tination inhibition test with psittacosis antigens.

Summary. Psittacosis agent was found to hemagglutinate chicken erythrocytes susceptible to agglutination by vaccinia virus. Negative results were obtained with vaccinia-negative cells. The agglutination of chicken cells by psittacosis was similar to mouse cells with reference to conditions for hemagglutination, sensitivity to inhibitors and the hemagglutination inhibition test. Differences between vaccinia and psittacosis hemagglutinins were found. No cross serologic relationship was found between psittacosis and vaccinia in the hemagglutination inhibition test. Positive hemagglutination inhibition tests were obtained with anti-trachoma sera using psittacosis hemagglutinin and either chicken or mouse cells.

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Effect of Actinomycin D on Morphine Tolerance.* (30187)

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There is indirect evidence suggesting that tolerance to morphine may be related to the production, in the central nervous system, of proteins or peptides capable of counteracting the effects of the drug(1-5). The purpose of the present study is to determine whether development of tolerance to the analgesic action of morphine can be prevented by drugs which inhibit protein synthesis.

Methods and materials. a) *Mice.* Tolerance was induced in Swiss albino mice (20 to 25 g, both sexes) obtained from Thomas E. Euers, Austin, Texas by the following schedule of subcutaneous injections of morphine sulfate: First day, 2 injections of 0.12 mg per mouse (4.8 to 6.0 mg/kg); second day, 3 injections of 0.22 mg per mouse (8.8 to 11 mg/kg) and third day, 2 injections of 0.28 mg per mouse (11.2 to 14 mg/kg). The interval between daily injections was 4 hours.

Response to noxious stimuli was tested by Haffner's method as modified by Bianchi and Franceschini(6). The mice were screened before the experiment and those which did not respond to the standard pressure applied to the base of the tail for 10 seconds were eliminated. The analgesic effect of 0.15 mg of morphine sulfate per mouse (6.0 to 7.5 mg/kg) was determined the day before starting the injection schedule to produce tolerance and on days 1, 3 and 4 during the establishment of tolerance. The results are expressed in terms of the per cent mice re-

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TABLE I. Effect of Actinomycin D on Morphine Tolerance Development in Groups of 30 Mice.

Treatment	% of mice responding to painful stimulus after morphine on days 1	3	4	P* (4 day)
Morphine	13.3	63.3	96.7	
+ Actinomycin .03 μg	13.3	56.7	90.0	—
+ " .08 μg	13.3	50.0	76.7	<.05
+ " .17 μg	13.3	33.3	70.0	<.01

* The probability of groups behaving identically calculated by χ^2 method.

sponding to the standard stimulus, that is, tolerant to morphine, 30 minutes after injection.

b) *Rats.* Sprague-Dawley rats, all male, weighing around 300 g were made tolerant to morphine by 3 subcutaneous injections every day for 14 days. The rats received injections of 10 mg/kg of morphine sulfate the first 2 days and dosage was increased progressively until 100 mg/kg was given on the last 2 days. The analgesic action of morphine was tested by the method of D'Amour and Smith(7). The results are expressed in terms of the reaction time in seconds to a standard radiant heat stimulus, indicated by the voltage scale of the instrument.

c) *Metabolic inhibitor.* Actinomycin D† was given intraperitoneally in distilled water. The doses are expressed in μg per animal.

Results. In the first experiment, 10 mice were given 0.33 μg of actinomycin in place of morphine according to the schedule described above. On the 1st, 3rd, and 4th days they were tested for their response to the standard stimulus before and after the challenging injection of morphine sulfate All animals responded before morphine injection and none of them responded after. These results indicate that actinomycin, when given alone, produced no analgesia and did not antagonize the effect of morphine.

In the next experiment, 3 groups of 30 mice each were given actinomycin D, 0.03, 0.08 and 0.17 μg with each injection of morphine according to the same dose schedule. A fourth (control) group received morphine only. Table I shows a definite trend towards decreasing tolerance development with increasing doses of actinomycin, and a χ^2 analysis demonstrated a significant decrease in tolerance at the higher doses of actinomycin.

In the next experiment larger doses of actinomycin were tried using the same morphine schedule, and actinomycin D, 0.5, 2.0 and 8.0 μg per mouse per day was given to groups of 20 or 30 mice. The results (Table II) again indicate antagonism of tolerance development related to the dose of actinomycin and highly significant differences between the actinomycin-treated and the control mice.

The decrease in tolerance was clearly related to the dose of actinomycin, and Fig. 1 shows the dose-response relationship for the pooled results of the last 2 experiments.

In the final experiment, 2 groups of 8 rats each were used. Preliminary testing of each group indicated that a statistically significant increase in reaction times at a stimulus intensity of 60 followed a single injection of 3 mg/kg of morphine. Both groups were then given morphine according to the schedule described above. In one group, each morphine injection was combined with 10 μg/kg of actinomycin D. On the 14th day, the response of all surviving rats at 2 levels of stimulus intensity was measured before

TABLE II. Effect of High Doses of Actinomycin D on Morphine Tolerance.

Treatment	% of mice responding to painful stimulus after morphine on days 1	3	4	N	P‡ (day 4)
Morphine	20.0	55.0	100	20	—
+ Actinomycin .5 μg	30.0	46.6	69.0	30	<.01
+ " 2.0 μg	30.0	38.0	50.0	30*	<.001
+ " 8.0 μg	25.0	15.8	42.9	20†	<.001

* 2 died. † 13 died. ‡ χ^2 method.

† Kindly supplied by Merck and Co.

TABLE III. Reaction Times in 2 Groups of 8 Morphine-Tolerant Rats Before and After 5 mg/kg of Morphine.

Treatment	Survivors (N)	Reaction time (sec ± S.D.) 80 v Before	80 v After	90 v Before	90 v After
Morphine	7	2.8 (±.9)	2.2 (±.6)	1.7 (±.7)	1.8 (±.4)
+ Actinomycin	5	2.5 (±.8)	4.2 (±.6*)	1.9 (±.3)	3.8 (±.9)

* P = .02

and after a single dose of 5 mg/kg of morphine. The stimulus intensities were selected so as to elicit in all animals a response within 10 seconds. The results (Table III) show complete tolerance to the analgesic effect of 5 mg/kg of morphine in the rats given morphine alone and a significant morphine effect (increase in reaction time) in the actinomycin treated group.

Discussion. These experiments have shown that 3 daily injections of increasing doses of morphine for 3-4 days produced tolerance in mice sufficient to abolish the analgesic effect of 6 mg/kg of morphine. Early reports indicated doubt as to the ability of mice to develop tolerance to morphine. More recent work is in agreement with our present findings(8).

The reduction in rate of development of morphine tolerance in the presence of the inhibitor of protein synthesis, actinomycin D, suggests that the animals became tolerant by synthesizing a protein or polypeptide which antagonizes morphine effects. It has been shown that, in the doses used in these experiments, actinomycin does not affect the resting level of RNA synthesis but opposes an increase in this process(9). Therefore, tolerance development may be related to development of a new RNA.

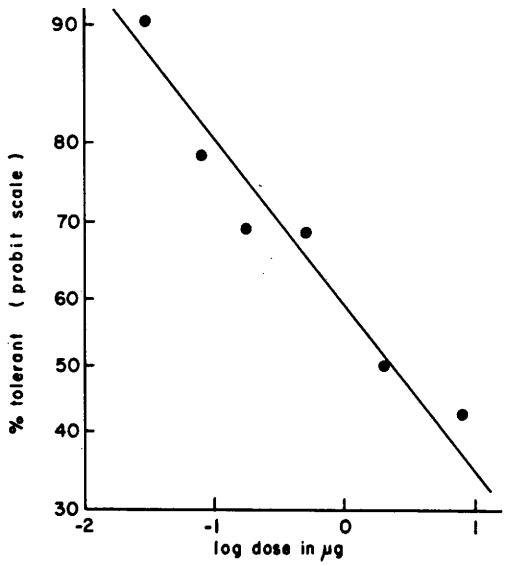

FIG. 1. Decrease in morphine tolerance by increasing doses of actinomycin D. Abscissa: dose in μg per mouse on log scale; ordinate: percentage of tolerant animals on probability scale.

Among the various mechanisms by which inhibition of the synthesis of new proteins might decrease tolerance development is the possibility of interference with the production of peptides such as substance P, bradykinin, or β-melanocyte-stimulating hormone which are capable of producing changes in the excitability of central synapses(2,4,5). Substance P has been shown to antagonize the analgesic effect of morphine(1), and β-melanocyte-stimulating hormone exerts an effect opposite to that of morphine on dorsal root potentials (3,4,5).

It is also known that certain types of learning are impaired by metabolic inhibitors(10,11). Morphine administration and the development of tolerance can be considered a form of learning, since it subjects certain neuronal cells to new information and results in an altered response. The decreased responsiveness to morphine may be dependent upon either cellular or integrative mechanisms.

Summary. The inhibitor of protein synthesis, actinomycin D, is capable of decreasing the rate of development of morphine tolerance in mice and rats. This effect may be the result of the suppression of the synthesis of new RNA and consequently of proteins or peptides which counteract the effects of morphine.

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A Rapid and Sensitive Assay of Muramidase.*† (30188)

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During our investigation on the purification of muramidases (lysozymes) from human and bovine milk a need was felt for a more rapid and sensitive technique for the enzyme assay. The procedure used by Smolelis and Hartsell(1) and the modifications presented by Jolles(2) and Shahani *et al*(3) were lengthy and showed large day-to-day variations. Litwack's modification(4) which measures the change in per cent transmittance (%T) between 30 and 60 seconds after enzyme addition was found to have insufficient sensitivity for samples below 2 μg of enzyme.

The Commission on Enzymes of the International Union of Biochemistry has recommended that enzyme assays be based, wherever possible, upon measurements of initial rates of reaction(5). Therefore a continuous, sensitive and rapid technique was developed for assaying muramidase activity which allowed measurement of the initial reaction rate. This modification helps to eliminate the greatest disadvantage of earlier assay techniques, *viz.*, end-product inhibition.

Methods and materials. The reagents used in this assay are as follows: (a) a 50 mg% suspension of ultraviolet-killed and lyophilized *Micrococcus lysodeikticus* cells (Difco) in M/15 phosphate buffer, pH 6.2; (b) 0.3 M NaCl; (c) Standard muramidase solutions prepared by dissolving crystalline egg-white lysozyme (Nutritional Biochemicals Corp.) in M/15 phosphate buffer. These materials are then added to a standard 4.2 ml Beckman pyrex cuvette in the following proportions: 1.5 ml of the cell suspension, 0.5 ml of the NaCl solution, and 1.0 ml of the enzyme solution. This makes an effective concentration of 0.05 M NaCl and 25 mg% cells in the enzyme assay mixture. This mixture is momentarily stirred and rate of clearance is measured in a Beckman DB spectrophotometer with an attached Sargent SR recorder. The initial transmittance of the assay mixture is approximately 10% when water is set at 100%T at 540 mμ.

For this method, no particular time interval is necessary for making the measurements. The only requirement is that the rate of clearance of the suspension by muramidase should be linear. The linearity can be easily determined from the recorder chart.

Results and discussion. Fig. 1 presents results taken directly from the recorder at

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