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Thymol Suppression of Protein Synthesis in Influenza Virus-Infected and Uninfected Chick Embryo Fibroblast Cells[1] (39759)

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Studies in this laboratory showed that thymol in moderate concentrations inactivated influenza viruses, herpesviruses, and several other lipid-enveloped viruses (1). The surface proteins of the influenza virus, however, retained appreciable activity when exposed to inactivating concentrations of thymol.

To seek information on the possible mode of action of thymol, a lipid-enveloped membrane system consisting of human erythrocytes in saline suspension was exposed to thymol (2). Thymol accumulated in the lipid membrane of the red cell and concentrated 50-fold intracellularly as a result of its association with hemoglobin in a 2:1 molecular ratio. From these data it appeared that thymol might act by penetrating the envelope of lipid-enveloped viruses and inactivate internal protein(s) essential for virus replication. Cell protein synthesis would also likely be inhibited by thymol by a similar mechanism.

In a test of the hypothesis it was found that thymol did not interfere with uptake of radioactive leucine into infected and uninfected cell cultures, but it profoundly reduced the incorporation of the isotope into protein.

Materials and methods. Virus and cell cultures. Influenza A/PR/8/34 (HON1) was obtained from Dr. Walter Dowdle, CDC, USPHS, Atlanta, Ga., and inocula used in these experiments were grown in rhesus monkey kidney cell culture. Chick embryo fibroblast cells (CEF) were prepared from 9- to 11-day-old embryonated eggs by trypsinization of minced embryos. Experiments were carried out in 150 × 25-mm plastic culture dishes (Falcon Plastics, Oxnard, Calif.).

Cell cultures grown in Eagles minimum essential medium (MEM) supplemented with 10% fetal calf serum and confluent, after 4- to 5-days incubation in 5% carbon dioxide atmosphere, were inoculated with 4.0 ml of a 1:10 dilution of virus (initial titer $10^{8.0}$ EID_{50}/0.2 ml. (The m.o.i. was approximately 8). After adsorption for 1.5 hr, the inoculum was poured off and 12–15 ml of Eagles MEM with 2% fetal calf serum was added.

Consecutive pulse and chase experiments with [^{14}C]- and [^{3}H]leucine with infected cells were begun at 16 hr after removal of inoculum. Uninoculated cultures from the same batch were used in parallel experiments. All experiments were carried out in a 37° incubator room; all fluids were brought to 37° before use. Before pulsing, plates were rinsed with Hank's balanced salt solution. After pulsing they were rinsed once with balanced salt solution before adding Eagles MEM with 2% fetal calf serum during the chase period. At the time of harvesting cells, chase fluid was removed and the cells were cooled on ice and harvested with a rubber policeman. Cell harvests were sonicated on ice three times for 20 sec at setting 43 (Biosonik). The sonicates were centrifuged for 10 min at 12,062*g* in a refrigerated centrifuge, and the supernatant was saved.

Pulse and chase experiments. Experiments 64, 70, 78, and 80 each consisted of two sets of infected and two sets of uninfected cell cultures. They were each processed as follows: The two sets of infected and the two sets of uninfected cells were pulsed for 15 min with [^{14}C]leucine and chased for 30 min for control purposes. Immediately thereafter each set of infected and uninfected cell cultures was pulsed and chased as above with [^{3}H]leucine. However, 0.66 m*M* (100 μg/ml) thymol was added to one set of in-

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fected and one set of uninfected cells during the tritium pulse. All experiments were carried out with the same molar concentrations of leucine.

Protein estimation. Sonicates precipitated with a 5% final concentration of trichloracetic acid were washed twice in distilled water, dissolved in dilute sodium hydroxide, and assayed for protein by the Lowry *et al.* procedure (3). Aliquots of dissolved precipitates were counted in a Beckman Model LS-250 liquid scintillation counter after the addition of 5 ml of Aquasol.

Hemagglutination titration. This procedure was performed by standard methods in plastic microtiter "U" plates obtained from Cooke Engineering Company, Alexandria, Va.

Polyacrylamide gel electrophoresis. Polyacrylamide gels, 7.5%, in 12.5-cm siliconized glass tubing were prepared in the laboratory or purchased from Bio-Rad Laboratories, Richmond, Va. Material was prepared in the laboratory following the procedure of Skehel and Schied (4). Viral proteins were dissociated in 4 *M* urea, 4% SDS, and 2% mercaptoethanol by heating at 90° for 2 min. Samples were run, 50 μl of sonicate/gel, in Bio-Rad gel electrophoresis apparatus Model 150 with Bio-Rad 0.1% SDS buffer mixture. Gels were run at 2 mA/gel (30–40 V) and required about 20 hr to complete a run as determined by progress of a tracking dye. Gels were sliced in a template and eluted in 0.5 ml of 0.1% SDS overnight at 37° with shaking. Scintillation counting was done after the addition of 5 ml of Aquasol in a Mark I Nuclear Chicago liquid scintillation counter. Each sample was counted three times for 10 min each. Counts per minute (cpm's) of the double isotope-labeled gels were converted to dpm's of the respective isotopes by a computer program prepared by one of us (M.W.N.).

Chemicals. Tritiated L-[4,5-^{3}H]leucine and L-[^{14}C]leucine were obtained from Schwartz-Mann, Orangeburg, N.J. The specific activities of the isotopes used in experiment 78 are described in the footnote of the table in the appendix that describes this experiment.

Thymol, N. F., was obtained from J. T. Baker Chemical Company, Phillipsburg, N.J. Thymol stock solution, 6.6 m*M* (1 mg/ml) or 3.3 m*M* (500 μg/ml), was freshly prepared by heating crystals in distilled water at 75–80° until dissolved.

Results. Effect of thymol on incorporation of leucine into viral and cellular proteins. The amount of leucine incorporation into protein for the several experiments is shown in Table I. The method of calculation is illustrated with data from experiment 78 in the appendix.

From Table I it was calculated that in the presence of thymol (0.66 m*M*) there was an 80% suppression of leucine incorporation into infected cells and a 73% suppression into uninfected cells. Statistical analysis of these data (see appendix) showed in both instances that the reductions were highly statistically significant ($P < 0.0005$). The possibility was considered that thymol which remained in the sonicate might cause the alterations in the separation of the proteins in the gel during electrophoresis. However, analysis of one sonicate showed that only 0.5 μg/ml of thymol remained (5), and the addition of thymol at a final concentration of 0.33 m*M* to a mixture of sonicate and SDS buffer before electrophoresis had no effect on the gel pattern.

The suppressive effect of thymol on protein synthesis by infected cell cultures from experiment 78 is shown in Fig. 1. The dashed lines show the pronounced reduction of incorporation of leucine in infected cells exposed to thymol. The solid line shows the incorporation in the control experiment. In other experiments the effect was progressively reduced with thymol concentrations of 0.33 and 0.17 m*M*.

Effect of thymol on the molecular weight of newly formed proteins. Incorporation of leucine into high molecular weight proteins was found to be less reduced than into low molecular weight proteins in the presence of thymol, in both infected and uninfected cultures, than in similar cultures not treated with thymol. Quantitatively the effect was slight and can be seen in Fig. 1. An analysis of experiments 70, 78, and 80 showed that 45.2% of the total dpm of gels of uninfected cells exposed to thymol were in the first 10 gel slices, while only 24.5% of the total dpm were in the first 10 gel slices of uninfected cells not exposed to thymol ($P < 0.025, t = 4.86$, one-tailed tests, 2 *df*). In infected cells

TABLE I. LEUCINE INCORPORATION INTO PROTEIN IN INFLUENZA VIRUS-INFECTED AND UNINFECTED CHICK EMBRYO FIBROBLAST CELLS IN THE PRESENCE AND ABSENCE OF THYMOL.

Cell group	Experiment number	Sample	First pulse (15 min) and chase (30 min) (μmole of leucine incorporated/mg of protein)[a]	Second pulse (15 min) and chase (30 min) (μmole of leucine incorporated/mg of protein)[b]	
Infected	64	1	5.60×10^{-4}	7.80×10^{-4}	
		2	4.03×10^{-4}	8.65×10^{-5}	(thymol)
	70	1	2.30×10^{-3}	2.02×10^{-3}	
		2	1.84×10^{-3}	3.52×10^{-4}	(thymol)
	78	1	9.25×10^{-4}	8.18×10^{-4}	
		2	9.66×10^{-4}	1.63×10^{-4}	(thymol)
	80	1	1.45×10^{-3}	1.02×10^{-3}	
		2	1.49×10^{-3}	2.19×10^{-4}	(thymol)
Uninfected	64	1	1.57×10^{-3}	2.20×10^{-3}	
		2	1.40×10^{-3}	2.92×10^{-4}	(thymol)
	70	1	2.90×10^{-3}	2.19×10^{-3}	
		2	3.20×10^{-3}	7.67×10^{-4}	(thymol)
	78	1	1.88×10^{-3}	1.43×10^{-3}	
		2	1.83×10^{-3}	4.73×10^{-4}	(thymol)
	80	1	2.21×10^{-3}	1.76×10^{-3}	
		2	2.68×10^{-3}	5.78×10^{-4}	(thymol)

[a] The first pulse was a control period using [^{14}C]leucine.

[b] The second pulse was an experimental period and a control period using tritiated leucine. Thymol, 0.66 m*M* (100 μg/ml), was present during the second pulse period of sample 2 in infected and uninfected cells of each experiment. Each experiment was, therefore, controlled both horizontally and vertically. The method of determining the effect of thymol on protein synthesis is described in the appendix.

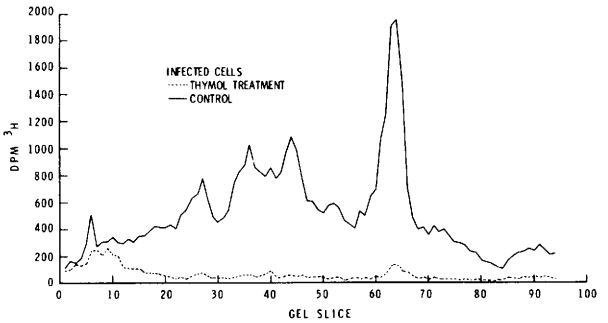

FIG. 1. Counts per minute of polyacrylamide gel (7.5%) of sonicate of CEF cell cultures infected with influenza A/PR/8 exposed to 0.66 m*M* (100 μg/ml) thymol during a 15-min pulse with tritiated leucine. Cells were harvested following a 30-min chase period (dashed line). Counts per minute of gel of sonicate of infected cells pulsed and chased with tritiated leucine without thymol (solid line). Data are from experiment 78.

exposed to thymol 33.3% of dpm were in gel slices 1–23 in contrast to 10.7% in gel slices 1–23 from infected cells not exposed to thymol ($P < 0.05$, $t = 4.15$, one-tailed test, 2 *df*).

Coelectrophoresis of a sonicate of influenza virus-infected CEF cells and purified A/PR8 virus showed viral peaks at gel slices 28, 36, and 63 (Fig. 2). A "P" protein was not present in the sonicate unless it occurred as a "shoulder" on the leading edge of the peak at gel slice 28. The "P" protein in any event would likely have been quantitatively less than the peak in Fig. 1, since this protein is difficult to demonstrate with PR8 virus. It seems most probable, therefore, that the slower moving proteins are cellular in origin and represent proteins whose synthesis is relatively more resistant to the effect of thymol than proteins of lower molecular weight.

Other evidence of viral protein identity

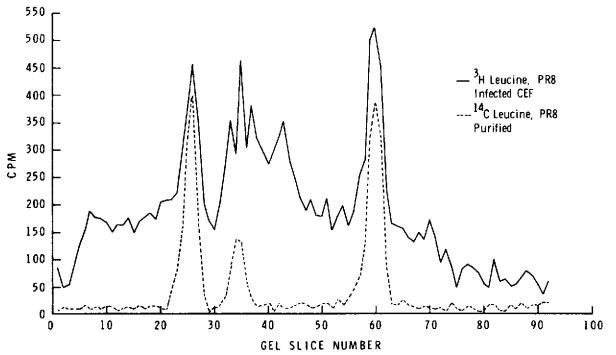

FIG. 2. Coelectrophoresis on 7.5% polyacrylamide gel of sonicate of PR8-infected CEF and purified PR8 virus. Solid line indicates cpm of sonicate following 15-min pulse with tritiated leucine followed by a 30-min chase. The dashed line indicates the cpm of [^{14}C]leucine-labeled PR8 virus purified by sucrose density gradient equilibration.

was limited to detection of hemagglutinating material in sonicates of infected cells. Three sonicates (experiments 70, 78, and 80) treated with thymol all had hemagglutination titers of 1:128, while three not treated with thymol had titers of 1:128, 1:512, and 1:1024. Uninfected cell sonicates had no measurable hemagglutinating activity.

Kinetics of the effect of thymol on leucine incorporation in infected and uninfected cell cultures. Sets of infected (Fig. 3) and uninfected (Fig. 4) cell cultures pulsed with tritiated leucine were harvested at 0 hr (pulse fluid was added and poured off immediately), during pulse at 7.5 min, at the end of the pulse period at 15 min, and at 45 min (after a 30-min chase). Second sets of infected and uninfected cell cultures were processed as above except that 0.33 m*M* (50 μg/ml) thymol was added during the pulse period. Table II shows the counts per minute per milligram of TCA-precipitable protein in the sonicates and the counts per minute of supernatants of TCA-precipitable protein in the sonicate adjusted to milligrams of protein. Counts of the supernatant will largely represent intracellular leucine.

Figure 3 is a graph of results of the study of infected cell cultures with and without thymol during the pulse and chase period. The counts per minute of supernatants in both experiments were similar. Incorporation of leucine into protein was consistently highest in cells not exposed to thymol. The findings in uninfected cells (Fig. 4) paralleled those in infected cells except that leucine penetrated uninfected cells in double the amounts of infected cells, and leucine incorporation was proportionately greater.

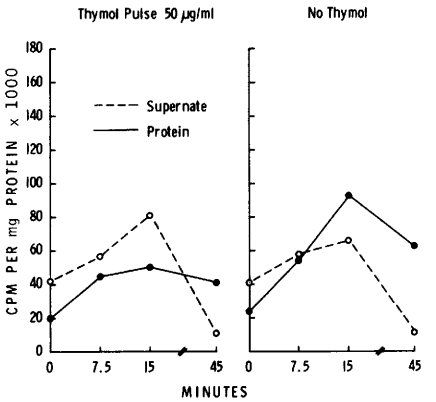

FIG. 3. Influenza virus-infected CEF cells exposed to thymol during pulse and chase with [^{3}H]leucine as described in Table II. Dashed line indicates cpm in supernatant of TCA precipitate of sonicate. Solid line indicates cpm of dissolved TCA precipitate.

In other experiments with uninfected cells, 0.66 m*M* thymol (100 μg/ml) was used. This concentration of thymol caused much greater suppression of leucine incorporation into TCA-precipitable material, but results were otherwise comparable to those with 0.33 m*M* (50 μg/ml) thymol.

These data appear to show that thymol does not block the entrance of leucine into cells, infected or uninfected, but that it acts intracellularly to cause a suppression of leucine incorporation into protein that increases throughout a 15-min pulse period.

Relative leucine incorporation in infected and uninfected cell cultures. The incorporation of leucine appeared to be uniformly less in chick embryo fibroblast cells 16 hr after infection with influenza A/PR/8 than in infected cells prepared from the same batch of chick embryos and tested simultaneously with infected cells. To examine this, the average micromoles of leucine per milligram of protein incorporated in 12 infected and 12 uninfected cell cultures in the absence of thymol were compared (data from Table I). Infected cells incorporated 1.209×10^{-3} and uninfected cells incorporated 2.105×10^{-3} μmole of leucine/mg of protein, or only 57.4% of uninfected cells ($t = 3.74$, df 22, $P < 0.01$, two-tailed test).

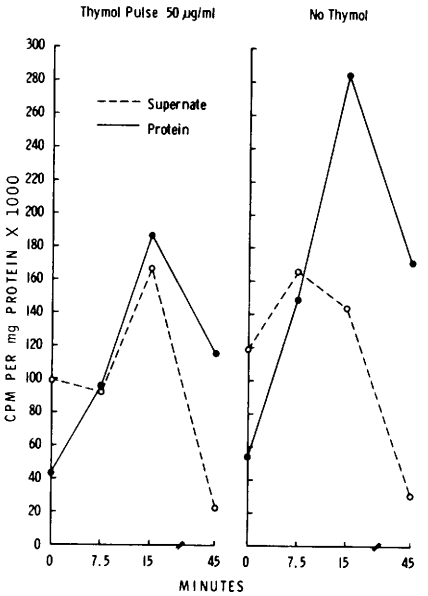

FIG. 4. As in Fig. 3 except that cells were uninfected.

Discussion. These data show a highly significant suppression of protein synthesis in influenza virus-infected and uninfected CEF cells exposed to 0.66 m*M* thymol (100 μg/ml) for 15 min. The effect was progressively less with thymol concentrations of 0.33 and 0.17 m*M*.

Periodic sampling during a 15-min exposure to 0.33 m*M* thymol revealed that thymol did not reduce the intracellular pool of leucine in infected or uninfected cell cultures, despite the pronounced suppression

TABLE II. EFFECT OF THYMOL ON INCORPORATION OF [^{3}H]LEUCINE BY INFLUENZA-INFECTED AND UNINFECTED CEF Cell Cultures.[a]

Cell group	Sample periods (min) 0	7.5	15	45
Infected (Influenza A/PR8)				
0.33 m*M* Thymol (50 μg/ml) during 15-min pulse				
Protein, mg/100 μl[b]	0.48	0.57	0.43	0.63
TCA precipitate, cpm/mg of protein[c]	20079	44784	50016	40961
TCA supernatant, cpm/mg of protein[d]	41802	57482	81209	11204
No thymol				
Protein, mg/100 μl	0.40	0.48	0.45	0.69
TCA precipitate, cpm/mg of protein	24152	54222	92911	62930
TCA supernatant, cpm/mg of protein	41325	58016	66457	11224
Uninfected				
0.33 m*M* Thymol (50 μg/ml) during 15-min pulse				
Protein, mg/100 μl	0.40	0.56	0.40	0.50
TCA precipitate, cpm/mg of protein	42540	96001	186730	116000
TCA supernatant, cpm/mg of protein	99000	92857	167850	23460
No thymol				
Protein, mg/100 μl	0.46	0.48	0.46	0.53
TCA precipitate, cpm/mg of protein	54108	149510	285300	172716
TCA supernatant, cpm/mg of protein	119826	166666	144304	31301

[a] Pulse 15 min, 66.6 μCi of [^{3}H]leucine/plate (15 ml); chase 30 min.
[b] mg of TCA-precipitable protein/100 μl of sonicate.
[c] cpm of dissolved TCA precipitate in 100 μl of sonicate divided by mg of protein/100 μl of sonicate.
[d] cpm of supernatant of 100 μl of sonicate after TCA precipitation divided by mg of protein/100 μl of sonicate.

APPENDIX. EXPERIMENT 78: COMPUTATION OF MICROMOLES OF LEUCINE INCORPORATED PER MILLIGRAM OF PROTEIN IN THE PRESENCE AND ABSENCE OF THYMOL IN INFLUENZA VIRUS-INFECTED AND UNINFECTED CHICK EMBRYO FIBROBLAST CELLS

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
	^{14}C (dpm/50 μl)	[^{14}C]Leucine incorporated (μmole/50 μl)	Pulse fluid: [cold leucine] + [^{14}C leucine] ÷ [^{14}C leucine]	Total leucine incorporated [μmole/50 μl; Col. (2) × (3)]	Protein (mg/50 μl)	Leucine incorporated [μmole/mg of protein; Col. (4) ÷ (5)]	^{3}H (dpm/50 μl)	[^{3}H]Leucine incorporated (μmole/50 μl)	Pulse fluid: [cold leucine] + [^{3}H leucine] ÷ [^{3}H leucine]	Total leucine incorporated [μmole/50 μl; Col. (8) × (9)]	Leucine incorporated [μmole/mg of protein Col. (10) ÷ (5)]
CEF, infected with influenza A/PR8											
Sample 1	7431	1.07×10^{-5}	19.4	2.08×10^{-4}	0.225	9.25×10^{-4}	49015	4.71×10^{-7}	391.6	1.84×10^{-4}	8.18×10^{-4}
Sample 2	5173	7.47×10^{-6}	19.4	1.45×10^{-4}	0.150	9.66×10^{-4}	6473 (Thymol pulse, 0.66 m*M*)	6.23×10^{-8}	391.6	2.44×10^{-5}	1.63×10^{-4}
CEF, uninfected											
Sample 3	17576	2.56×10^{-5}	19.4	4.97×10^{-4}	0.265	1.88×10^{-3}	100308	9.65×10^{-7}	391.6	3.78×10^{-4}	1.43×10^{-3}
Sample 4	17767	2.59×10^{-5}	19.4	5.02×10^{-4}	0.275	1.83×10^{-3}	34518 (Thymol pulse, 0.66 m*M*)	3.32×10^{-7}	391.6	1.30×10^{-4}	4.73×10^{-4}

Note 1. Col. (2) For samples 1 and 2, specific activity of [^{14}C]leucine was 312 mCi/mmole. $312 \times 2.22 \times 10^{6} = 6.9264 \times 10^{8}$ dpm/μmole of [^{14}C]leucine. Col. (2) = Col. (1) ÷ 6.9264×10^{8}. For samples 3 and 4, specific activity of [^{14}C]leucine was 309 mCi/mmole. $309 \times 2.22 \times 10^{6} = 6.8598 \times 10^{8}$ dpm/μmole of [^{14}C]leucine. Col. (2) = Col. (1) ÷ 6.8598×10^{8}. Col. (8) Specific activity of [^{3}H]leucine = 59 Ci/mmole. $59 \times 10^{3} \times 2.22 \times 10^{6} = 1.0398 \times 10^{11}$ dpm/μmole. Col. (8) = Col. (7) ÷ 1.0398×10^{11}.

Note 2. Col. (3) Pulse fluid with [^{14}C]leucine.

Leucine (9.87% of normal) = 5.889×10^{-4} mmole/15 ml
[^{14}C]Leucine (10 μCi) = 0.320×10^{-4} mmole/15 ml
Total leucine = 6.209×10^{-4} mmole/15 ml.

Col. (9) Pulse fluid with [^{3}H]leucine.

Leucine (8.9% of normal) = 4.96×10^{-4} mmole/15 ml
[^{3}H]Leucine (75 μCi) = 0.127×10^{-4} mmole/15 ml
Total leucine = 4.9727×10^{-4} mmole/15 ml.

APPENDIX – *Continued*

Statistical Analysis of Data from Table I on the Basis of the Null Hypothesis that, if Thymol Had no Effect, the Complex Fraction Below Would Have a Value of $R = 1$.

$$\frac{\dfrac{\text{Leucine incorporation, second pulse (thymol) Sample 2}}{\text{Leucine incorporation, first pulse Sample 2}}}{\dfrac{\text{Leucine incorporation, second pulse Sample 1}}{\text{Leucine incorporation, first pulse Sample 1}}} = R.$$

$$t = \frac{1 - (\text{mean of } R \text{ of experiments } 64, 70, 78, 80)}{\text{SE}}$$

Infected cells:

$$t = \frac{1 - \dfrac{0.155 + 0.218 + 0.192 + 0.209}{4}}{\text{SE}}$$

$t = 55$; $df = 3$; one tailed; $P < 0.0005$.

Uninfected cells:

$$t = \frac{1 - \dfrac{0.149 + 0.317 + 0.399 + 0.271}{4}}{\text{SE}}$$

$T = 17.2$; $df = 3$; one tailed; $P < 0.0005$.

of protein synthesis in thymol-treated cultures. These data suggest that thymol does not prevent entrance of leucine into cells but acts at some intracellular site to interfere with protein synthesis. Based on the evidence that thymol is taken up by red cell membranes and associates with hemoglobin (2), it is possible that thymol penetrates cell membranes and becomes associated with enzymes in the rough endoplasmic reticulum and perhaps elsewhere, where it interferes with synthesis of viral and cellular proteins.

Effects of thymol described by others are consistent with the foregoing concept of its mode of action. Seeman and Weinstein (6–8) found that thymol along with a variety of local anesthetics, tranquilizers, alcohols, and antihistamines stabilized erythrocytes against lysis by hypotonic saline. This action appeared to result from the accumulation of these substances in the red cell membrane with a resulting expansion of the membrane and increased cell volume that would accommodate more fluid within the cell. This effect on red cells correlated with the anesthetic properties of the compounds. Since these agents, like thymol, would also presumably concentrate intracellularly and interact with intracellular proteins, such an interaction might play a role in these effects.

In studies of cell-free membranes, thymol was found to stimulate or, more often, suppress the activity of some membrane-bound enzymes (9, 10). These actions could result from the direct interaction of thymol with the enzymes such as that described with hemoglobin. It would also follow that anesthetic agents that accumulate in cell membranes or influence the activity of enzymes might also possess activity against lipid-enveloped viruses, some perhaps even greater than that of thymol.

An incidental finding in this study was the observation that CEF cells infected with influenza virus, at 16 hr after inoculation, incorporated only 57% of the amount of leucine that was incorporated by uninfected cells. In infected cells, values for leucine incorporation included both cellular and viral proteins, but it appeared that the largest proportion of newly formed protein was of viral origin. Hightower and Bratt (11) found, at 6-hr postinfection, that Newcastle disease virus-infected CEF cells incorporated about 55% as much radioactive amino acid as uninfected cells. They estimated that 20% was cell protein while 35% was viral protein. On the other hand Lamb and his associates (12) found that in Sendai virus-infected CEF cells, newly synthesized protein was 205% that in uninfected cells, about equally divided between host cell and viral protein.

Summary. Thymol in a concentration of 0.66 m*M* (100 μg/ml) during a 15-min exposure suppressed incorporation of leucine into protein in chick embryo fibroblast cells uninfected and infected with influenza A/PR/8/34 by 73 and 80% respectively. Lesser concentrations of thymol showed progressively less effect. Formation of high molecular weight protein, probably cellular in origin, was less suppressed than that of lower molecular weight proteins in both infected and uninfected cell preparations.

Periodic sampling during simultaneous pulsing with [^{3}H]leucine and exposure to 0.33 m*M* thymol, showed that leucine penetrated thymol-treated, infected, and uninfected cells, to about the same extent as cells not exposed to thymol, indicating an intracellular site of effect of suppression of leucine incorporation rather than a membrane effect.

Influenza virus-infected cells incorporated into protein only 57% of the amount of leucine incorporated by uninfected CEF.

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