribosomal. If ribosomal RNA is indeed synthesized in nucleoli (*13*), fraction II probably represents, at least in part, nucleolar RNA. RNA from fraction III sediments in the 10 to 40*S* region of the sucrose gradient, and its profile shows two ill-defined peaks, of varying magnitude depending on the preparation; newly made RNA of fraction III is also scattered from 10 to 40*S*. Analysis of the nucleotide composition of all the RNA of fraction III and of the newly made component reveals a low ratio of guanine plus cytosine to adenine plus uracil, compatible with a mixture consisting of about ⅔ DNA-like RNA and ⅓ RNA of ribosomal type.

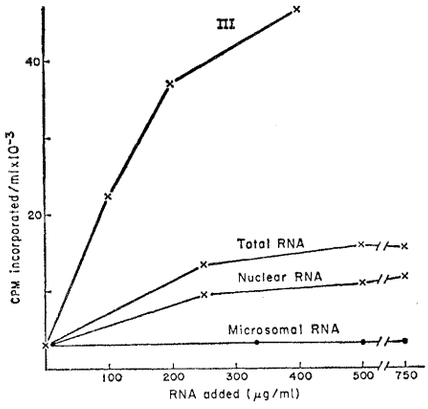

Fig. 1. Stimulation of amino acid incorporation into protein by various preparations of RNA. Cell-free extracts (*S*-30) of *E. coli B* were prepared and "preincubated" (to eliminate the activity of *E. coli* mRNA) according to the procedure of Matthaei and Nirenberg (*21*), with glutathione (0.01*M*) instead of β-mercaptoethanol in the preincubation mixture. Incubation with and without added RNA was performed at 35°C in 0.5 ml of mixture containing: *S*-30 (3 mg of protein), 3 m*M* ATP, 0.2 m*M* GTP, 10 m*M* phosphoenol-pyruvate, 30 μg/ml of pyruvate kinase, 0.012*M* KCl, 0.05*M* NH_4Cl, 0.012*M* $MgCl_2$, 0.1*M* tris, *p*H 7.5, 0.04 m*M* each of 20 amino acids not including serine and valine; 0.4 μc/ml of C^{14}-serine (120 μc/μmole) and C^{14}-valine (150 μc/μmole). After 60 minutes of incubation the proteins were precipitated in 0.5*N* perchloric acid and washed (*22*); radioactivity was measured in a Tricarb liquid scintillation counter. Duplicate determinations were performed and control values in nonincubated preparations were substracted. The figure shows counts per minute of C^{14}-labeled amino acids incorporated in the absence of RNA and in the presence of different RNA preparations added at various concentrations.

These results indicate that successive extractions at increasing temperatures separate RNA from spleen and lymph nodes into three distinct fractions: fraction I, extracted at 20°C without detergent, represents cytoplasmic RNA; fraction II, extracted at 45°C in the presence of SDS, consists mainly of nuclear ribosomal RNA, probably being of nucleolar origin; fraction III, extracted only at 65°C, is rich in RNA having a rapid rate of synthesis, a relatively fast turnover, and a base composition close to that of DNA. The RNA of fraction III might therefore represent chromosome-bound newly made mRNA.

The RNA's from these three fractions were tested for their ability to stimulate the incorporation of amino acids into proteins. Fraction I was inactive, even at high concentrations, fraction II had some template activity, and fraction III was highly active (Fig. 3). The activity of this last fraction was identical when RNA was extracted from either spleen or lymph nodes, and it was much greater than that of all other RNA preparations prepared from these organs (Fig. 1).

Isolated nuclei were also extracted with phenol at increasing temperature. RNA extracted at 65°C had again the best template activity, but the stimulation was of somewhat lower magnitude, possibly as the result of some degradation of RNA during the isolation of nuclei.

Stimulation of protein synthesis by RNA of fraction III had the following characteristics: (i) Incorporation of amino acids was proportional to the concentration of RNA added to the incubation mixture up to 400 to 500 μg/ml, at which point it reached a plateau. (ii) RNA-induced protein synthesis increased linearly with time for about 30 minutes and reached a maximum after 45 minutes of incubation. (iii) Maximum incorporation varied somewhat depending on the preparation of fraction III, and in the best cases amounted to 100 pmole of amino acid per milliliter, which corresponded to a 35-fold enhancement of the incorporation of the unstimulated *E. coli* system. This stimulation of protein synthesis is of higher magnitude than that obtained with other RNA fractions of mammalian origin. (iv) The stimulatory activity of fraction III RNA was totally abolished by chloramphenicol, puromycin, and ribonuclease. Furthermore, the activity of fraction III was unaffected by deoxyribonuclease; this eliminated the possibility that this activity resulted from contamination by a small amount of denatured DNA, which under certain conditions stimulates cell-free protein synthesis (*14*). The possibility also existed that the activity of fraction III merely resulted from partial denaturation of RNA by heat, since there seems to exist an inverse correlation between the extent of internal hydrogen bonding of various RNA species and their template activity;

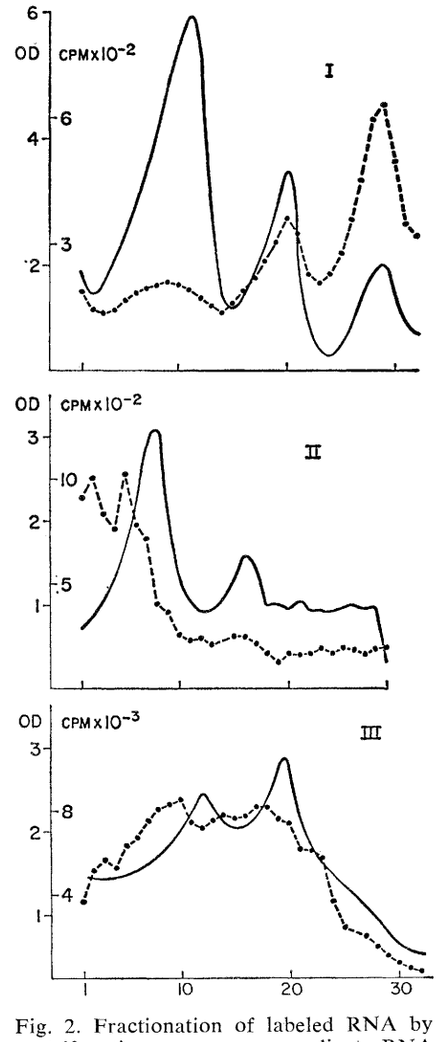

Fig. 2. Fractionation of labeled RNA by centrifugation on sucrose gradient. RNA fractions obtained after 20 minutes of labeling in vivo (4 mc P^{32}) were centrifuged (SW 25 Spinco rotor) at 25,000 rev/min for 11 hours at 15°C on sucrose gradients (5 to 20 percent) prepared in 0.05*M* tris buffer (*p*H 7) containing 0.1*M* NaCl and SDS (0.5 percent). After collection of 1-ml fractions, $O.D._{260\ m\mu}$ (solid line) and radioactivity (dotted line) were measured.

however, fraction I RNA isolated as described and then treated as fraction III RNA (65°C for 6 minutes, 0.5 percent SDS) was no more active than unheated fraction I. Thus it appears that the high template activity of fraction III can indeed be attributed to nuclear mRNA.

RNA from fraction III was fractionated further by sucrose-gradient centrifugation into a light (10 to 23*S*) and a heavy (24 to 40*S*) portion; each was tested individually for template activity; these portions were equally active, an indication that fraction III includes template RNA of a wide range of molecular weight. There is an apparent contradiction between the relatively larger molecular weight of this RNA and the lower molecular weight of an RNA fraction observed on spleen microsomes, which also has the characteristics of mRNA (*5*). This difference is compatible either with a breakdown of mRNA molecules occurring during the isolation of microsomes, or with a physiological splitting that might occur during the transfer of mRNA from its nuclear site of synthesis to its microsomal site of function.

The function of an RNA molecule as a genetic messenger implies the specificity of the proteins synthesized under its direction. To determine if template RNA from spleen and lymph nodes indeed directs the synthesis of new proteins, different from those of *E. coli*, the relative rate of incorporation of various amino acids was measured in the *E. coli* system in the presence of RNA of fraction III. The RNA from f_2 bacteriophage, which induces in this system the specific synthesis of the coat protein of the phage (*15*), was used as a control. Serine, threonine, and proline are found in *E. coli* proteins in the ratio indicated in Table 3. The RNA from f_2 phage directed the incorporation of serine, threonine, and proline in the same relative amounts in which they are found in the coat protein. The RNA from fraction III, whether from spleen or lymph nodes, directed the incorporation of these same amino acids in a ratio entirely different from that in which they are found in *E. coli* proteins (Table 3). Since the experiment with RNA from f_2 bacteriophage has shown that in this system the incorporation of each of these three amino acids takes place exclusively according to the coding specificity of the mRNA added, and is not limited by other factors, it can be concluded that RNA from fraction III is indeed directing the synthesis in vitro of proteins different from those of *E. coli*. The relative amounts of these three amino acids in γ-globulins are indicated in Table 3 solely for the purpose of comparison, and these values cannot as such be taken as evidence for the synthesis of γ-globulins in this system. Techniques of immunological precipitations have been used to ascertain whether the synthesis of some fragments of γ-globulins does indeed occur under those conditions; the results, however, have been so far inconclusive, mainly because of an important unspecific binding of radioactive products to the control precipitates.

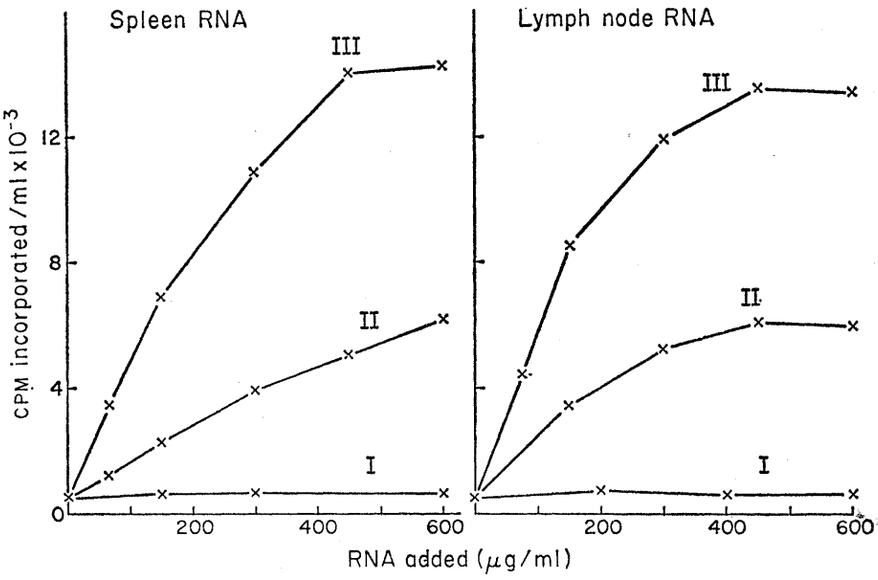

Fig. 3. Effect of RNA from fractions I, II, and III on amino acid incorporation into protein. Conditions were as described for Fig. 1.

The transfer of immunological information by RNA-rich preparations has recently been reported (*16–18*). Whether the synthesis of specific antibodies observed in these experiments had been directed by any mRNA present in the RNA preparations is, however, doubtful. In certain of the experiments reported, the RNA was highly degraded, as judged from its sedimentation profile (*16*), and it is now apparent that the preparations were contaminated with antigen in amounts sufficient to account for the observed induction of antibody synthesis (*19*). In another type of experiment, the best results (*18*) were obtained with RNA preparations which have no or negligible template activity and which are strongly contaminated with proteins (*20*), thus possibly carrying antigenic determinants.

An evaluation of the role of mRNA,

Table 3. Effect of RNA from spleen, lymph nodes, and f_2 bacteriophage on the relative incorporation of serine, threonine, and proline into protein. The components were incubated as for Fig. 1, except that the radioactive amino acid was either serine (120 μc/μmole), threonine (160 μc/μmole), or proline (186 μc/μmole). The molar amounts of each amino acid incorporated are expressed as a function of the value obtained for serine, arbitrarily taken as 1. The RNA from f_2 bacteriophage (*25*) (150 μg/ml) and fraction III RNA from spleen or lymph node (300 μg/ml) were added as indicated. The relative amount of amino acids naturally occurring in *E. coli* proteins (*26*), γ-globulin (*27*), and f_2 bacteriophage coat protein (*28*) are given for comparison (numbers shown in parentheses).

Amino acid	Relative amounts of amino acids					
	In *E. coli*	Incorp. in presence of Fr. III RNA		γ-Globulin	Incorp. in presence of f_2 RNA	In f_2
		Spleen	Lymph node			
Serine	(1)	1	1	(1)	1	1
Threonine	(1.36)	0.58	0.65	(0.71)	0.69	0.68
Proline	(1.07)	.63	.73	(.68)	.48	.49

and therefore of the genome, in the control of antibody specificity, depends on the availability of RNA with high template activity, devoid of significant protein contamination and obtained in an undegraded form. The characteristics of RNA from fraction III seem to fulfill these requirements.

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13 August 1965

Collagenase: Effect on the Morphogenesis of Embryonic Salivary Epithelium in vitro

Abstract. *Salivary epithelium in culture, under conditions which promote morphogenetic branching, grows as a simple disc in the presence of collagenase, or is "depatterned" midway in the morphogenetic course by a short exposure to a collagenase.*

The characteristic morphogenesis of salivary epithelium of mouse embryos in vitro is dependent upon closely associated salivary mesenchyme, either in direct combination or across a membrane filter (*1*). In the trans-filter situation collagen fibers appear at the epithelial surface, and evidence has been presented which is in conformity with the hypothesis that the collagen is synthesized by the mesenchyme and polymerized on the epithelial side after crossing the filter in soluble form (*2*). The question arises whether collagen at epithelial surfaces has morphogenetic significance. The question is fortified by the observation that collagen fibers of characteristic periodicity are particularly abundant in association with the surface of the morphogenetically inactive stalk of the 13-day rudiment; they are much less abundant in the bulb region and seem to be absent at the ends of morphogenetically active adenomeres (*3*). Further, autoradiographically demonstrable label, introduced into the mesenchyme as tritiated proline and conforming in distribution to ultrastructurally identifiable collagen, is removed by collagenase (*2*). Experiments were performed, therefore, to test whether collagenase treatment would affect the morphogenetic pattern of the epithelium.

Two kinds of experiments were carried out: (i) continuous treatment by inclusion of collagenase in the medium; (ii) treatment for a short period during morphogenesis, followed by subsequent culturing in the absence of collagenase. Both procedures gave results suggesting that a collagenase-sensitive material, presumably collagen, plays a role in salivary morphogenesis.

The procedure for obtaining salivary rudiments from 13-day mouse embryos, and for separating their epithelial and mesenchymal components by means of trypsin, has been described (*4*). The epithelia were clotted in the cup of a membrane filter assembly (*5*), and a piece of salivary capsular mesenchyme was placed immediately opposite on the upper or platform side of the filter. The whole assembly was in contact with a subjacent drop of nutrient medium (Eagle's plus 10 percent horse serum and 10 percent chick embryo juice) in the well of a culture dish (*5*). The cultures were incubated at 38°C in a 5 percent CO_2-gassed, high-humidity incubator.

Three collagenase preparations were used: (i) Worthington crude ("CLS"); (ii) Worthington crystalline A (CLSP-A, monomer); (iii) Worthington crystalline B (CLSP-B, dimer). In the continuous exposure experiments crude collagenase was incorporated into the medium of standard cultures, including mesenchyme, at concentrations ranging from 0.006 to 0.4 mg/ml. The highest concentration proved toxic as judged by darkening and fragmentation of the epithelium; the lowest was weakly active in affecting morphogenesis. Intermediate concentrations in the range of 0.025 to 0.1 mg/ml were most effective. Crys-

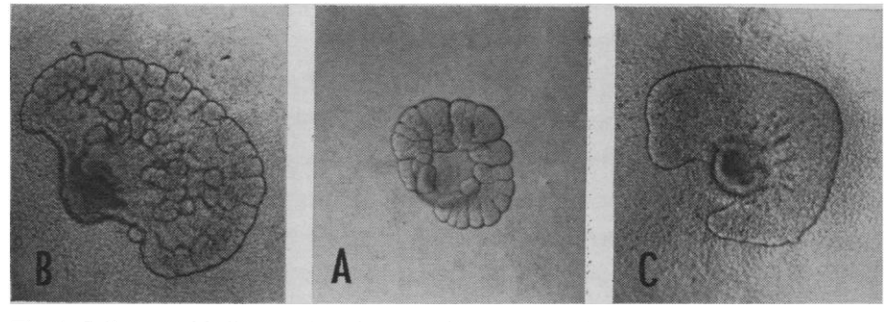

Fig. 1. Salivary epithelium undergoing morphogenesis in vitro. (*A*, center) At 48 hours, untreated; (*B*) at 72 hours, treated with Tyrode's solution at 48 hours; (*C*) at 72 hours, treated with collagenase at 48 hours.