development and for adapting to novel nutritional conditions. It seems reasonable to suppose that the periodicity in other biochemical activities of the microspores may be regulated by mechanisms similar to that governing thymidine kinase. If so, then, in the ultimate sense at least, the problem of mitosis is a problem of regulated gene action.

Summary.—The brief appearance of thymidine kinase activity prior to DNA synthesis in microspores of *Lilium* is due to a *de novo* synthesis of protein. The chain of events leading to such synthesis begins with the formation of RNA; any of the steps in the sequence may be blocked by an appropriate inhibitor.

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*POLYRIBOSOMES IN NORMAL AND POLIOVIRUS-INFECTED HELA CELLS AND THEIR RELATIONSHIP TO MESSENGER-RNA**

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An extensive body of evidence has accumulated in the past two years in support of the hypothesis that protein synthesis is directed by strands of "messenger-RNA" (m-RNA) which are attached to ribosomal particles.[1, 2] From the analysis of attachment to ribosomes of nucleic acids and synthetic polyribonucleotides which stimulate protein synthesis *in vitro*,[3–5] as well as a study of the ribosomal components active in the synthesis of protein in *E. coli*,[6] it appeared that aggregates containing more than one ribosome existed. Examination of the ribosomal complement of rabbit reticulocytes led to the clear demonstration that a unit composed of several (probably 5) ribosomes was responsible for the majority of the protein synthesis.[7–9] This unit was resistant to treatment with sodium desoxycholate, a salt commonly used to dissolve membrane material from animal cell sources, but was readily subject to reduction in size by pancreatic ribonuclease. The conclusion was drawn that the polyribosome or polysome, as the large structure has been called, probably consisted of a strand of messenger-RNA (m-RNA) to which a number of ribosomes were simultaneously attached. A similar conclusion has been reached independently by Wettstein *et al.*,[10] who have studied functioning ribosomal aggregates from rat liver.

An examination of the RNA responsible for protein synthesis in cells derived from whole animals is complicated by the difficulty in using radioisotopes in satis-

factory concentrations. HeLa cells, however, provide a favorable material since (1) they represent a homogenous population of animal cells growing exponentially with a generation time of 18–24 hr; (2) information is available about the rates and types of RNA synthesized by these cells;[11] and (3) poliovirus (an RNA virus) multiplies well under controlled conditions in these cells. The experiments presented here demonstrate that there are, in the cytoplasm of HeLa cells, polysomes of various sizes which contain a fraction of RNA with characteristics of m-RNA. In addition it is shown that in a poliovirus-infected cell, where the functioning m-RNA is probably the virus RNA itself[5] (2×10^6 mol. wt.), polysomes of very large size are assembled.

Materials and Methods.—Cells and virus: The techniques of cell growth in suspension cultures and of virus infection and purification have previously been described.[12]

Cell fractionation and analytical procedures: Cytoplasmic extracts of $4–8 \times 10^7$ HeLa cells were made by centrifuging the cells out of growth medium (3 min at 800 *g*), washing twice with Earle's saline,[12] and resuspending in 2 ml of a standard buffer used for rabbit reticulocytes[8] (RSB = $10^{-2}M$ tris pH 7.4, 10^{-2} *M* KCl and $1.5 \times 10^{-3}M$ $MgCl_2$). The cells were allowed to swell in this hypotonic buffer for 5 min and were given 6–10 strokes in a tight-fitting Dounce homogenizer, after which the nuclei were separated by centrifugation (10 min at 800 *g*). Sodium desoxycholate was added to a final concentration of 0.5% and the extracts were immediately layered on 5–20% W/W sucrose gradients in RSB. After ultracentrifugation, fractionation of the gradients was accomplished as previously described,[11] and the contents of the gradient were continuously analyzed for UV absorbance at 260 mμ using a Gilford recorder. Accurate analysis of 0.2 to 0.3 $O.D._{260}$ units dispersed into 4–5 ml is possible with this technique. Extraction, sedimentation analysis, and determination of base composition of RNA have been described.[11]

Use of radioactive precursors: C^{14}-labeled amino acids (23.6 μc/umole carbon) and algal protein (1000 μc/mgm) hydrolysate were obtained from New England Nuclear Corporation, and P^{32} was obtained from Oak Ridge. Incorporation of labeled precursor was halted by pouring the culture over an equal volume of frozen, crushed 0.14 *M* NaCl.

Results.—When cytoplasmic extracts of HeLa cells were subjected to sedimentation analysis, a large amount of UV-absorbing material was found to sediment as a broad band representing S values from 150 to 400. Figure 1 shows that these structures, presumably polyribosomes, were active sites of protein synthesis since they contained over 50 per cent of the radioactivity incorporated in a 1 min pulse of C^{14} valine, while the 74S peak, which represents single ribosomes, was not labeled. The specific activity was uniform from about 180 to 300S with the greatest number of counts in a 200S peak. It can be estimated[8, 10] that a structure of 200S probably contains 7 or 8 ribosomes. The 1 min label period was chosen because in preliminary experiments it was found that the total acid-precipitable radioactivity in the polysome region after 3 or 6 min was not significantly greater than after 1 min, whereas the acid-precipitable radioactivity at the top of the gradient increased. This implies that the length of time required for the manufacture and release of most peptide chains in HeLa cells has an upper limit of about 1 min, which conforms with the estimate of Dintzis[13] that a hemoglobin chain requires 1 to 1.5 min for completion and release.

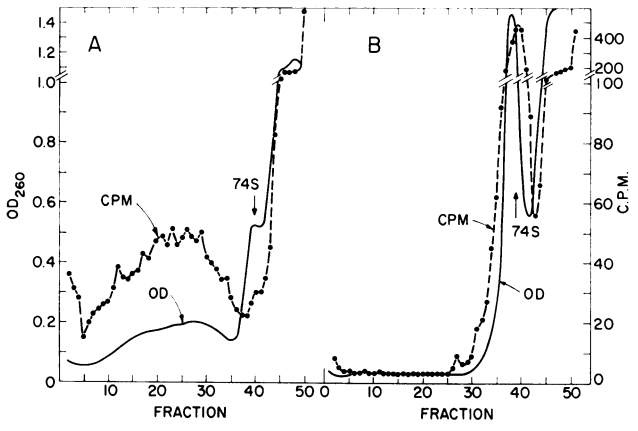

FIG. 1.—Sedimentation analysis of cycloplasmic extracts of HeLa cells. Logarithmically growing HeLa cells at 4×10^5 cells/ml were collected from 200 ml of culture by centrifugation at 25–30° and resuspended at 37° in Eagle's medium[12] (dialyzed serum) containing $^1/_{40}$ the normal amino acid concentration. After 45 min incubation at 37° the cells were labeled for 1 min by adding 10 μc of valine C^{14} (115 μc/μmole). Then the cytoplasmic fraction was prepared as described under *Methods*. Before sedimentation analysis in sucrose gradients (55 min at 24,000 rpm, SW 25 rotor of model L Spinco ultracentrifuge), one half of the cytoplasmic extract was treated with 1γ/ml crystalline pancreatic ribonuclease (Worthington) for 10 min at 0° (*B*). The other half remained untreated (*A*). The gradients were fractionated and UV absorbancy was determined. Acid-precipitable radioactivity was measured after each fraction was made I N in KOH and allowed to stand at room temperature for 10 min; the fraction was then precipitated with trichlororacetic acid (TCA) to give an excess of 5% TCA. S values were approximated according to the method of Martin and Ames[23] from comparison of mobilities of various peaks on the strip chart from the UV recorder, which was fed at a constant flow by a finger pump. This was necessary because the desoxycholate used in the preparation of cytoplasmic fractions changed the surface tension of the top of the gradient causing changes in drop size. The range of S values in the structures which incorporate amino acids is 150–400S with a peak at about 200S. The marker used for comparison is the single ribosome peak, which is 74S in HeLa cells.[18]

Several lines of evidence indicate that the polyribosome is a structure composed of a number of single 74S ribosomes which sediment together in a sucrose gradient because they are closely packed on a strand of RNA. Figure 1*A* and 1*B* demonstrate that the polyribosomes are broken down to 74S units by a short treatment with ribonuclease at 4°. The recently incorporated C^{14} amino acid is retained on the 74S ribosomes, which are themselves not destroyed by the mild ribonuclease digestion. Another experiment suggesting that the integrity of the polyribosome in the HeLa cell is dependent on an unstable fraction of RNA is shown in Figure 2. The interruption of a continuing supply of newly formed RNA by the addition of actinomycin D to a growing culture results in the loss of polyribosomes revealed by a disappearance of UV-absorbing material and a corresponding loss of protein-synthesizing capacity from the heavy regions of a sucrose gradient. (Actinomycin D is an antibiotic which immediately stops RNA synthesis in HeLa cells[11] as well as in other animal and bacterial cells.[14]) It has been shown in a number of systems,[15] including HeLa cells,[12] that ribosomal RNA once formed is stable. Hence, a decay in the rate of protein synthesis after actinomycin treatment is an indication of the existence of another fraction of RNA which is responsible for protein synthesis. Levinthal *et al.*[16] have shown in *B. subtilis* that in the presence of actinomycin the m-RNA is unstable, and have used this system to measure its rate of decay and the accompanying decrease in the rate of protein synthesis. The data of Figure 2

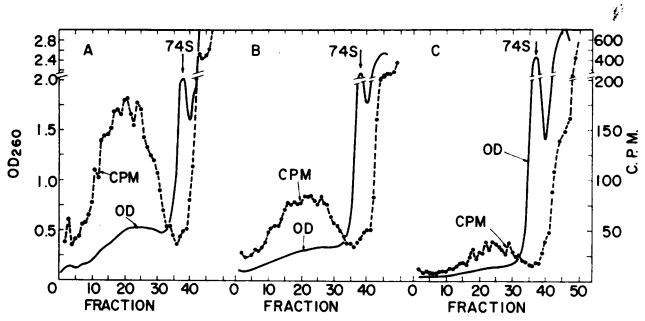

FIG. 2.—Fate of cytoplasmic polyribosomes after treatment of HeLa cells with actinomycin D. Actinomycin D (5γ/ml) was added to a 200 ml culture of HeLa cells, and 3 (*B*) and 7 (*C*) hr afterward the cytoplasm of the cells was examined for polysomes as in Fig. 1, except that the cells were labeled with 6 μc of each of 8 amino acids (valine, isoleucine, leucine, arginine, proline, tyrosine, histidine, and lysine). A 100 ml culture not treated with actinomycin served as control (*A*). The sedimentation analysis was at 24,000 rpm for 60 min. The data presented in (*B*) and (*C*) for both OD_{260} and radioactivity have been corrected for the small variations in the efficiency of the cytoplasmic extraction procedure, by determining the total absorbancy (OD_{260}) of each extract and multiplying each value by an appropriate correction factor.

show that in the cytoplasm of the actinomycin-treated HeLa cell, the rate of protein synthesis, hence presumably the m-RNA, decays with a half life of 3–4 hr.

Direct evidence of an RNA in polyribosomes distinct from ribosomal RNA is presented in Figure 3 and Table 1. After exposure to P^{32} for 40 min, cells were

TABLE 1

COMPOSITION OF VARIOUS NUCLEIC ACID FRACTIONS FROM HELA CELLS

Material Analyzed	Per Cent of Nucleic Acid as A	U(T)	G	C
Polysomal RNA, pulse labeled, 6–12S	25.4	27.3	21.0	26.9
Polysomal RNA, pulse labeled, 12–22S	26.6	27.7	21.5	24.2
Ribosomal RNA, 28S	20.5	19.3	28.3	31.9
16S	23.2	23.2	25.2	28.0
DNA	29.1	27.5	22.0	21.4

Methods and data for ribosomal RNA and DNA as described previously.[11] Data for polysomal RNA represent averages of 4–6 analyses. A = adenylate or adenine; U = uridylate; T = thymine; G = guanylate or guanine; C = cytidylate or cytidine.

fractionated and polysomes separated. Sedimentation analysis of RNA derived by phenol extraction from the labeled polysomes revealed (Fig. 3) that the radioactivity was distributed broadly (6 to 25S) with a peak around 10S. This distribution definitely does not correspond to the 28 and 16S ribosomal RNA. The base composition of the 10S material as determined by the distribution of P^{32} among the products of alkaline hydrolysis of the RNA (Table 1) was also unlike ribosomal RNA, and the guanylic acid + cytidylic acid (G + C) content resembled more the G + C content of the DNA than that of ribosomal RNA. The inequality of G and C may be an indication that, as in bacteriophage T4,[17] only one strand of DNA is copied to produce the m-RNA. If both strands were copied, not only should the G + C equal that of DNA, but the G and C contents should themselves be equal. Although the unequal distribution of G and C might be due to nonrandomness of nearest neighbors in DNA coupled with unequal specific activities in the pools of different ribonucleoside triphosphates, this appears to be unlikely. No important discrepancies arising from nearest neighbor exclusion have been found in the base analyses of several types of high molecular weight RNA that have been analyzed by P^{32} distribution and spectrophotometrically.[18]

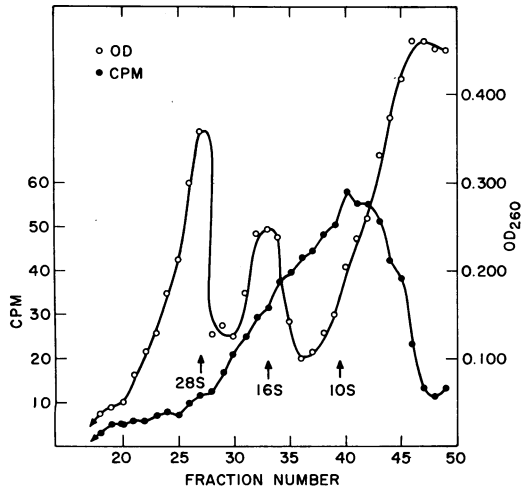

FIG. 3.—Sedimentation analysis of RNA from polyribosomes. After incubation at 37° for 45 min in PO_4-free Eagle's medium, a culture (600 ml at 4 × 10^5 cells/ml) of HeLa cells was labeled for 40 min with 20 mc of carrier-free P^{32}. A cytoplasmic extract was prepared in 6 ml of RSB, layered onto 3 sucrose gradients, and centrifuged at 24,000 rpm for 70 min. Polyribosomes of from 180–400S were collected and centrifuged at 50,000 rpm × 90 min. The RNA from the pellets was extracted and analyzed as previously described.[6]

To summarize, the evidence for a strand of m-RNA to which individual ribosomes attach to form a polyribosome consists of (1) the instability of the polyribosome to mild RNAse treatment; (2) the disappearance of polyribosomes in actinomycin-treated cultures; and (3) the existence in polyribosomes of a fraction of RNA distinct from ribosomal RNA which is labeled rapidly and has a G + C content resembling that of DNA.

Effect of poliovirus infection on polyribosomes: Infection of HeLa cells by poliovirus (an RNA-containing virus) has been observed to cause a decrease in the total protein synthesis and an interruption in host cell RNA synthesis.[19, 20] Since these effects might be an expression of changes in the cellular polysomes, the effect of viral infection on polysomes in growing cells was studied. Cultures were infected while in logarithmic growth, and aliquots were examined for polyribosomes 1.5 and 3.5 hr after infection. At the earlier time (Fig. 4), there was a small decrease (~25 per cent) in the number of polyribosomes as measured both by UV absorbance and amino acid incorporation, but by 3.5 hr (about 0.5 to 1 hr after the beginning of viral RNA and protein synthesis[21]) there was a drastic reduction (>90 per cent)

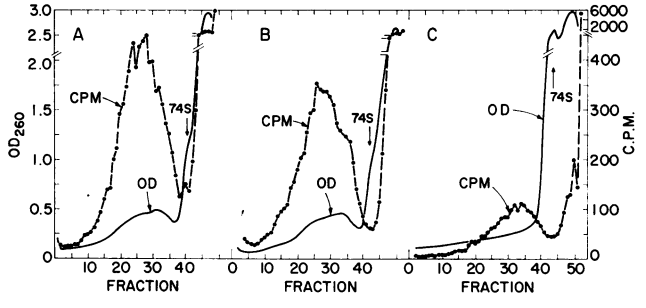

FIG. 4.—Effect of poliovirus infection on the cytoplasmic polyribosomes of HeLa cells. A 200 ml culture of growing HeLa cells was infected by the addition of purified virus as previously described[19] and cytoplasmic extracts were examined for polyribosomes 1.5 (*B*) and 3.5 (*C*) hr after infection. Labeling was with 30 μc of algal protein hydrolysate (1000 μc/mgm) in each culture. A 100 ml uninfected culture (*A*) was the control.

in the number of polyribosomes. This effect was considerably greater than that observed with 3 hr of actinomycin treatment (Fig. 2), indicating that polio infection has a direct destructive effect on polyribosomes, and thus on cellular protein synthesis, which exceeds the effect of simply preventing a new supply of m-RNA. Previous observations[20] have indicated that in polio-infected cells there appears late in infection a ribonuclease-like activity which liberates acid soluble products from preformed cellular RNA. The destruction of polysomes described here could represent an earlier manifestation of this activity.

In order to determine if polysomes specific to virus-infected cells could be observed, cultures which were to be infected were first treated with actinomycin. Actinomycin is known not to affect viral replication,[19] but does markedly reduce the number of normal polysomes (Fig. 2). Figure 5 shows that actinomycin

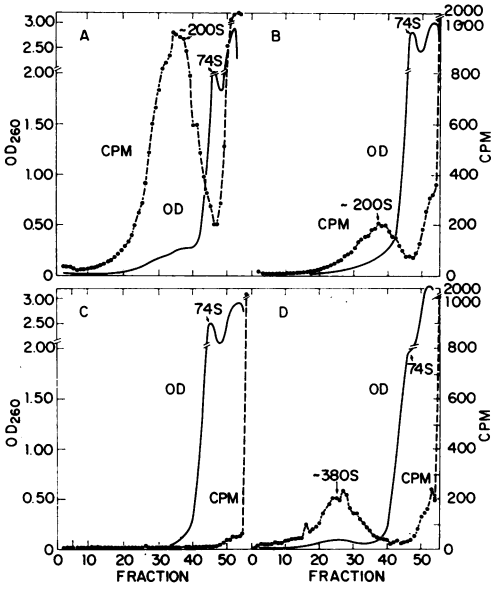

FIG. 5.—The appearance of polyribosomes specific to the poliovirus-infected cell. A culture of 400 ml of HeLa cells (4×10^5 cells/ml) was treated for 3 hr with actinomycin D (5γ/ml) and then concentrated to 4×10^6 cells/ml still in presence of 5γ/ml actinomycin. Three fourths of the culture was then infected with poliovirus (~50 PFU/cell) while one fourth continued as the uninfected control. Two hr after the beginning of incubation of the concentrated cell suspension the control (*A*) and the first infected sample (*B*) were analyzed for polysomes as in Fig. 1. Samples were also taken at 2.75 hr (*C*) and 3.75 hr (*D*) after the beginning of infection. The radioactive label used was the algal hydrolysate as in Fig. 4.

treatment plus the first 2 hr of poliovirus infection lowers the background of normal polysomes more than either treatment alone. Then, at a time when viral molecules are being formed,[21] a new variety of polyribosome is observed which has an average S value of 380 to 400 compared to the average value of 200S in the uninfected cell. These large structures appear both as UV-absorbing material and protein synthesizing units in the 400S region of the gradients.

Discussion.—The present experiments are concerned with (1) the presence and properties of a messenger-RNA fraction from animal cells; (2) the role of m-RNA in organizing polyribosomes; and (3) the basis for the inhibition of cellular protein synthesis by poliovirus.

The newly recognized species of RNA (Fig. 3 and Table 1) associated with the polyribosomes is definitely not ribosomal RNA (r-RNA) and shares a number of characteristics with bacterial messenger-RNA—preferential labeling by short exposure to radioisotope, heterogeneous size from 6 to over 20S, attachment to structures that are synthesizing protein, and a base composition which reflects

that of cellular DNA. It appears extremely likely that this represents cytoplasmic m-RNA. Comparison of this material with rapidly labeled RNA from the nucleus of the HeLa cell[11] reveals several differences. Thus, the size of the labeled material from the nucleus is predominantly 45S, whereas RNA of this size seems to be missing from the polyribosomes. It has been shown, however, that most of the large 45S material in the nucleus is ribosomal precursor, which is later specifically converted into 28 and 16S ribosomal RNA. Perhaps the 6 to 20S RNA in cytoplasmic polyribosomes also derives from a heavier nuclear precursor. Another apparent difference between the rapidly labeled nuclear RNA and the cytoplasmic m-RNA relates to stability. A large fraction of the radioactivity in the nuclear fraction disappears with a half life of 15 to 20 min after actinomycin treatment, while polyribosomal protein synthesis and presumably cytoplasmic m-RNA decay with a half life of 3 hr after exposure to actinomycin. These differences may indicate that there exists a class of nuclear messenger-RNA with very different properties from cytoplasmic m-RNA. Alternative explanations could be that the instability of a portion of the nuclear RNA is due to (1) RNA that is made and degraded without relation to protein synthesis, (2) inherent instability of ribosomal precursor RNA subsequent to actinomycin treatment, or (3) inability of m-RNA to become attached to ribosomes and thus become stabilized in the presence of actinomycin. Clarification of these questions will be possible only when the rapidly labeled nuclear fraction can be separated into its component parts. Meanwhile, it appears quite clear that a cytoplasmic messenger RNA exists and can be isolated from polyribosomes.

As for the manner in which polyribosomes arise and function, it seems likely that groups of ribosomes are formed into polyribosomes by attaching to m-RNA. If this is correct, the length of the m-RNA chain should be the controlling element in the size of the polyribosome. In fact, the polyribosomes from poliovirus (RNA = 35S) infected cells have an average S value of about 400, while the majority species of cellular polyribosomes is about 200S, consistent with a smaller m-RNA size (6 to 25S) (Fig. 3). This interpretation, derived from sedimentation studies, is supported by electron microscopic analysis of polyribosomes of different sedimentation values.[22] The electron micrographs show that most of the polyribosomes from the virus-infected cells contain many more ribosomes than the polyribosomes from uninfected cells.

The following considerations can be made about the order of appearance of m-RNA and of ribosomal RNA in cytoplasmic polysomes. If an average polyribosome contains 6 to 8 ribosomes (1.2 to 1.6 $\times$ 10^7 m.w.u. of RNA) and a 10 to 12S piece of m-RNA (2.5 $\times$ 10^5 m.w.u. of RNA), there would be in the polysome about 50 times more r-RNA than m-RNA. The m-RNA associated with polyribosomes is renewed with a half life of 3 hr, while r-RNA doubles only once in 24 hr. Hence, the steady state flow of total radioactivity carried by isotopically labeled RNA into the polyribosome would favor the r-RNA ($50 \times 3/24 \cong 6$).

Following a pulse label with P^{32}, radioactivity in RNA is found almost entirely in the messenger fraction, as defined by its distinct size and base composition. The steady state situation then has clearly not been reached by a 40 min labeling with P^{32}. This indicates that polyribosomes are formed by new m-RNA strands recruiting preformed ribosomes either in the nucleus or the cytoplasm. This is

consonant with the finding that approximately 1 hr elapses before a large fraction of the 45 and 35S nuclear RNA becomes 28 and 16S ribosomal RNA. In the poliovirus-infected cell, polyribosomes are also presumably formed by the attachment of free ribosomes to a newly introduced messenger, which must be the viral RNA itself, since this is the only RNA formed in substantial amounts in virus-infected, actinomycin-treated cells.[12] This hypothesis, which was first formulated on the basis of findings with phage-infected bacteria,[1] is being tested by analyzing the types of RNA in the virus specific polysomes.

Experiments with virus-infected cells may help solve an important problem which could previously not be resolved. It appears that inhibition of synthesis of normal cellular proteins, due to the dual action of actinomycin and of viral infection, is sufficiently complete so that most of the proteins synthesized in actinomycin-treated infected cells by 3 hr after infection are specified by the viral RNA. This may allow the eventual determination of the number and types of protein which are encoded by the viral RNA.

Note added in proof: Recent experiments by M. Scharf and L. Levintow (personal communication) have demonstrated that at least 50% of the amino acid label which is incorporated into protein on the specific polyribosomes from poliovirus-infected cells has the immunologic specificity of the subunits of the poliovirus capsid.

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*PYRIMIDINE-SPECIFIC ANTIBODIES WHICH REACT WITH DEOXYRIBONUCLEIC ACID (DNA)**

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Communicated by E. L. Tatum, March 7, 1963

Progress in a program to synthesize antigens which might be used to elicit antibodies specific for nucleic acid components has been reported.[1] In the foregoing paper, the properties and biochemical applications of an antiserum complementary to purin-6-oyl residues were outlined. It was demonstrated, using the complement-fixation reaction, that this "anti-purinoyl" antibody cross-reacted with denatured DNA, but not with native DNA, from several species.

This communication illustrates the principle of synthesis of polyfunctional antigens containing pyrimidine residues and describes conjugates which contain 5-acetyluracil and uracil residues, respectively, attached through the $N_1(N_3)$ position, to serum albumins. Some of the characteristics of rabbit antibodies to these conjugates are also delineated. The interaction between "anti-pyrimidyl" antisera and DNA can be demonstrated by complement fixation; the reaction between antibody to the 5-acetyluracil conjugate and DNA has been shown in addition by passive cutaneous anaphylaxis in the guinea pig. Possible uses for these antisera in the investigation of the biological functions of nucleic acids are discussed.

Materials and Methods.—Pyrimidine-protein conjugates: These substances were synthesized by adaptation of the elegant methods developed by Shaw and co-workers[2-4] for synthesis of pyrimidines and (N_1)-pyrimidyl amino acids, and for *N*-terminal amino acid analysis of proteins.

Polyfunctional (5-acetyluracil-1-) bovine serum albumin (5-AcU-BSA): "Ethoxyacrylamide," mp 90–91°, was synthesized from urethane, diketene, and triethylorthoformate by following the published[2, 3] procedure. To a cold solution of 2.3 gm BSA (0.03 mMole, Pentex Fraction $\overline{V}$) in 50 ml of water plus 20 ml 0.1 *N* NaOH (pH 11.2), 750 mg "ethoxyacrylamide" (3.6 mMoles) was added in small portions. After agitation for 5 min, 10 ml tetrahydrofuran and another 20 ml of 0.1 *N* NaOH were added, and the mixture was stirred at 37° for an hour. Adjustment of the pH to 10.3 by addition of another 4 ml of NaOH was followed by further stirring for 18 hr at this temperature. The mixture was then dialyzed for 24 hr against running tap water. The clear, orange solution (pH 7.2) was treated dropwise with 1 *N* HCl to bring the pH to 2.2. After standing for 10 min, the mixture was brought to pH 5.0 with the addition of 10% Na_2CO_3. The off-white, precipitated conjugate was centrifuged in the cold, and was dissolved in 25 ml 0.15 *M* $NaHCO_3$ to give a clear, rust-red solution. Addition of 27 ml 0.15 *M* HCl to pH 4.9 caused re-precipitation of the conjugate. After centrifugation in the cold, the conjugate was dissolved in 30 ml 0.1 *M* phosphate buffer, pH 7.0. It was dialyzed against cold tap water for 24 hr, and then lyophilized to yield 2.2 gm of a light tan-colored product.

The conjugate in phosphate buffer, pH 7.0, exhibited an absorption peak at 286 mμ, and at a concentration of 248 μg/ml had an O.D. of 1.38. On the assumption that an average 5-acetyl-