in 0.0125*M* acetate buffer at *p*H 4.5 with purified β-glucuronidase of beef liver (*11*), which cleaves acyl as well as acetal bonds. Free retinoic acid was rapidly released by the active enzyme but not by the boiled control. When saccharolactone, presumably a specific inhibitor of β-glucuronidase (*12*), was added, the rate of release of retinoic acid from the metabolite and of phenolphthalein from pure phenolphthalein β-glucuronide was comparable at several concentrations of inhibitor (Table 1).

Finally, the molar ratio of glucuronic acid to retinoic acid in the metabolite, as measured by the naphthoresorcinol test (*13*), was 1.4. The ratio may be greater than unity as a result of small amounts of bilirubin glucuronide which contaminated this preparation. Therefore, we suggest that the major metabolic product of retinoic acid in rat bile is retinoyl β-glucuronic acid (Fig. 2).

The appearance of retinoyl β-glucuronide in the bile is not surprising. Bilirubin and perhaps vitamin K are also excreted as glucuronides in the bile (*14*) and many steroid glucosiduronates are found in urine. Although some glucuronides and glucosiduronates are not absorbed from the gut (*15*), retinoyl β-glucuronide, like estriol β-glucosiduronate (*16*), apparently is absorbed and participates in an enterohepatic circulation (*6*). In normal animals, fraction III is excreted mainly in the feces but also appears in the urine (*17*).

The rapid conjugation of retinoic acid with glucuronic acid in the liver, and perhaps in other organs, excretion of the metabolite in the bile, and its partial reabsorption from the intestine clarify some puzzling aspects of retinoic acid metabolism: the rapid disappearance of free retinoic acid from the liver and other tissues (*5*), and the presence of water-soluble metabolites of retinoic acid in the liver and intestine (*5*, *10*, *18*). Whether retinoyl β-glucuronide is biologically active per se in growth and metabolism, is cleaved by tissue β-glucuronidases to free retinoate, or is merely an excretion product is still uncertain.

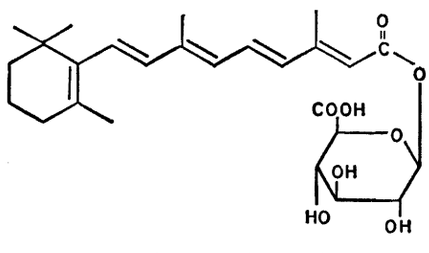

Fig 2. Retinoyl β-glucuronic acid.

PERCY E. DUNAGIN, JR.
E. HARRIS MEADOWS, JR.
JAMES ALLEN OLSON
Department of Biochemistry, University of Florida College of Medicine, Gainesville

References and Notes

1. J. F. Dowling and G. Wald, *Proc. Natl. Acad. Sci. U.S.* **44**, 648 (1958).
2. S. Mahadevan and J. Ganguly, *Biochem. J.* **81**, 53 (1961).
3. A. A. Dmitrovski, *Biokhimiya* **26**, 109 (1961); T. D. Elder and Y. J. Topper, *Federation Proc.* **20**, 196 (1961); *Biochim. Biophys. Acta* **64**, 430 (1962); S. Mahadevan, S. K. Murthy, J. Ganguly, *Biochem. J.* **85**, 326 (1962); S. Futterman, *J. Biol. Chem.* **237**, 677 (1962).
4. P. E. Dunagin, Jr., R. D. Zachman, J. A. Olson, *Biochim. Biophys. Acta* **90**, 432 (1964).
5. I. M. Sharman, *Brit. J. Nutr.* **3**, viii (1949); J. E. Dowling and G. Wald, *Proc. Natl. Acad. Sci. U.S.* **46**, 587 (1960); E. R. Redfearn, *Arch. Biochem. Biophys.* **91**, 226 (1960); L. Jurkowitz, *ibid.* **98**, 337 (1962); S. Krishnamurthy, J. G. Bieri, E. L. Andrews, *J. Nutr.* **79**, 503 (1963).
6. R. D. Zachman and J. A. Olson, *Nature* **201**, 1222 (1964); *J. Lipid Res.* **6**, 27 (1965).
7. Obtained from Rolfsmeyer Farm, Madison, Wis.
8. J. Hirsch and E. H. Ahrens, Jr., *J. Biol. Chem.* **233**, 311 (1958).
9. In chromatographic procedures on paper or thin-layer plates, R_F is the distance from the origin to the center of the spot divided by the distance from the origin to the solvent front. Thus $0 \leqslant R_F \leqslant 1$.
10. K. Yagishita, P. R. Sundaresan, G. Wolf, *Nature* **203**, 410 (1964).
11. Product of Sigma Chemical Co., St. Louis.
12. G. A. Levvy, *Biochem. J.* **50**, xv (1951); G. A. Levvy and C. A. Marsh, *Advan. in Carbohydrate Chem.* **14**, 381 (1959).
13. W. Deichmann, *J. Lab. Clin. Med.* **28**, 770 (1943).
14. R. Schmid, in *The Metabolic Basis of Inherited Disease*, J. B. Stanbury, J. B. Wyngaarden, D. S. Frederickson, Eds. (McGraw-Hill, New York, 1960), p. 226; J. D. Taylor, G. J. Miller, L. B. Jaques, J. W. T. Spinks, *Can. J. Biochem. Physiol.* **34**, 1143 (1956).
15. F. R. Smith, D. F. Tapley, J. E. Ross, *Biochim. Biophys. Acta* **69**, 68 (1963).
16. E. Diczfalusy, C. Franksson, B. Martinson, *Acta Endocrinol.* **38**, 59 (1961).
17. K. Nath and R. D. Zachman, personal communication.
18. K. Yagishita, P. R. Sundaresan, G. Wolf, *Federation Proc.* **23**, 294 (1964); M. Zile and H. F. DeLuca, *ibid.*, p. 294.
19. We thank O. Wiss of Hoffman-LaRoche Co., Basel, Switzerland, for the 6,7-C^{14}-retinoic acid, G. Wolf of the Massachusetts Institute of Technology for the details of chromatographic system II prior to publication, and D. Tapley of Columbia University for the saccharolactone. R. D. Zachman and Marta Singer assisted during several phases of this investigation. Supported by NIH grant-in-aid AM-1278.

10 December 1964

Vaccinia Virus Directed RNA: Its Fate in the Presence of Actinomycin

Abstract. *RNA similar in base composition to viral DNA is formed in the cytoplasm of HeLa cells infected with vaccinia virus. This RNA is found both in polyribosomes and in a broad 30S to 74S region in a sucrose density gradient. When infected cells are exposed to actinomycin, the amount of this DNA-like RNA in the 30S to 74S region is unchanged. Under the same conditions, 70 percent of the polyribosome-associated DNA-like RNA is degraded to acid-soluble fragments within 2 hours, and protein synthesis is reduced by 90 percent.*

RNA with a base composition similar to that of viral DNA is formed in the cytoplasm of HeLa cells after infection with vaccinia virus (*1*). This RNA, which we call D-RNA, associates with ribosomes to form polyribosomes (*2*), which are the sites of synthesis of viral proteins (*3*). On the basis of these findings and in analogy with other systems (*4*), notably bacteria infected with T-phage (*5*), it seems likely that the D-RNA is the intermediate or "messenger" for viral protein synthesis. In experiments designed to measure the functional half-life of D-RNA, it was found that when RNA synthesis was blocked by the addition of actinomycin D (C_1, IV) to cell cultures, the formation of immunologically detectable viral proteins in infected cells was promptly interrupted. Under similar conditions in uninfected cells protein synthesis continued for several hours (*6*). In an effort to understand the basis for this difference, we have compared the stability of polyribosomes in uninfected and infected cells and found that the bulk of the polyribosomes in infected cells are rapidly degraded when RNA formation is blocked. These studies reveal that a significant fraction of the D-RNA is not in polyribosomes and is not degraded in the presence of actinomycin.

Suspension cultures of 3 to 4 × 10^5 HeLa S3-1 cells per milliliter of growth medium (*1*) were exposed for 1 or 2 hours to uridine-2-C^{14} or P^{32}-phosphate

to label the newly formed RNA. At the indicated time intervals, cytoplasmic fractions were prepared as already described (*3*). The cells were chilled and ruptured with a Dounce homogenizer at a concentration of 3 to 8 × 10^7 cells per milliliter in 2 ml of RS buffer ($10^{-2}M$ tris HCl at *p*H 7.8, containing $10^{-2}M$ KCl and $1.5 \times 10^{-3}M$ $MgCl_2$). Nuclei and unbroken cells were removed by low-speed centrifugation, and the supernatant was brought to 0.25 percent sodium deoxycholate to solubilize cell membranes. The extract was then layered onto a linear sucrose gradient (5 to 20 percent) containing a 5 ml "cushion" of 70 percent sucrose, and centrifuged for 4¼ hours at 25,000 rev/min in a Spinco SW-25 rotor. Under these conditions single ribosomes and smaller components were resolved in the linear portion of the gradient, and the polyribosomes were concentrated in the 70 percent sucrose layer at the bottom.

The cytoplasmic fraction of HeLa cells contains soluble RNA, 45*S* and 60*S* ribosomal subunits, 74*S* single ribosomes, and polyribosomes as seen in the optical density tracings, Figs. 1 and 2. Most of the 74*S* ribosomes are polyribosome-associated and are released by treatment of extracts with ribonuclease (5 μg/ml) for 10 minutes at 0° to 4°C, conditions which degrade the polyribosomes completely. In the cytoplasm of uninfected cells the RNA synthesized during a 1-hour exposure to uridine-2-C^{14} is present in polyribosomes, 45*S* ribosomal subunits, and soluble RNA (Fig. 1*A*). The base composition of newly formed RNA in the 45*S* and polyribosome regions was determined with extracts of cells which had been incubated for 2 hours with P^{32} before fractionation and centrifugation. The newly synthesized RNA in the polyribosomes presumably includes cell template RNA and soluble RNA as well as ribosomal RNA, and its base composition before actinomycin treatment is consistent with that of a mixture of RNA species (Table 1). The radioactive RNA in the 45*S* region has a base composition similar to that of 16*S* ribosomal RNA (Table 1). When RNA synthesis is blocked for 30 minutes by the addition of actinomycin (5 μg/ml) (94 percent inhibition within 15 minutes), there is less than a 20-percent decrease in the absorbance at 260 mμ in the polyribosome region with a corresponding increase in single ribosomes (Fig. 1*B*). After inhibition of RNA synthesis for 90 minutes, more than 80 percent of the polyribosomes remain. The increase in radioactivity (Fig. 1*B* as compared to 1*A*) reflects that portion of the RNA formed in the nucleus, prior to inhibition, which is transferred

Table 1. Base composition of newly formed RNA in polyribosomes and 45*S* region of density gradient. Suspension cultures of 4 × 10^5 cells/ml were incubated with P^{32}-phosphate (0.7 mc/100 ml) and ruptured immediately or after a 30-minute exposure to actinomycin (5 μg/ml). Infected cultures received P^{32} from 4 to 6 hours after infection. After centrifugation of the cytoplasmic fractions, samples from the 45*S* and polyribosome regions of the gradients were extracted with phenol and analyzed (*13*).

Sample	Moles/100 moles nucleotides				
	AMP*	UMP	GMP	CMP	Sum of GMP and CMP
Uninfected polyribosomes					
Before Ac	25.6	24.3	24.3	25.8	50.1
30 minutes after Ac	24.3	22.6	29.0	24.1	53.1
Infected polyribosomes					
Before Ac	32.7	29.8	18.9	18.7	37.6
30 minutes after Ac	33.6	29.7	17.6	19.0	36.6
Uninfected 45*S*					
Before Ac	23.6	23.7	27.2	25.5	52.7
30 minutes after Ac	21.9	26.6	29.2	22.4	51.6
Infected 45*S*					
Before Ac	32.0	29.7	19.9	18.5	38.4
30 minutes after Ac	34.4	27.6	19.4	18.7	38.1
Vaccinia DNA †	32.3	33.5 ‡	17.6	16.6	34.2
HeLa DNA	29.3	29.9 ‡	20.3	20.5	40.8
4*S* Soluble RNA	17.4	21.1	31.1	30.4	61.5
16*S* Ribosomal RNA	22.2	23.1	29.4	25.4	54.8

* AMP, CMP, GMP, and UMP are adenylic, cytidylic, guanylic, and uridylic acids, respectively; Ac, actinomycin. † The values for 4*S* and 16*S* RNA have been reported (*1*) and those for vaccinia and HeLa cell DNA were kindly provided by C. Patch. ‡ Thymidylic acid.

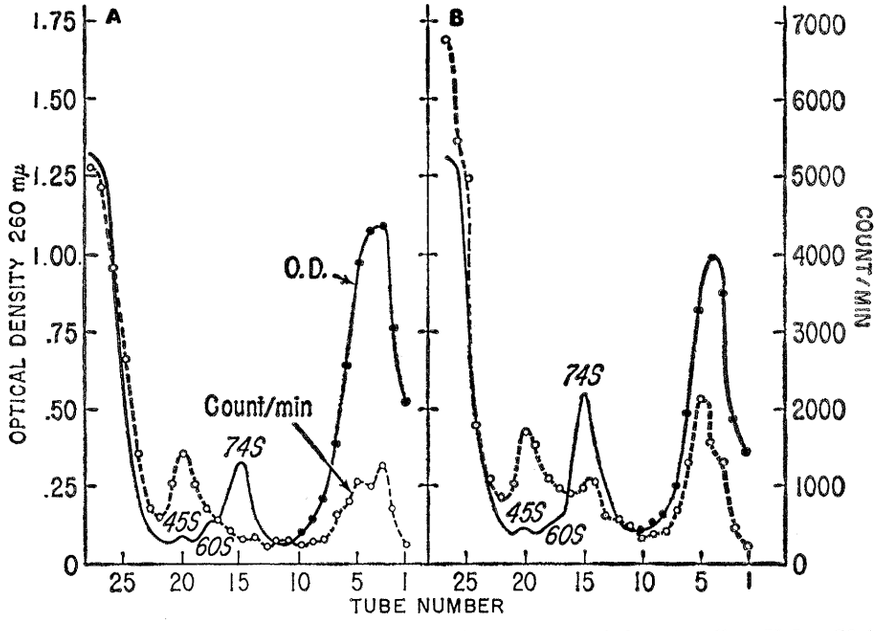

Fig. 1. Sedimentation of cytoplasmic fractions from uninfected cells. HeLa S3-1 cells at 37°C in suspension culture received uridine-2-C^{14} (specific activity, 24 μc/μmole; final concentration, $2 \times 10^{-6}M$). One hour later the 400-ml culture was divided, and one-half (*A*) was immediately chilled, and the cells were disrupted. The second portion (*B*) was incubated with actinomycin (5 μg/ml) for 30 minutes before the cells were disrupted. The extracts were centrifuged on sucrose gradients. The tubes were then punctured and the optical density of the effluent was monitored automatically. The collected fractions were chilled, precipitated with 2 mg carrier RNA and 5 percent trichloroacetic acid, and counted (*1*). The automatically monitored optical density tracing at the 20 to 70 percent sucrose interface is partially obscured by light scattering owing to mixing of the sucrose solutions in the flow cell, and the absorbance (260 mμ) of individual fractions from this region was determined manually.

to the cytoplasm and incorporated into ribosomes and polyribosomes in the presence of actinomycin (*7*). That polyribosomes in HeLa cells are not rapidly degraded in the presence of actinomycin has been reported previously (*8*); this slow degradation is consistent with the observation that protein synthesis in the cell continues for several hours after RNA formation has been interrupted (*6, 9*).

In cells infected with vaccinia virus, inhibition of RNA synthesis by actinomycin is associated with a rapid loss of polyribosomes. Infected cells were incubated with uridine-2-C^{14} 5 to 6 hours after infection, a time when all newly formed cytoplasmic RNA of size greater than 4*S* is D-RNA and radioactive ribosomal RNA is not present (Table 1) (*1*). The polyribosomes contain 60 percent of the radioactive D-RNA, and the balance sediments as a broad peak in the 30 to 74*S* region of the gradient (Fig. 2*A*). After a 30-minute exposure to actinomycin, both the absorbance and acid-precipitable radioactivity in the polyribosome region are reduced by more than 50 percent with a concomitant absorbance increase in 74*S* single ribosomes (Fig. 2*B*). However, there is no corresponding rise in radioactivity in the 30 to 74*S* or 4*S* regions of the gradient, an indication that the polyribosome-associated D-RNA is reduced to acid-soluble fragments.

The extent of D-RNA degradation to acid-soluble material was determined in cells which were exposed to uridine-2-C^{14} 5 to 6 hours after infection. Actinomycin was then added. At 30-minute intervals cytoplasmic fractions were prepared, and 0.5 percent sodium dodecylsulfate (SDS) was added to dissociate the RNA from protein (*10*). The extracts were centrifuged through sucrose density gradients, and the amount of acid-precipitable radioactivity present in the 10 to 30*S* peak characteristic of D-RNA was determined as described (*1*). During the first 30 minutes after RNA synthesis is blocked, one-third of the D-RNA is lost (Table 2). This value is in agreement with the observation that 60 percent of the D-RNA is in polyribosomes of which 55 percent are degraded during a 30-minute exposure to actinomycin. The rate of breakdown decreases with time, and after 2 hours of inhibition 62 percent of the D-RNA and 30 percent of the polyribosomes remain.

The base composition of the polyribosome-associated D-RNA, as well as that of the D-RNA in the 30 to 74*S* region, is unchanged after a 30-minute (or 90-minute) exposure to actinomycin (Table 1). However, the capacity of the remaining polyribosomes to direct protein synthesis is markedly reduced. Cells were exposed for 1 minute to C^{14}-labeled amino acids 7½ hours after infection as described previously (*3*). At this time in the infectious cycle viral protein synthesis has replaced cell protein formation (*6*). Cytoplasmic fractions were then prepared and centrifuged as described above. Acid-precipitable radioactivity is present in the polyribosomes, but not in the single ribosomes or the 30 to 74*S* region of the gradient (Fig. 3*A*). For comparison, cells which had been treated with actinomycin 6 to 7½ hours after infection were also incubated for 1 minute with radioactive amino acids, fractionated, and centrifuged. Protein synthesis was reduced by 90 percent, although considerably more than 10 percent of the initial absorbancy in the polyribosome region remained (Fig. 3*B*). The polyribosomes which remained contained D-RNA and were completely degraded, releasing single ribosomes, when treated with ribonuclease. When protein synthesis in uninfected cells was similarly measured,

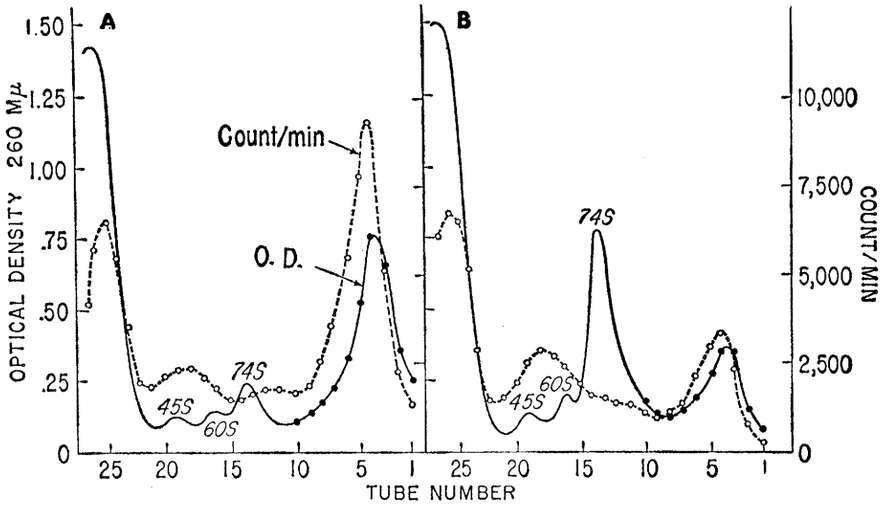

Fig. 2. Sedimentation of cytoplasmic fractions from infected cells. A suspension culture was infected (*1*) and exposed to uridine-2-C^{14} from 5 to 6 hours after infection. The culture was then divided, and one-half was treated with actinomycin (5 μg/ml) for 30 minutes. Cytoplasmic fractions were prepared and centrifuged as for Fig. 1. *A*, Before actinomycin; *B*, after actinomycin.

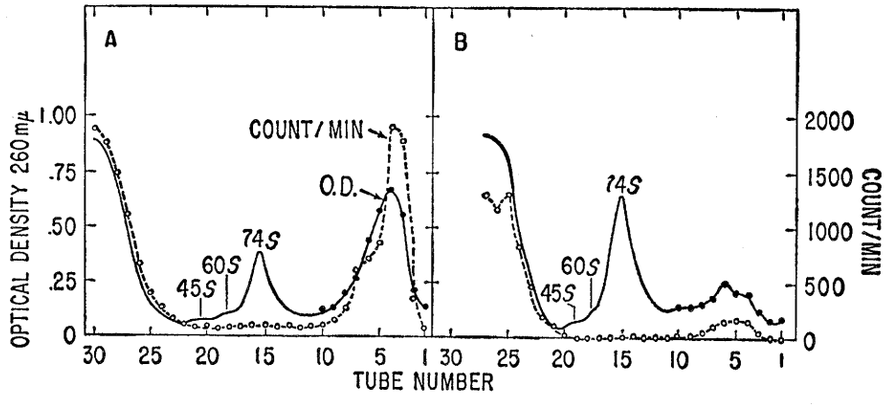

Fig. 3. Inhibition of protein synthesis in actinomycin-treated infected cells. At 6 hours after infection 5 μg of actinomycin per milliliter was added to one (*B*) of a pair of cultures. Ninety minutes later both cultures were centrifuged, all but 15 ml of media was removed, and the cells were resuspended in the remaining volume. The concentrated cell suspension was exposed for 1 minute at 37°C to 25 μc of C^{14}-labeled amino acids in the form of algal protein hydrolysate, and the incorporation was stopped by pouring the suspension onto frozen media. The cells were washed with chilled media and resuspended in RS buffer for fractionation and analysis as described in the text and in the legend to Fig. 1.

acid-precipitable radioactivity was also present only in the polyribosomes and at the top of the gradient, and there was a 20-percent reduction in incorporation of amino acids after a 90-minute exposure to actinomycin (5 μg/ml).

In cells infected with either bacteriophage or vaccinia virus, the formation of infectious virus requires the synthesis of several proteins (*11*). It has been suggested that the sequential synthesis of the different phage proteins may be controlled by the turnover of distinct "messenger RNA's" and the resulting degradation and reassembly of polyribosomes (*12*). A similar type of regulation may operate in vaccinia-infected cells. In these cells, a large fraction of the D-RNA is present in polyribosomes, and more than half of it is degraded to acid-soluble fragments when RNA renewal is blocked by actinomycin. The polyribosomes containing the D-RNA which is degraded in the presence of actinomycin presumably take part in the synthesis of virus structural proteins since the formation of immunologically identifiable viral proteins is interrupted by the presence of actinomycin. Those polyribosomes which remain after exposure to actinomycin are relatively inactive in protein synthesis. In addition to the D-RNA which associates with ribosomes to form polyribosomes, a portion of the D-RNA sediments in the 30 to 74*S* region of a density gradient. Its stability in the presence of actinomycin may be due to association with 45*S* ribosomal subunits or with protein, since its sedimentation coefficient is reduced to 16*S* after deproteinization with phenol or SDS treatment. The D-RNA not in polyribosomes does not direct viral protein synthesis. It may be a precursor of polyribosomes, or involved in the complex sequence of events associated with viral replication.

Table 2. Extent of D-RNA degradation. Infected cells received uridine-2-C^{14} as described (Fig. 1). At the indicated times after addition of 5 μg of actinomycin per milliliter, 5×10^7 cells were fractionated, and SDS (final concentration, 0.5 percent) was added to the cytoplasmic extracts, which were centrifuged (25,000 rev/min, 14½ hours, 15°C) in 5 to 30 percent linear gradients of sucrose dissolved in .01*M* acetate buffer, *p*H 5.1, containing 0.1*M* NaCl and 0.5 percent SDS. The values are the average of two experiments and were obtained by summing the acid-precipitable radioactivity in the 10 to 30*S* region.

Time after adding actinomycin (min)	Acid-precipitable D-RNA remaining	
	(count/min)	(%)
0	14400	100
30	9675	67
60	9625	67
120	8875	62

A. J. SHATKIN
E. D. SEBRING
N. P. SALZMAN
Cell Biology Section, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland

References and Notes

1. N. P. Salzman, A. J. Shatkin, E. D. Sebring, *J. Mol. Biol.* **8**, 405 (1964).
2. Y. Becker and W. K. Joklik, *Proc. Natl. Acad. Sci. U.S.* **51**, 577 (1964).
3. M. D. Scharff, A. J. Shatkin, L. Levintow, *ibid.* **50**, 686 (1963).
4. F. Lipmann, in *Progress in Nucleic Acid Research*, N. Davidson and W. E. Cohn, Eds. (Academic Press, New York, 1963), vol. 1, p. 135; C. Levinthal, D. P. Fan, A. Higa, R. A. Zimmerman, *Cold Spring Harbor Symp. Quant. Biol.* **28**, 183 (1963).
5. E. Volkin and L. Astrachan, *Virology* **2**, 149 (1956).
6. A. J. Shatkin, *Nature* **199**, 357 (1963).
7. M. Girard, S. Penman, J. E. Darnell, *Proc. Natl. Acad. Sci. U.S.* **51**, 205 (1964).
8. S. Penman, K. Scherrer, Y. Becker, J. E. Darnell, *ibid.* **49**, 654 (1963).
9. E. Reich, R. M. Franklin, A. J. Shatkin, E. L. Tatum, *ibid.* **48**, 1238 (1962).
10. W. Gilbert, *J. Mol. Biol.* **6**, 389 (1963).
11. S. S. Cohen, in *Perspectives in Virology III*, M. Pollard, Ed. (Harper & Row, New York, 1963); G. Appleyard, J. C. N. Westwood, H. T. Zwartouw, *Virology* **18**, 159 (1962).
12. M. Sekiguchi, S. S. Cohen, *J. Biol. Chem.* **238**, 349 (1963).
13. E. D. Sebring, N. P. Salzman, *Anal. Biochem.* **8**, 126 (1964).

11 February 1965

Bird Mortality after Spraying for Dutch Elm Disease with DDT

Abstract. *In Hanover, New Hampshire, where elms were sprayed with DDT, 151 dead birds were found; 10 dead birds were found in Norwich, Vermont, where no DDT was used. Chemical analyses of dead birds, observation of symptoms of DDT poisoning, and a population decline after spraying all indicate severe mortality among certain species in Hanover.*

In an attempt to control Dutch elm disease, the use of DDT (*1*) has become widespread in the United States. While its effectiveness in controlling the disease is uncertain and controversial (*2*), evidence of bird mortality following its use has become extensive (*3–10*). Because no studies have been conducted in the northeastern states (*4*), it has been presumed that abnormal mortality does not occur in this area (*5*); observation of dead birds following annual DDT spraying in Hanover, New Hampshire, however, suggested that this presumption was incorrect. We therefore undertook a study (*11*) to evaluate the effects of DDT on local birds, using the unsprayed town of Norwich, Vermont, 1 mile (1.6 km) west of Hanover, as a reference area.

About 2300 of Hanover's elms, occurring on 670 acres (271 hectares), were sprayed with 1285 lb (583 kg) of DDT by a Rotomist on 15–18 April 1963; dosage averaged 1.9 lb/acre (2.1 kg/hectare), but varied widely because of uneven elm distribution. Such a dormant (prefoliar) spray has been used in Hanover for about 15 years. DDT was replaced by Methoxychlor (*1*) in 1964 and was similarly applied.

From April through July of 1963 and 1964 we counted all birds identified by sight or sound (*12*) in ecologically comparable study areas of Hanover and Norwich. Concurrently, with the aid of residents, dead or dying birds were collected in both towns. Analyses for DDT, DDE, DDD (*1*), fat, and water (*13*) were performed on 106 birds (*14*), including those exhibiting tremors (*15*) prior to death, birds representing a variety of species and feeding habits, some showing injuries, and five reference robins, *Turdus migratorius*, from unsprayed areas. The brain, liver, breast muscle, heart, gonads, and remainder of 48 robins, and whole carcasses of 58 other birds of 25 species, were analyzed.

During 1963, 151 specimens (29 with tremors) of 34 species were found in Hanover; 10 dead birds (none with tremors) were found in Norwich. In 1964, 72 birds (6 with tremors) came from Hanover, while 8 (none with tremors) came from Norwich. Most dead birds, especially small, obscure ones such as sparrows or warblers, will not be found, so these data presumably represent a minor fraction of the total dead birds (*6, 16*).

The robin population in Hanover by 1 June 1963 had fallen 70 percent below the original 1 May population;