ACTION OF ACTINOMYCIN D ON ANIMAL CELLS AND VIRUSES

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Actinomycin D is a brightly colored, peptide-containing antibiotic, discovered by Vining and Waksman.[1] It is exceedingly toxic to higher organisms and strongly inhibits the growth of a number of natural[2] and experimental tumors as well as of a variety of gram-positive bacteria. The structural and chemical properties of actinomycins have been elaborated in great detail.[3]

It has been reported that actinomycin is strongly bound to DNA, but not to RNA, in solution[4] and remains bound in the course of ultracentrifugation,[4] electrophoresis,[5] and dialysis.[6] Kirk[6] demonstrated that actinomycin could inhibit the activity of an enzyme fraction obtained from bacteria capable of incorporating radioactive deoxynucleoside triphosphates into DNA. She concluded that the inhibition of the biosynthesis of RNA, DNA, and protein which she had observed in Micrococcus could be attributed to interference with the action of the DNA polymerase,[7] the enzyme presumably responsible for normal DNA replication.

We have previously reported that the primary action of actinomycin on mammalian cells in culture is a specific, selective inhibition of cellular RNA synthesis.[8] This paper sets forth the detailed experimental findings which have led to the above conclusion, and describes the biochemical and biological properties of cells exposed to the action of actinomycin D. The experiments show: (1) that the biosynthesis of normal cellular RNA may be completely uncoupled by actinomycin from the biosynthesis of both DNA and protein, for prolonged periods; (2) that the synthesis of RNA normally produced by the cell strains we have examined may be *totally* suppressed by actinomycin; (3) that the growth of Mengovirus, whose genetic material consists of RNA, is unaffected by actinomycin concentrations much higher than those which inhibit RNA synthesis (99.9%) by host cells.

Materials and Methods.—Mouse fibroblasts (strain L-929) were maintained, propagated, and infected, under conditions and with the viral strains previously described.[9, 26]

Autoradiography was performed on cultures grown on coverslips. These were maintained and infected in parallel with other monolayer cultures used for experiments in which viral and/or cellular growth were being observed. ^{3}H-Cytidine (all radioactive substances were obtained from New England Nuclear Corp., Boston, Mass.) was used to follow synthesis of DNA and RNA (final concentration 0.5 μg/ml, 0.5 μc/ml). The period of exposure to radioactive cytidine was varied from 2–10 hours, and the radioactivity incorporated into acid-insoluble material measured by the methods of Doniach and Pelc[10] as in previous work.[9] Following fixation and extraction with dilute perchloric acid, replicate coverslips were mounted for autoradiography (*a*) without further treatment or (*b*) following exposure to DNase or RNase (1.5 hours, 37°C, Tris buffer pH 8.0, 0.001 M), permitting estimation of the proportion of radioactivity present in DNA and RNA.

Suspension cultures of mouse fibroblasts, strains L-929 and L-2, were grown according to McLimans *et al.*[11] and Siminovitch *et al.*[12] respectively. Suitable concentrations of actinomycin were added to the cells growing at initial population densities of 2.5–6 $\times$ 10^{5} ml. After 30 minutes radioactive precursors were added to the following final concentrations: either D,L-leucine-4,5-^{3}H, (1.0 μc/ml., 0.0002 or 0.0004 M) and thymidine ^{3}H-methyl, (0.25–0.5 μc/ml., 0.5–2.0 μg/ml) or ^{3}H-uridine, (0.25–0.5 μc/ml, 0.8–1.0 μg/ml). Aliquots of the cultures were withdrawn at appropriate intervals. The cells were washed twice with saline, three times with 0.25 N perchloric acid, once with ethanol-ether (1:3) and with ether. Radioactivity of RNA was determined by plating, at infinite thinness, centrifuged aliquots of an alkaline hydrolysate (0.5 N KOH; 18 hr, 37°)

which had been neutralized with perchloric acid; that of DNA by plating similar aliquots of an acid-hydrolysate (0.5 N perchloric acid, 70°, 1 hr) neutralized with the theoretical amount of KOH. The insoluble residue remaining after acid hydrolysis was washed with ethanol-ether and ether, and an aliquot was used for measuring incorporation of leucine into protein. Most of the determinations of radioactivity were performed in a gas-flow counter; in later experiments the Tri-Carb Liquid Scintillation Spectrometer was used for this purpose. Actinomycin D was generously supplied by Dr. K. Folkers of Merck, Sharp and Dohme Laboratories, Rahway, N. J.

Results.—Effects of actinomycin on cell growth: The effect of progressively increasing concentrations of actinomycin in growing cultures is shown in Figure 1. It is seen that a very low level* of the drug suffices to inhibit cell division completely. Marginal concentrations permit some cell division, following which the mitotic activity of the culture stops.

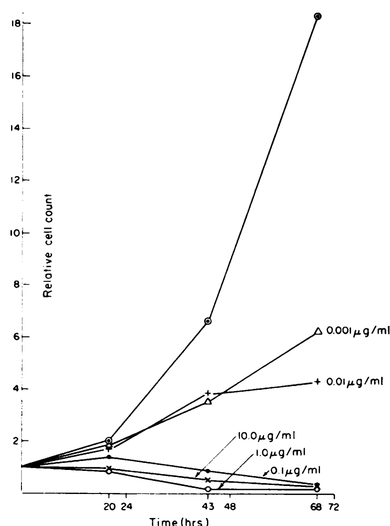

FIG. 1.—Growth inhibition of L-cell monolayers by actinomycin D. The antibiotic was added at time 0 to each of a series of monolayer cultures in exponential phase of growth as in ref. 9.

Cytological effects of actinomycin: High levels of actinomycin (>0.5 µg/ml) are tolerated by cells for at least 24 hours. After this, cells begin to detach from the surface of the Petri dish and are lost to the medium. Those which remain progressively lose their stainable RNA, cytoplasmic as well as nucleolar. The cytoplasm shrinks, while the nucleus frequently undergoes considerable enlargement. These observations resemble those reported by Goldstein *et al.*[13]

When a growing culture is exposed to ^{3}H-cytidine, radioactivity is incorporated into an acid-insoluble form and the extent of this incorporation may be estimated by autoradiographic techniques. Normally a portion of the incorporated radioactivity can be rendered acid-soluble by treating the fixed cells with deoxyribonuclease, the remainder being sensitive to ribonuclease.

In the presence of actinomycin D (>0.5 µg/ml) ^{3}H-cytidine is incorporated almost wholly into the nucleus and the acid-insoluble radioactivity is removed by an incubation with DNase (Fig. 2). The rate of incorporation of ^{3}H-cytidine into ribonuclease-sensitive material may be reduced to 0.1% of normal following 8 hours' exposure to a high level of actinomycin (2 µg/ml for the cell densities employed in this experiment[14]).

Macromolecule synthesis by cells exposed to actinomycin D: The data in Figures 3–5 illustrate the effects of different concentrations of actinomycin on RNA, DNA, and protein synthesis in growing suspensions of L-cells. It is apparent that RNA synthesis is very sensitive to the antibiotic. Concentrations of actinomycin which inhibit incorporation into RNA by more than 90% permit substantially normal rates of DNA and protein synthesis for extended periods, although biosynthesis of the latter two components is ultimately inhibited by the higher level of drugs used.

It may be noted that the incorporation of radioactive leucine into protein is

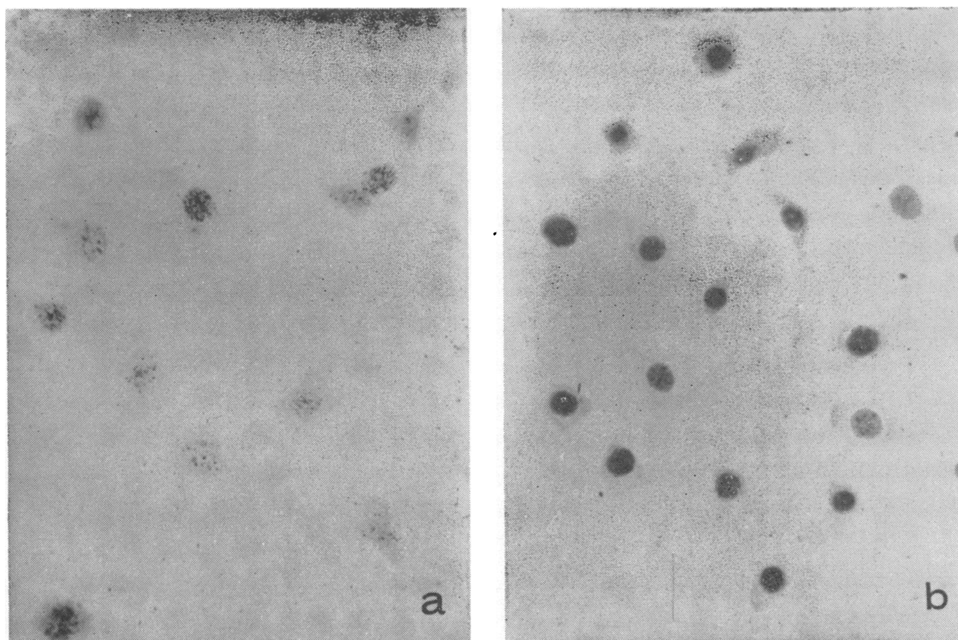

FIG. 2.—Autoradiography of L-cells exposed to actinomycin D (1μg/ml) and ^{3}H-cytidine for 2.5 hr (for conditions see text). Film exposed for 72 hr following: (a) incubation in buffer, (b) digestion with deoxyribonuclease.

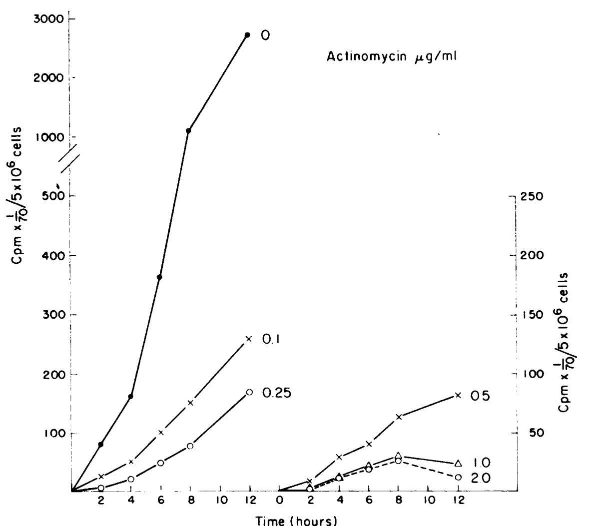

FIG. 3.—Incorporation of ^{3}H-uridine into RNA in cultures exposed to actinomycin D. Note separate scale on right for highest concentrations of antibiotic.

probably not due merely to processes involving exchange of protein residues with amino acids in the cellular pool. In a separate experiment, the rate of incorporation of radioactive leucine in a culture containing actinomycin (1 μg/ml) was reduced by 14% relative to the control. The degree of reduction of this surviving incorporation by Puromycin,† a specific inhibitor of protein synthesis, was 47% in the actinomycin-treated and 41.5% in the control cultures.‡

Effect of actinomycin on multiplication of RNA- and DNA-containing animal viruses: (a) *RNA viruses:* Mengovirus is an RNA-virus. Previous experiments,[9] in which Mitomycin was used to destroy the host cellular genome, have shown that the growth of Mengovirus is not affected under conditions in which host cell DNA- and RNA-biosyntheses are no

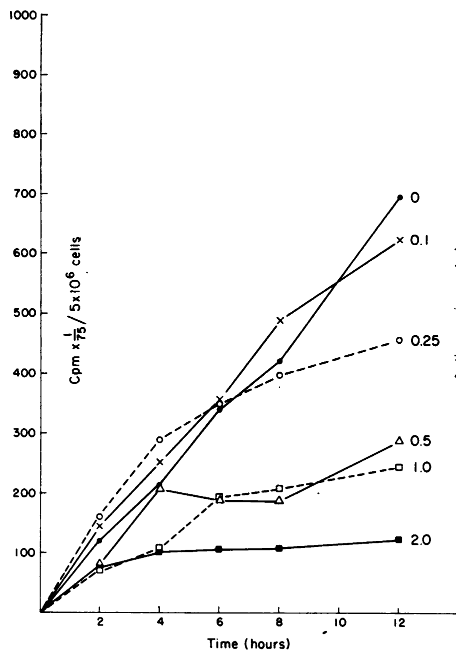

FIG. 4.—Incorporation of ^{3}H-thymidine into DNA in L-cells exposed to actinomycin D.

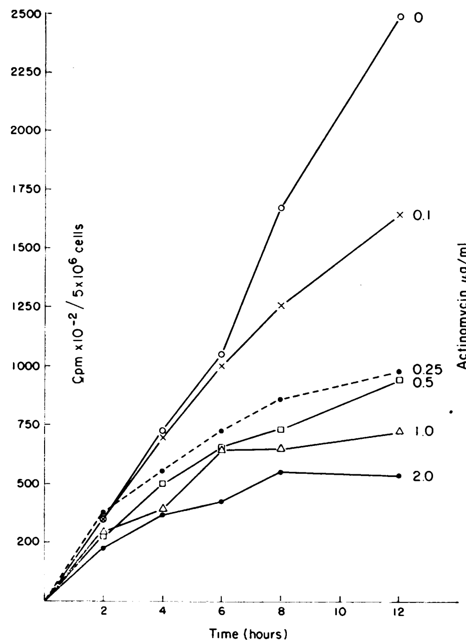

Fig. 5.—Incorporation of ^{3}H-leucine into protein in L-cell cultures containing various concentrations of actinomycin D.

longer detectable due to the extensive damage to the governing templates.

The data in Table 1 show that even very high concentrations of actinomycin do not inhibit Mengovirus growth. It is seen that when the antibiotic is applied with the virus, and maintained throughout the viral growth cycle, normal virus yields are obtained. Equally normal yields result when the host cells are pretreated with actinomycin 24 hours prior to infection. (Under these conditions actinomycin may be presumed to be present in the cell at the time of infection owing to its concentration in the cell nucleus where it is quantitatively, but not irreversibly, bound to deoxyribonucleoprotein.[15])

TABLE 1

EFFECT OF ACTINOMYCIN ON MENGOVIRUS MULTIPLICATION

(a) Compounds added during adsorption and present throughout = *S*. (b) Compounds added for 8 hr and virus added 24 hr after compounds = *P*. Yields at 12 hr.

	Cells/plate	Virus/plate	Virus/cell
Control 0	1.3×10^5	6.0×10^4	0.46
12 hr		1.1×10^8	846
A/S 0.5 µg/ml		1.2×10^8	962
5.0 µg/ml		1.4×10^8	1080
10.0 µg/ml		1.3×10^8	1000
A/P 0.5 µg/ml	7.7×10^4	2.4×10^8	3180
5.0 "	8.6×10^4	8.5×10^7	988
10.0 "	9.0×10^4	6.5×10^7	722

Poliovirus growth is resistant to actinomycin (Table 2). Preincubation of host cells with actinomycin did not significantly affect poliovirus yields under conditions in which RNA biosynthesis of uninfected cells (measured by incorporation of

uridine-2-^{14}C into acid-insoluble, alkali-labile form) is at least 95% inhibited. These results were not changed by addition of actinomycin at other times before, during, or after adsorption of virus.

TABLE 2

EFFECT OF ACTINOMYCIN ON POLIOVIRUS MULTIPLICATION

Time after infection (hr)		pfu/ml
2	Control	9×10^6
$6^1/_2$	"	1.3×10^9
2	+ actinomycin	8×10^6
$6^1/_2$	"	1.4×10^9

A suspension (2×10^7/ml) of HeLa cells (strain S-III) was exposed to acti omycin (5 μg/ml) for 30 min at 37°, infected at a multiplicity of 100 with poliovirus type I for 20 min in the presence of the antibitiotic. Following centrifugation and washing with medium containing actinomycin (5 μg/ml), the cells were resuspended (at 5×10^6/ml) and permitted to produce virus. The level of actinomycin (5 μg/ml) was maintained throughout. Uninfected cultures in parallel were used to measure inhibition of incorporation of uridine-2-^{14}C into RNA. These showed the following inhibition with respect to control: 0–4 hr, 95%; 4–$6^1/_2$ hr, 100%.

(b) *Vaccinia virus:* The results summarized in Table 3 show that the growth of vaccinia virus is inhibited by actinomycin, high concentrations of which completely suppress virus yield. Since washing the host cells following an 8-hour exposure to actinomycin probably removes little, if any, of the drug,[14] the infecting vaccinia DNA may be assumed to compete with the resident cellular DNA for the residual bound actinomycin. Any drug bound to the infecting DNA might then be expected to inhibit the expression of virus-controlled functions and the growth of virus by preventing the formation of vaccinia-specific RNA. The results show that this expectation is fulfilled. It is also seen that when the antibiotic is added to the incubation medium simultaneously with the virus a given concentration of actinomycin causes a greater inhibition of virus growth than the same amount applied to the host cells for 8 hours one day preceding infection.

TABLE 3

EFFECT OF ACTINOMYCIN ON VACCINIA GROWTH

Experiment carried out as described for Mengovirus (cf. Table 1). Yield taken at 24 hr.

	Cells/plate	Virus/plate	Virus/cell	Comments
Control 0	2.5×10^5	1.6×10^5	0.64	
24 hr		1.5×10^7	60.0	
A/S 0.1 μg/ml		1.4×10^5	0.56	
0.5 "		3.5×10^4	0.14	
5.0 "		2.2×10^4	0.088	
10.0 "		2.4×10^4	0.096	
A/P 0.1 μg/ml	2.2×10^5	1.8×10^6	8.2	Typical vaccinia CPE
0.5 "	2.5×10^5	1.6×10^6	6.4	"
5.0 "	1.9×10^5	3.0×10^4	0.16	Little or no CPE
10.0 "	2.7×10^5	7.5×10^4	0.28	No CPE

Preincubation of intact virus with actinomycin, followed by dialysis, caused no inhibition of virus growth, as was previously the case with Mitomycin.[9] This finding does not rule out the possibility that some actinomycin was actually bound to the viral DNA. Since the amount of viral DNA is small in relation to that of the host cell, any actinomycin complexed to the infecting genome would probably be taken up preferentially by the much larger quantity of cellular DNA present, permitting viral multiplication to proceed. This interpretation is consistent with our finding that the division of the host cell is inhibited by $^1/_{20}$th the concentration of actinomycin required to cause significant impairment of vaccinia growth.

Discussion.—The results reported herein demonstrate that actinomycin D at appropriate concentrations selectively suppresses RNA synthesis in L cells. Concentrations of the antibiotic which diminish RNA synthesis by 99% permit substantial rates of protein and DNA synthesis for prolonged periods. In its susceptibility to Puromycin the synthesis of protein observed under these conditions appears to resemble that which occurs normally in the cell. It is obvious that these results are not compatible with any formulation which requires the participation of a short-lived intermediate, continuously regenerated by the cellular genome, in protein synthesis. These observations thus reinforce the conclusions drawn from previous studies of induced enzymes in bacteria,[17] and suggest that in mammalian, as in bacterial cells, the synthesizing units which govern the production of a given protein display considerable, if not absolute, stability. The biosynthesis of alkaline phosphatase in bacteria, which was seen to survive the action of Mitomycin, is thus analogous with that of hemoglobin in rabbit reticulocytes, which is unaffected by high concentrations of Mitomycin and actinomycin.[18] From these and from other experiments which will be published in the near future it appears reasonable to conclude that the genetic information required for protein synthesis is embodied in stable ribosomal templates, capable of protracted independent function but subject, especially in bacteria, to superimposed control mechanisms.

The inhibition of cellular RNA synthesis by actinomycin, in bacterial[16] and mammalian cells,[18] is complete (>99%) at high concentrations of the antibiotic, and includes all fractions of the cellular RNA—nuclear, ribosomal, and "soluble" RNA. Thus the entire complement of the RNA of uninfected cells appears to be produced in an actinomycin-sensitive, and DNA-dependent reaction, none resulting from autonomous replication. Therefore, it is of particular interest that the growth of Mengovirus and poliovirus appear completely resistant to actinomycin, as was previously also seen for Mitomycin. Both lines of evidence suggest that the biosynthesis of the RNA of these RNA viruses is a process fundamentally different from that of the biosynthesis of RNA of the normal cell, and that this biosynthesis does not require the participation of either the host DNA, or of a DNA molecule which might be formed as an intermediate in the expression of viral genetic potentialities. It might be anticipated that this abnormal RNA synthesis would require the formation of a new enzyme following infection with RNA viruses.

The findings reported herein are supported by experiments with enzyme fractions, from mammalian[19] and bacterial[20] sources, capable of catalyzing the DNA-dependent synthesis of RNA. In these investigations actinomycin powerfully inhibits the synthesis of polyribonucleotides, confirming the expectations based on *in vivo* experiments. The action of the antibiotic presumably results from its binding to DNA, and since normal rates of DNA biosynthesis persist following substantial inhibition of RNA synthesis it seems probable that the enzymes catalyzing DNA-biosynthesis and DNA-dependent RNA biosynthesis differ significantly in their respective stereochemical relationship to the DNA molecule.

In these experiments, as in previous ones[9] in which growth of vaccinia virus has been observed, we have been able to confirm the findings of Cairns,[27] who demonstrated cytoplasmic DNA synthesis in cells infected with vaccinia. In cells exposed to actinomycin, whether by pretreatment before infection, or simultaneously with the infecting virus, production of significant amounts of virus could always be

correlated with the characteristic cytopathogenic effect which its growth produces and with the presence of cytoplasmic centers of DNA synthesis (as shown by autoradiographically demonstrable ^{3}H-thymidine incorporation into acid-insoluble material). Conversely, conditions which inhibited virus yield also inhibited the appearance of the cytopathogenic effect and cytoplasmic DNA synthesis. It may be significant that cellular DNA synthesis is relatively resistant to actinomycin, which preferentially inhibits RNA synthesis, whereas vaccinia DNA-synthesis is relatively sensitive. This apparently anomalous result could be explained if the synthesis of new enzymes were required before such a virus specific DNA-synthesis could proceed. The production of these enzymes, which would depend on an antecedent RNA synthesis, would display the actinomycin sensitivity characteristic of a DNA-dependent RNA synthesis. Thus the over-all process of vaccinia DNA-synthesis would appear as sensitive as that of the complete virus and resemble that of a DNA-dependent RNA synthesis.

The observations recorded in this and in preceding papers[9, 21] are of significance in relation to the radiosensitivity of the "capacity"[25] of host cells to support viral growth. Mitomycin C, which selectively causes destruction of cellular DNA and therefore of host cell genes, does not affect the growth of animal[9] and bacterial[22] RNA viruses; and temperate[23] as well as virulent[24] DNA bacteriophages may replicate and express their genetic potentialities following pretreatment of the host cells to the point of complete loss of genetic potency in all genetic tests. Actinomycin, by inhibiting DNA-dependent RNA synthesis, blocks expression of the genetic potentialities of the host. Nevertheless, the growth of several viruses at least is unaffected by this agent.

Thus, the integrity and functioning of cellular DNA can be abolished without seriously affecting virus growth. The radiosensitivity of host cell capacity for virus production cannot, therefore, represent damage only to genetic functions of the host cell, but would represent effects of primarily nongenetic origin. Irradiation of host cells prior to infection may be visualized as damaging genetic as well as nongenetic cellular entities. Damage to the latter might be expected to cause corresponding impairment of subsequent virus growth, since any affected radiosensitive structures of the host (e.g., transfer RNA, important enzymes, etc.) required for virus growth could not be repaired or replaced due to simultaneous damage to the controlling gene.

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Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

* The effect of a given level of actinomycin is dependent on amount of uptake (this being a function of length of exposure at any given concentration[15]) and cellular population density.[6]

† 10 μg/ml.

‡ Analogous experiments[18] have been performed with several gram positive organisms whose growth and RNA biosynthesis are susceptible to inhibition by actinomycin. As was first shown by Kirk,[6] these bacteria resemble mammalian cells in that protein synthesis may continue at approximately 25% of normal rates for about $^2/_3$ of a generation time at concentrations which inhibit RNA and DNA biosynthesis by about 95% and 50% respectively.

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FURTHER IMPLICATIONS OF THE DOUBLET CODE

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It is commonly assumed at present that the order of groups of nucleotides in RNA determine the order of amino acids in the protein which is assembled by using that RNA as a template. The "biological code" is the correlation of groups of nucleotides ("words") with amino acids. If more than one word corresponds to an amino acid, the words are said to be "degenerate"; if more than one amino acid corresponds to a word, the word is said to be "ambiguous." If no amino acid corresponds to a word, the word is designated "nonsense." If a group of three nucleotides designates an amino acid, the code is triplet and there are 64 possible words. If a group of two nucleotides designates an amino acid, the code is called a doublet and there are 16 possible words. It is evident that a doublet code must contain apparently ambiguous words since there are 20 amino acids which must be designated by 16 words.

At present the various experimental data which indicate the correspondence of amino acids to code words are usually interpreted in terms of a triplet code, but a number of inconsistencies appear in this interpretation. All of the words indicated by studies of the amino acid incorporation[1-7] stimulated by synthetic polynucleotides contain at least one uracil (U). If the templates for protein synthesis were