*BIOSYNTHESIS OF RNA IN ANTIBODY-PRODUCING TISSUES**

By Bernard Mach and Pierre Vassalli

LABORATORY OF BIOCHEMICAL GENETICS, THE ROCKEFELLER UNIVERSITY, AND DEPARTMENT OF PATHOLOGY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE, NEW YORK

Communicated by E. L. Tatum, July 20, 1965

The formation of antibodies is, in several respects, a unique type of protein synthesis: it is an induced process which requires, to achieve the synthesis of highly specific proteins, the production of a large number of new, specialized cells. Thus, an antigenic stimulation results in rapid cellular proliferation and differentiation in spleen and lymph nodes, and induces the synthesis by plasma cells of specific antibody molecules, whose unique structure accounts for their selective affinity for the inducing antigen.

These features, and in particular the problem of the control of antibody specificity, confer a special interest on the role of RNA biosynthesis and function in antibody-producing tissues. The present report concerns the biosynthesis of RNA in spleen and lymph nodes of rats immunized with *H. pertussis*, an antigen which produces in this species an unusually strong immune response. The isolation of a messenger RNA fraction and the study of its template activity have been reported elsewhere.[1]

Materials and Methods.—Sprague-Dawley rats of 200–300 gm were immunized by the injection of a mixture of killed *H. pertussis* (Eli Lilly Co.) and sheep red blood cells in the peritoneal cavity (i.p.) (4×10^9 *H. pertussis*, and 0.2 ml packed RBC) and in each footpad (1.5×10^9 *H. pertussis*, and 0.025 ml packed RBC); secondary immune response was initiated by injection of antigen at one fifth the initial dose.

H^3-uridine, H^3-uracil, H^3-D-L-leucine, C^{14}-L-serine, and C^{14}-L-valine were purchased from New England Nuclear Corp.; H^3-thymidine was obtained from Schwarz Biochem, and carrier-free P^{32} from Oak Ridge National Laboratory. Polyvinylsulfate and 1,5-naphthalenedisulfonate were purchased from Eastman Organic Chemicals.

Studies on in vivo synthesis of DNA, RNA, and proteins: Rats were killed either 8 hr following the i.p. injection of H^3-thymidine (25 μc; 100 μc/μmole), or 2 hr following the i.p. injection of H^3-uracil (50 μc; 25 μc/μmole) and H^3-D-L-leucine (30 μc; 20 μc/μmole). Spleen and lymph nodes (axillary and popliteal) were removed, weighed, and quickly frozen. Tissues were homogenized in 0.1 *M* Tris buffer pH 7 and precipitated in 0.25 *N* perchloric acid. The incorporation of radioactive precursors into DNA, RNA, and proteins was determined as described elsewhere.[2]

Extraction of RNA: · Frozen tissues were homogenized at 4°C in a mixture of equal volumes of buffer (0.1 *M* Tris pH 5, 0.5% sodium dodecyl sulfate, and 0.5% naphthalenedisulfonate) and fresh phenol (Mallinckrodt, A. R., 88%, without preservative). The mixture was shaken for 6 min at 65°C and rapidly chilled. Following centrifugation and removal of the aqueous phase, phenol and interphase were re-extracted at 20°C with fresh buffer, and the pooled aqueous phases were treated twice more with half a volume of phenol at 20°C. RNA was precipitated once for 2 hr at 0°C in 0.1 *M* NaCl with 2.5 vol of chilled ethanol, and three times in 2 *M* K acetate with one third volume chilled ethanol for at least 2 hr at −20°C. Repeated precipitations under these conditions have been shown to free RNA from contamination by inorganic P^{32} (ref. 3), and oligodeoxyribonucleotides.[4] The RNA pellet was dissolved in distilled water and stored at −20°C. When it was necessary to remove sRNA, additional precipitations in 2.5 *M* NaCl and in 0.2 *M* $MgCl_2$[5] were performed. For studies of labeled RNA, rats were killed at various times following intravenous injection of P^{32} (3–5 mc) or H^3-uridine (200 μc, 10 μc/μmole).

Fractionation of RNA by sucrose gradient centrifugation: RNA preparations were centrifuged in a SW25 Spinco rotor at 25,000 rpm for 11 hr (except when otherwise indicated) at 15°C on sucrose gradients (5–20%) prepared in 0.005 *M* tris buffer (pH 7) containing 0.1 *M* NaCl and 0.5%

sodium dodecyl sulfate. After collection of 1-ml fractions and measurement of OD at 260 mμ, 1.5 mg of yeast RNA was added to each tube and, following precipitation in the cold in 0.25 *N* perchloric acid, the acid-precipitable radioactivity was measured in a Tricarb liquid scintillation counter.

Determination of nucleotide composition: After hydrolysis in 0.3 *N* KOH for 18 hr at 37°C, nucleotide composition was determined as described elsewhere,[1] using the ion-exchange chromatography procedure of Katz and Comb.[6]

Preparation of cytoplasmic and microsomal RNA: Spleens were homogenized in buffer (0.05 *M* Tris, pH 7.4, 0.25 *M* sucrose, 0.004 *M* $MgCl_2$, 0.025 *M* KCl, and 3 μg polyvinylsulfate/ml) with five strokes in a teflon-glass Potter homogenizer kept in ice. Cytoplasmic RNA was extracted from the supernatant obtained after a centrifugation at 1000 *g* for 10 min. Microsomes were isolated by centrifugation of the homogenate at 3000 *g* for 10 min, followed by a centrifugation of the supernatant for 120 min at 103,000 *g*. Microsomal RNA was extracted from the pellet. Cytoplasmic and microsomal RNA were extracted as described above, except that the first phenol extraction was performed at 20°C rather than at 65°C.

Determination of template activity: Sucrose gradients were divided into six fractions according to the sedimentation profile of RNA; the fractions from six gradients were pooled and RNA of each fraction precipitated with ethanol. The ability of RNA from these fractions to stimulate the incorporation of amino acids into proteins was measured in an *E. coli* cell-free system, as described elsewhere.[1]

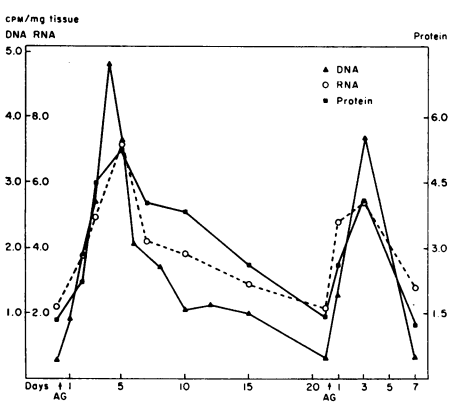

FIG. 1.—Rate of synthesis of DNA, RNA, and proteins in lymph nodes during the primary and secondary immune response. Each point represents the average of the results obtained from 2–6 animals.

Results.—Rate of synthesis of DNA, RNA, and proteins in the immune response: Figure 1 shows that, following a first or a second injection of antigen (Ag), the rate of synthesis of DNA, RNA, and proteins expressed per mg of lymph node tissue increased sharply and then declined gradually, faster, however, in the secondary response than in the primary; the secondary response was accompanied by a strikingly fast increase in the weight of lymph nodes (up to 2 times in the first 24 hr), so that the total synthesis per whole organ was considerably enhanced. The results observed in the spleen were somewhat comparable, but erratic values were occasionally observed, probably reflecting individual variations in the immune conditions of the animals prior to the antigenic challenge.

On the basis of these results, the 9th or 10th day of the primary response and the 2nd or 3rd day of the secondary response were chosen for further studies. In the first case, massive cellular proliferation has already subsided, and the RNA synthesized by differentiated cells might be more selectively related to antibody formation; in the second case, on the contrary, cellular proliferation is at its maximum.

Rapidly synthesized RNA of spleen and lymph nodes: All studies were performed on both spleen and lymph nodes; since the results were comparable, they will not be presented independently. Sucrose gradient centrifugation showed that RNA extracted from these tissues sediments like rat liver RNA, with characteristic 30*S*, 18*S*, and 4*S* peaks. The sedimentation profile of rapidly labeled RNA of nonimmunized animals was widely scattered throughout the gradient, with a predominance of low

mol wt components (Fig. 2*a*). In contrast, the sedimentation profile of newly made RNA obtained from immunized animals after 20 min of labeling already showed some correlation with the peaks of total cellular RNA; in addition, an important amount of radioactive RNA with sedimentation coefficient higher than 30*S* was detected (Fig. 2*b*, *c*). Rapidly synthesized RNA sedimenting faster than the 4*S* peak (Table 1) had a GC/AU ratio of 1.34, compatible with a mixture of 65–70 per cent of rRNA and 30–35 per cent of RNA with a DNA-like GC/AU ratio.

The base composition of rapidly synthesized RNA as well as its sedimentation profile suggested that, during the immune response, the synthesis of rRNA occurs at an unusually rapid rate. Since rRNA seems to be formed from high mol wt precursors,[7, 8] it appeared that the heavier fraction of newly made RNA observed after 20 min of labeling might represent rRNA precursors. Shorter centrifugation times, and a shorter time of radioactive labeling, were used to investigate this possibility (Fig. 3). After only 10 min of labeling, an important fraction of newly made RNA sedimented considerably faster than 30*S* rRNA. Ten min later, there were two distinct peaks of heavy, newly synthesized RNA; as was seen in Figures 2*b* and *c*, the fraction of highest radioactivity showed a peak around 35–40*S* and overlapped with 30*S* rRNA. These profiles closely resemble those of rRNA precursors described in mammalian cells in culture.[7, 8] The base composition of rapidly labeled RNA of high mol wt (30*S* and above) confirmed that this RNA is of the ribosomal type (Table 1).

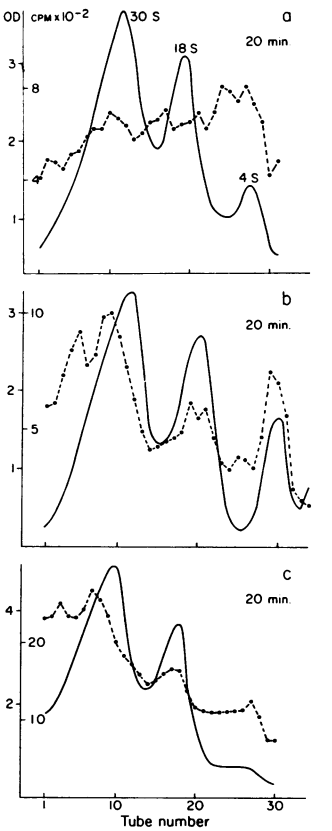

FIG. 2.—Sucrose density gradient analysis of RNA from whole cells after 20 min of radioactive labeling. RNA from nonimmunized (*a*) and immunized (*b* and *c*) animals; in (*c*), sRNA has been removed.

TABLE 1

NUCLEOTIDE COMPOSITION OF RAPIDLY SYNTHESIZED RNA FROM WHOLE CELLS AND MICROSOMES

	U(T)	G	C	A	GC/AU
RNA from whole cells, 20 min P^{32}					
More than 10*S* (spleen)	25.1	32.7	24.4	17.7	1.34
" " " (lymph nodes)	25.0	32.1	25.2	17.7	1.34
More than 30*S* (lymph nodes)	20.9	35.4	29.2	14.5	1.82
RNA from spleen microsomes, 50 min P^{32}					
2–10*S*, minus sRNA	25.2	28.2	21.8	24.6	1.00
18*S* peak	20.6	32.1	26.6	20.6	1.42
30*S* peak	18.7	35.3	29.5	16.5	1.84
rRNA from spleen microsomes, OD					
18*S* peak	19.9	32.8	25.4	21.8	1.40
30*S* peak	18.0	34.5	28.7	18.7	1.72
Rat DNA* (electroph. separation)	28.4	21.4	11.4	28.6	0.755

* Chargaff, E., in *The Nucleic Acids*, ed. E. Chargaff and J. H. Davidson (New York: Academic Press, 1955), vol. 1, p. 356.

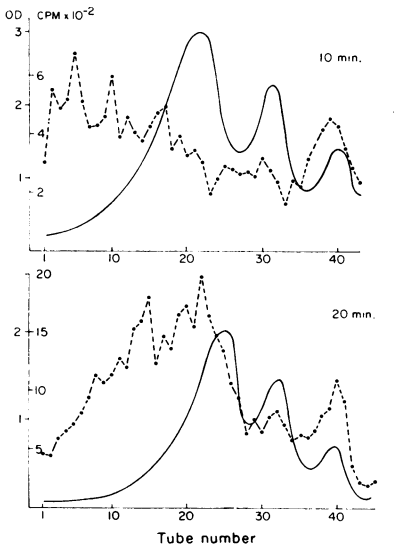

FIG. 3.—Sucrose density gradient analysis of RNA from whole cells after 10 and 20 min of radioactive labeling. Gradients were centrifuged for 8 hr.

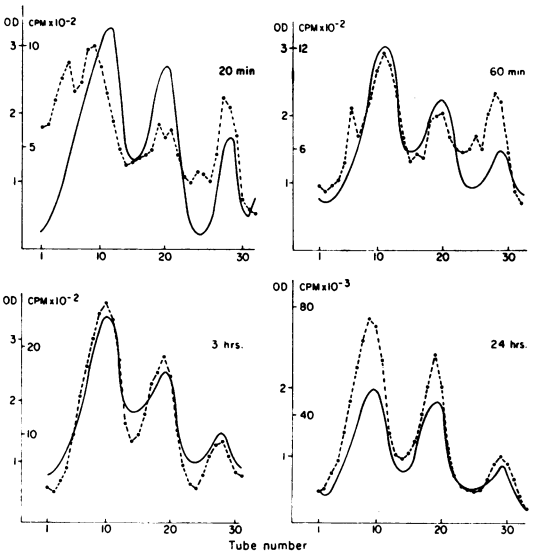

FIG. 4.—Sucrose density gradient analysis of RNA from whole cells after various periods of labeling.

The sedimentation profiles of rapidly labeled RNA also showed low mol wt RNA (<12*S*) of high specific activity. After removal of sRNA by salt precipitation, the low mol wt region still had a high specific radioactivity (Fig. 2*c*). This revealed the presence of a rapidly synthesized RNA of low mol wt distinct from sRNA.

When the period of labeling was progressively increased, the sedimentation profile of radioactive RNA gradually became identical with that of the bulk of rRNA (Fig. 4). After only 1 hr of radioactive labeling the superposition was almost complete, which is in agreement with an unusually fast rate of rRNA synthesis. It was also apparent that, after more than 1 hr of labeling, the specific activity of the 2–12*S* RNA fraction was no longer higher than that of rRNA; this suggests that the low mol wt RNA fraction observed after removal of sRNA has a relatively fast turnover.

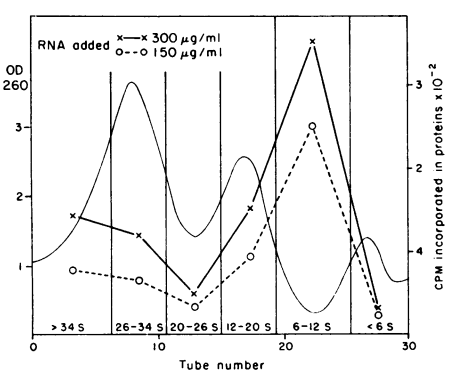

FIG. 5.—Template activity of various fractions of RNA from whole cells. Following sucrose gradient centrifugation, fractions were separated as indicated. The ability of 150 and 300 μg of RNA from each fraction to stimulate protein synthesis in an *E. coli* system is expressed in cpm of C^{14}-valine incorporated into protein.

These experiments on the kinetics of labeling of spleen and lymph node RNA were performed during both the primary and the secondary phase of the immune response; essentially similar results were observed.

Template activity of RNA fractions: Following centrifugation on sucrose gradi-

ents, total cellular RNA was divided into six fractions on the basis of the sedimentation profile. Figure 5 shows the template activity of these six fractions, determined by their ability to stimulate the incorporation of amino acids into proteins in an *E. coli* cell-free system; template activity is expressed per mg of RNA added to the incubation mixture. Although the highest activity was observed with RNA from the 6–12*S* region of the gradient, it is obvious that other fractions, which contain much larger amounts of RNA, are also active; the total template activity of cellular RNA is therefore scattered over a wide range of mol wt. The lack of activity of the lightest RNA fraction might result from the presence at the top of the gradient of some oligonucleotides, which are known to inhibit the template activity of RNA in this system.[4]

Kinetics of labeling of RNA from spleen microsomes: The first radioactive RNA observed on the microsomes was of low mol wt (Fig. 6). It was followed by a fraction which corresponded on the gradient to 18*S* rRNA, and eventually radioactivity also appeared on the heavy ribosomal RNA peak. After 50 min of labeling there were three distinct peaks of radioactive RNA, the lightest of which had the highest specific radioactivity. After longer periods of labeling, however, the low mol wt radioactive RNA did not continue to accumulate, in contrast to the two heavier radioactive RNA peaks; this indicated a faster turnover. The two radioactive peaks which sediment like rRNA had a base composition similar to that of the corresponding 30*S* and 18*S* rRNA, respectively (Table 1). The radioactive peak of

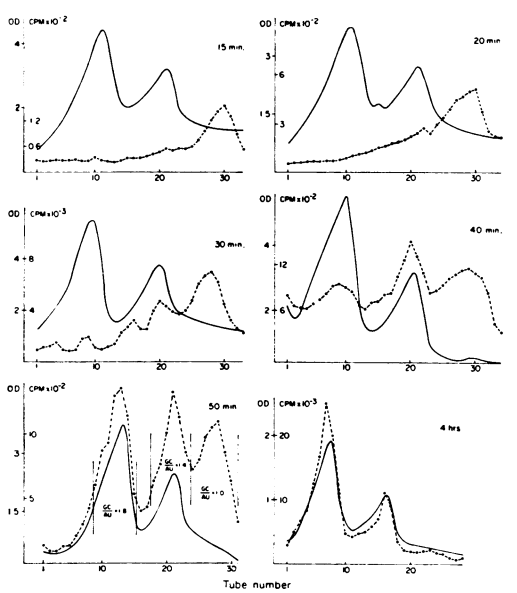

FIG. 6.—Sucrose density gradient analysis of RNA from spleen microsomes after various periods of labeling. sRNA was removed from all preparations by salt precipitation.

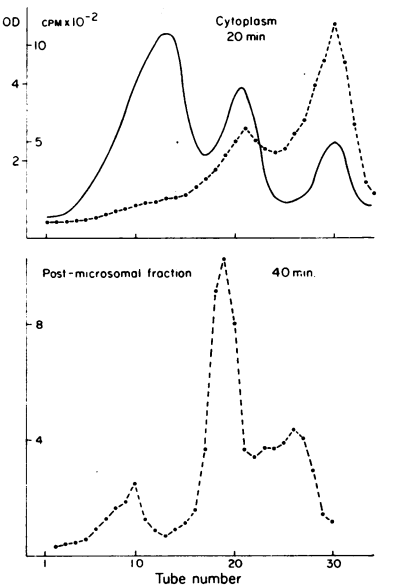

FIG. 7.—Sucrose density gradient analysis of RNA from cytoplasmic extracts. Cytoplasmic RNA was obtained from the same spleen homogenate as microsomal RNA of Fig. 6, 20 min. Labeled RNA of the post-microsomal fraction was extracted from the pellet obtained after high-speed centrifugation of the microsomal supernatant.

lower mol wt was found to have a base composition markedly different from that of rRNA and closer to that of DNA, with a GC/AU ratio of 1. All these studies were performed, with similar results, during the primary and the secondary phase of the immune response.

A rapidly labeled RNA fraction, which is neither rRNA nor sRNA, can thus be detected on the microsomes: this light fraction, whose heaviest components overlap on the gradient with the 18*S* ribosomal peak, has the following properties: (1) It is the first newly synthesized RNA to appear on the microsomes and during at least the first hour of labeling it has a higher specific activity than rRNA. (2) It has a higher turnover rate than rRNA. (3) Its base composition is relatively close to that of DNA. (4) It is found in the same region of the gradient as the RNA fraction of whole cells with the highest specific template activity. On the basis of these characteristics, this low mol wt RNA found on the microsomes appears to be messenger RNA.

Labeled RNA in the postmicrosomal fraction of the cytoplasm: RNA from the microsomes was compared with cytoplasmic RNA obtained from the same homogenate after 20 min of labeling; the cytoplasmic extract already contained radioactive 18*S* rRNA, which could not yet be detected on the microsomes (Fig. 7). This suggested that the postmicrosomal fraction contained rRNA in the process of being transferred to the microsomes. When the microsomal supernatant was analyzed after various periods of labeling, it was indeed found to contain newly made rRNA. Following centrifugation of the postmicrosomal fraction at 140,000 *g* for 4 hr, the supernatant no longer contained radioactive rRNA, which was recovered in the pellet (Fig. 7). In addition, the postmicrosomal fraction contained labeled RNA of lower mol wt which could also be extracted from the pellet after centrifugation at 140,000 *g* (Fig. 7); this rapidly labeled RNA was identical in sedimentation profile and base composition to the mRNA observed on the microsomes.

Discussion.—The injection of antigen results in an important increase in RNA synthesis in spleen and lymph nodes; under those conditions, the rapidly labeled RNA differs markedly from that of nonimmunized animals. It is commonly believed that, in mammalian tissue as well as in bacteria, rapidly labeled RNA represents mainly mRNA. During the immune response, however, the sedimentation pattern of rapidly labeled RNA reveals the existence of heavy precursors of rRNA and shows, after only 20 min of labeling, a good correlation with the profile of rRNA; furthermore, the nucleotide composition of this newly made RNA indicates a predominance of rRNA. It appears, therefore, that following immunization, the most important fraction of rapidly synthesized RNA is not of the messenger type, but of the ribosomal type; this unusually rapid rate of synthesis of rRNA is further illustrated by the strikingly fast accumulation of labeled rRNA on the microsomes.

The predominance of RNA of the ribosomal type in the rapidly synthesized RNA of spleen and lymph nodes is presumably a consequence of the rapid rate of cell division and differentiation associated with the immune response, a condition which requires the production of a large number of new ribosomes. The predominant synthesis of rRNA is observed even relatively late in the immune response; since it is known from autoradiographic studies that RNA synthesis in cells which are still in the process of differentiation far exceeds RNA synthesis in mature plasma cells,[9] one

can expect that newly made mRNA of antibody-producing cells will always be blurred by rRNA synthesized by less mature cells, even when these are present in small number.

Even though newly made rRNA appears on spleen microsomes at an unusually rapid rate, the first labeled RNA to reach the microsomes is an RNA fraction distinct from rRNA and sRNA and which has the characteristics of mRNA. Newly made mRNA can thus be selectively identified on the microsomes even under conditions where its identification in extracts of whole cells or nuclei is made difficult by a large excess of rapidly labeled RNA of ribosomal type. Since early detection of mRNA on microsomes or polysomes has also recently been described in the case of HeLa cells[10] and liver,[11] a rapid appearance at the site of protein synthesis is probably a general property of mammalian mRNA.

The relatively low mol wt of mRNA observed on microsomes could represent an artifact due to the degradation which might occur during the isolation of microsomes, when RNA is not protected from the effect of nucleases. It was found, however, that even when RNA was extracted from whole organs immediately homogenized in phenol and detergent, the 3–12*S* region of the gradient contained an RNA fraction distinct from sRNA, and with the characteristics of mRNA: a high specific radioactivity after short periods of labeling, a fast turnover, and a high template activity. Since low mol wt mRNA is thus observed even when the conditions of extraction prevent the action of nucleases, it appears that the small size of mRNA observed on the microsomes is probably not the result of degradation. It can be calculated, on the basis of a triplet code, that the main fraction (3–12*S*) of microsomal mRNA could direct the synthesis of proteins with mol wt ranging from 2,000 to 30,000. The heavier fraction of mRNA, which is masked under the 18*S* ribosomal RNA peak, is compatible with the synthesis of proteins as large as the H chains of γ-globulins (60,000 mol wt), inasmuch as they can be considered as a unit of synthesis.

It was found that the template activity of total cellular RNA was not restricted to the low mol wt active fraction, but that RNA of higher mol wt accounted for an important part of the total template activity; this suggested the existence in the cell of mRNA of high mol wt. It has indeed been possible, by successive phenol extractions at increasing temperatures, to isolate selectively an RNA fraction which sediments between about 10*S* and 35*S* and which has the characteristics of mRNA, including a strikingly high template activity;[1] RNA extractable only at elevated temperatures is thought to represent newly made messenger RNA still bound to the chromosomes. It thus appears that mRNA might exist in the cell in two distinct forms, one of relatively large mol wt representing mRNA as it is synthesized on the DNA, and one of lower size representing messenger as it functions at the site of protein synthesis. These observations suggest that mRNA could be synthesized as a larger precursor, possibly polycistronic, and that somewhere during the transfer from its site of synthesis to its site of function, a physiological process of splitting occurs, resulting in smaller template molecules. Such a mechanism would present some analogy with the formation of 30*S* and 18*S* rRNA from larger precursors.

It appears from the study of the postmicrosomal fraction that newly made rRNA is not transferred from the nucleus to the microsomes as free RNA, but rather in a sedimentable form, which could represent newly made ribosomes or ribosomal subunits, as recently observed in the case of HeLa cells in culture.[10] In addition, the

postmicrosomal fraction contained rapidly labeled mRNA, which was also found to exist in a sedimentable form; newly made mRNA might thus be transported to its site of function in association with subcellular particles.

Summary.—RNA from spleen and lymph nodes of the rat has been characterized by a study of its sedimentation properties, kinetics of radioactive labeling, nucleotide composition, and template activity. Immunization results in an unusually rapid rate of synthesis of rRNA and rRNA precursors, which account for a large part of newly synthesized RNA of these tissues. The first newly made RNA to reach the microsomes is a fraction of low molecular weight with the characteristics of mRNA. There is also evidence for the existence in the cell of mRNA of higher mol wt, which probably corresponds to mRNA as it is synthesized in the nucleus.[1] Newly made rRNA is transferred in the cytoplasm as subcellular particles; it appears very rapidly on the microsomes, 18*S* RNA preceding 30*S* RNA.

The authors are indebted to Dr. E. L. Tatum for his encouragement and to Cecil Zuber for her excellent technical assistance.

Abbreviations used are rRNA, ribosomal RNA; mRNA, messenger RNA; sRNA, soluble RNA; mol wt, molecular weight.

* This work was supported by the Jane Coffins Childs Foundation and USPHS grant A-1395.

[1] Mach, B., and P. Vassalli, *Science*, in press.

[2] Reich, E., R. M. Franklin, A. J. Shatkin, and E. L. Tatum, these PROCEEDINGS, **48**, 1238 (1962).

[3] Holland, J. J., these PROCEEDINGS, **50**, 436 (1963).

[4] DiGirolamo, A., E. C. Henshaw, and H. H. Hiatt, *J. Mol. Biol.*, **8**, 479 (1964).

[5] Harel, L., J. Harel, A. Boer, J. Imbenotte, and N. Carpeni, *Biochim. Biophys. Acta*, **87**, 212 (1964).

[6] Katz, S., and D. G. Comb, *J. Biol. Chem.*, **238**, 3065 (1963).

[7] Scherrer, K., H. Latham, and J. E. Darnell, these PROCEEDINGS, **49**, 240 (1963).

[8] Rake, A. V., and A. F. Graham, *Biophys. J.*, **4**, 267 (1964).

[9] Mitchell, J., *Australian J. Exptl. Biol. Med. Sci.*, **42**, 347 (1964).

[10] Girard, M., H. Latham, S. Penman, and J. E. Darnell, *J. Mol. Biol.*, **11**, 187 (1965).

[11] Munro, A. J., and A. Korner, *Nature*, **201**, 1194 (1964); Trakatellis, A. C., A. E. Axelrod, and M. Montjar, *J. Biol. Chem.*, **239**. 4237 (1964)

---

*REPLICATION OF VIRAL RNA, VIII. STUDIES ON THE ENZYMATIC MECHANISM OF REPLICATION OF MS2 RNA**

BY PIET BORST† AND CHARLES WEISSMANN

DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE

Communicated by Severo Ochoa, July 28, 1965

Evidence was presented in the preceding paper[1] that the bulk of the RNA[2] synthesized from radioactive nucleoside triphosphates by RNA synthetase[3] consists of parental type, "plus" MS2 RNA strands, showing that the enzyme is concerned with replication of the viral RNA. Previously it had been found that after the usual isolation procedure, involving extraction with phenol and precipitation with ethanol, about half of the newly formed radioactive RNA was resistant to RNase[4] and had properties identical to those of the MS2-specific double-stranded RNA