References and Notes

1. E. S. Russell, in *Aspects of Synthesis and Order in Growth*, D. Rudnick, Ed. (Princeton Univ. Press, Princeton, N.J., 1955), chap. 5.
2. ———, in *Methodology in Mammalian Genetics*, W. J. Burdette, Ed. (Holden-Day, San Francisco, 1963), pp. 217–32.
3. S. E. Bernstein and E. S. Russell, *Proc. Soc. Exptl. Biol. Med.* **101**, 769 (1959).
4. S. Bernstein, *Science* **137**, 428 (1962).
5. ———, *Rad. Research* **20**, 695 (1963).
6. J. E. Till and E. A. McCulloch, *Ann. N.Y. Acad. Sci.*, in press.
7. E. A. McCulloch and J. E. Till, *Rad. Research*, in press.
8. E. A. McCulloch, *Rev. Franc. Etudes Clin. Biol.* **8**, 15 (1963).
9. H. I. Kohn and R. F. Kallman, *Rad. Research* **5**, 309 (1956).
10. J. E. Till and E. A. McCulloch, *ibid.* **18**, 96 (1963).
11. ———, *ibid.* **14**, 213 (1961).
12. E. A. McCulloch and J. E. Till, *ibid.* **16**, 822 (1962).
13. ———, *J. Cell. Comp. Physiol.* **61**, 301 (1963).
14. We have found that the facility with which the colonies may be counted in WW^v mice is improved by irradiation of the hosts with 200 rad prior to the injection of cells. This treatment does not introduce any appreciable mortality.
15. E. S. Russell, S. Bernstein, E. C. McFarland, W. R. Modeen, *Rad. Research* **20**, 677 (1963).
16. G. Keighley, E. S. Russell, P. H. Lowy, *Brit. J. Haematol.* **8**, 429 (1962).
17. M. W. Thompson, E. S. Russell, E. C. McFarland, in *Genetics Today*, S. J. Geerts, Ed. (Pergamon, London; Macmillan, New York, 1963), vol. 1, Abstracts, pp. 185–186.
18. W. R. Bruce and E. A. McCulloch, *Blood* **23**, 216 (1964).
19. C. W. Gurney, L. G. Lajtha, R. Oliver, *Brit. J. Haematol.* **8**, 461 (1962).

20. We thank Dr. E. S. Russell for making available the mice of differing *W* genotype, and Miss R. Wyncoll, R. Course, J. Hicks, A. Galberg, R. Kuba, F. Mik, and P. Csordas for technical assistance. Supported by the Defence Research Board (grant 9350–14), the National Cancer Institute of Canada, the Medical Research Council, Canada (grant MA-1420), and the National Research Council of Canada (grant T-1714).

24 February 1964

Immunologically Determined and Competent Cells Are Affected Differentially by Actinomycin D

Abstract. *A differential effect of actinomycin D on the capacity of mouse peritoneal fluid cells to form antibody after cell transfer has been found. Actinomycin treatment in vitro during transfer inhibited the delayed response toward a first antigen, administered before transfer, while the competence of cells to respond to a second antigen, given after transfer, was not affected.*

Cells specified in their capacity to produce antibody towards an antigen which they had encountered previously, may be called immunologically determined. They are contrasted with immunologically competent cells, which are able to respond to any new antigen they might encounter later on. In a system which allows both cellular states, determination and competence, to be measured within the same cell population, it is possible to do experiments designed to differentiate these two properties at their cellular chemical basis. In the work reported here, actinomycin has been used because of its known interaction with DNA, leading to an inhibition of DNA-directed RNA synthesis (*1*). It will be shown that antibody formation resulting from cellular determination is abolished after an incubation of cells with actinomycin, while competence is retained.

Mouse peritoneal fluid cells, harvested, washed, and transferred to lethally irradiated isogenic recipient mice, can produce antibodies of two different specificities: (i) against an antigen that has been given to the cell-donor mice before transfer, and (ii) against a different antigen that is administered to the recipient mice immediately after transfer. The first antigen may be given to the donor mice as early as 3 months before transfer. Antibody synthesis commences in the recipient mice after a lag period of 4 days after transfer, although the first antigen has not been administered again. Thus the retention by these cells of the capacity to begin antibody production against the antigen encountered earlier, not as in a secondary response, but without further antigenic stimulation, demonstrates immunological determination. The capacity of the same cell population to respond to the second antigen, given only after transfer, reflects the presence of immunological competence. During transfer in vitro the cell populations were treated with actinomycin, which thus acts on the cellular state of determination with respect to the first antigen, and on competence with respect to the second. Since peritoneal fluid cells are a heterologous population, it is not known whether determination and competence are properties of the same or of different cells. Facts and arguments leading to the concept of immunological determination will be summarized.

All mice were C3H/HeICR females 8 to 10 weeks old at the beginning of experiments. The two serologically unrelated *Escherichia coli* phage species, $T6r^+$ and T7, were used interchangeably as the first and the second antigen, respectively. The immune peritoneal fluid cells were produced in female C3H mice which received the following sequence of treatments: (i) a dose of 800 rad whole body x-radiation (40.5 rad/min, 190 kv, 20 ma, filtered by 0.5 mm Cu, 1 mm Al, target distance 50 cm); (ii) within 4 hours after the irradiation an intravenous injection of 40 to 60×10^6 spleen cells from previously immunized isogenic mice, prepared 4 days after these mice had received the second of two intraperitoneal injections of 10^{10} plaque-forming units of bacteriophage (the first antigen) 3 to 5 weeks apart; the first injection had been given in complete Freund's adjuvant (Difco); (iii) an intraperitoneal injection of 10^{10} plaque-forming units of phage (first antigen) in 0.2 ml complete Freund's adjuvant. Ascitic fluid developed within 10 weeks after a single injection of phage (T7) in adjuvant, or within 2 to 4 weeks after a second injection (T6), given 4 weeks after the first. The cells were sedimented from the ascitic fluid, resuspended with the help of 0.025 percent Trypsin (Difco), and washed three times by a sucrose-layer technique. Thereby the amount of antibody carried over into the recipients, extra- or intracellularly, became too small to interfere with the measurement of antibody synthesized by the cells after transfer (Fig. 1 and ref. *7*). The incubation with actinomycin D was between the second and third washings, in a water bath at 37°C for 40 min. It was terminated by chilling the tubes with cells in ice water (*2*). The cell concentration during the incubation was 23×10^6 cells per milliliter. Within each experiment, cells incubated with or without actinomycin were portions of the same preparation of peritoneal fluid cells. Cell suspension medium was Hanks solution containing 2 percent polyvinylpyrrolidone (PVP, Cal-BioChem.) For the 37°C incubations, the bicarbonate-CO_2 buffer concentration was increased fivefold. The bottom layer in sucrose-centrifugation was a mixture of 2 volumes of Hanks-PVP and 1 volume of 0.6 molar sucrose in water.

The mice injected with immune peritoneal fluid cells received 850 rad, and then were injected intravenously with 10.5 to 42×10^6 peritoneal fluid cells per mouse, to which 7 to 10×10^6 un-

treated isogenic bone marrow cells for each mouse had been added, to avoid radiation death. Immediately afterwards 10^{10} plaque-forming units of the phage serving as the second antigen were given intraperitoneally without adjuvant. The mice harboring peritoneal fluid cells were bled at intervals by the technique of Stone (*3*). Serum samples were frozen in a bath cooled by a mixture of solid CO_2 and ethanol and stored at −36°C.

The general bacteriophage technique followed the description by Adams (*4*). The phages T6r$^+$ and T7 were grown by the agar overlay method, and purified by mouse or rabbit antiserums against *E. coli*, and by differential centrifugation. High-titer stocks were stored in phosphate buffer (*5*). For the phage neutralization assay, serial fivefold dilutions of serum samples in nutrient broth were incubated with bacteriophage for 24 hours at 2°C. The reaction mixtures had a volume of 0.14 ml and contained approximately 60 plaque-forming units of each of the two phage species. The whole volume was plated with *E. coli* B; after overnight incubation at 25°C, T7 and T6 plaques can easily be distinguished and counted separately. The serum titer is that dilution which results in 50 percent phage neutralization, in comparison to blanks. Titers were determined by interpolation in von Krogh graphs, as described by Barlow (*6*). The lowest serum dilution used was 7.

The measurement of determination is based on a new property found to be associated with mouse peritoneal fluid cells induced in the way just described under the influence of an antigen. A detailed account of the experiments which led to the concept of immunological determination has been presented (*7*), and only a summary will now be given.

Lymphocytes, macrophages, and polymorphonuclear neutrophils are suspended in antibody-containing peritoneal fluid; the cell concentration is usually of the order of 3 to 8 × 10^6 cells per milliliter. After washing and transfer to irradiated or unirradiated isogenic recipients, antibody is formed without any further addition of antigen. This antibody formation has the following characteristics.

1) Differentiated antibody-producing cells are essentially not present at the time of harvest and transfer, a conclusion justified because little or no antibody is produced for about 4 days after transfer. The antibody titer, however, rises dramatically between the 4th and the 15th day, and thereafter stays steady for several weeks (Fig. 1). During the 4-day delay the transferred cells undergo a change preparatory to antibody formation. This point is illustrated by a comparison with cell populations containing differentiated antibody-forming cells, such as spleen or lymph-node suspensions (*8*). Spleen and lymph-node suspensions can produce antibody immediately after transfer, apparently without needing to adapt to their new environment; they will even do so in allogenic recipients, provided these have not been immunized against cells of the donor strain. Immune peritoneal fluid cells, by contrast, fail to produce antibody in an allogenic transfer situation, because the recipient's homograft reaction destroys the grafted cells before these get a chance to complete differentiation and commence antibody synthesis.

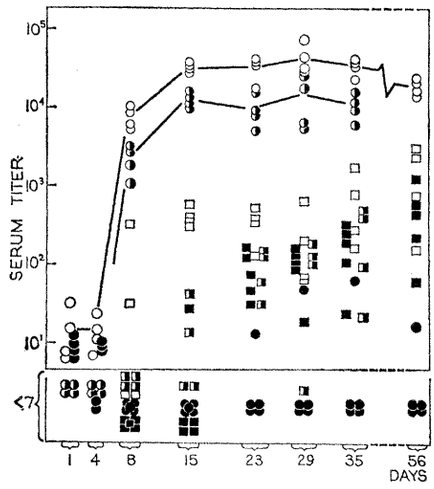


Fig. 1. Time course of the appearance of antibody (experiment 1) against the first antigen (cellular determination) (T7, circles) and the second antigen (cellular competence) (T6, squares). Open symbols: untreated cells; black symbols: cells treated during transfer with 1.4 × $10^{-6}M$ actinomycin D; 40 × 10^6 cells transferred in both these groups. Half-shaded symbols: untreated cells, diluted 1/5. Average values for T7, untreated cells, are connected by lines.

2) The transferred population of peritoneal fluid cells must contain the progenitors of antibody-producing cells, because (i) destroying their viability by freezing and thawing, x-radiation (600 rad), or by a homograft reaction in the recipients abolishes later antibody production; (ii) impairment of the immunological competence of the recipients by whole body x-radiation, just prior to cell transfer, does not only not prevent antibody formation after transfer, but leads to significantly increased titers; (iii) when peritoneal fluid cells are implanted into Algire-type diffusion chambers, antibody will be produced inside the chambers (*9*).

3) Antigen in quantities sufficient to produce antibody in unirradiated hosts is not present in the transfer system, as concluded from the viability requirement for the transferred cells, stated under 2. The strongest argument is from the experiment where no antibody formation ensued, when cells were transferred to nonirradiated (allogenic) C57BL mice, a strain found to be as responsive to the injection of bacteriophage antigen as the C3H cell-donor strain (*7*). No assumption is made, at this point, concerning the presence or absence of small amounts of cell-associated antigen.

Competence has frequently been measured by injecting cell suspensions into lethally irradiated recipients, and giving antigen to the recipients after cell transfer—or to the cells during transfer (*10*). It has been found to be associated not only with spleen and lymph node cell populations, but also with nonspecifically induced mouse peritoneal fluid cells (*11*). Transferred spleen and lymph node cells have been shown to be the actual progenitors of antibody-forming cells in the recipients, by means of Algire-type diffusion chambers (*12*), and by using allotypic markers on antibody molecules (*13*). Because of the lethal whole body x-radiation, given prior to cell transfer, the recipients are unable to form antibody by themselves, a point which is checked by suitable controls. When peritoneal fluid cells are transferred, the recipients have to be given isogenic bone marrow cells besides, to replace the hematopoietic system. It has been found that these do not, even in a dose ten times higher than is used in the present work, confer competence upon the recipients (*14*).

A total of 49 irradiated mice in six separate experiments received bone marrow cells and antigen, but no peritoneal fluid cells; these served as controls. The serums of most of these mice were assayed through days 40 to 49 after the injections. Of 17 animals injected with T6 phage, none developed

Table 1. Ratios of log of antibody titer due to the first antigen (cellular determination) to that due to the second antigen (cellular competence) of individual mice from three experiments. The day mentioned indicates the day of bleeding after transfer. In experiment 2, T7 was the first antigen, and T6 the second. In experiments 3 and 4 the order was reversed. Actin, actinomycin D; Ag, antigen.

No. cells per mouse (10^6)	Actin. (10^{-6})	log 1st Ag titer / log 2nd Ag titer				
		Experiment 2, day 28				
42	—	4.9/1.9,	5.2/1.9,	5.0/1.6,	5.0/2.1,	4.7/1.5
10.5	—	4.1/1.5,	4.6/1.4,	4.6/1.4,	4.6/1.8	
42	3.6	<0.8/1.8,	<0.8/1.9,	<0.8/2.1,	<0.8/2.1	
42	1.4	2.8/1.4,	<0.8/1.9,	<0.8/2.1,	<0.8/1.9,	<0.8/1.3
		Experiment 3, day 23				
42	—	2.8/2.6,	2.7/<0.8,	2.7/2.5,	2.4/2.3	
42	1.8	<0.8/2.2,	<0.8/2.5,	<0.8/2.3		
42	0.6	2.2/2.6,	2.1/2.9,	2.2/2.2		
		Experiment 4, day 28				
36	—	2.9/0.9,	3.0/2.4,	1.8/1.2,	3.9/2.3	
36	1.4	<0.8/2.9,	<0.8/1.8,	<0.8/2.5,	<0.8/1.8,	<0.8/2.8
36	0.8	<0.8/2.4,	<0.8/2.4,	<0.8/2.9,	<0.8/2.8,	<0.8/2.4

detectable antibody. Out of 32 mice injected with T7 phage, one showed anomalous behavior to phage neutralization (20 percent phage survival at 1/35 dilution; higher survival at the 1/7 dilution); another had a titer of 12 on day 28, which had disappeared on days 35 and 42. The remaining 30 mice had no detectable antibody activity to T7. In the experiments to be described, the appearance of antibody against the second antigen is therefore considered to be due to the competence of the transferred peritoneal fluid cells. Two experiments were excluded from the data, because antibody against the second antigen was near or below the lower limit of the assay after peritoneal fluid cell transfer, in both the actinomycin-treated and the untreated groups.

As an additional control, bacteriophages (2×10^9 plaque-forming units per milliliter) were incubated with 7 μM actinomycin under the same conditions as cells, and separated from the actinomycin by high-speed centrifugation. Neither their virulence nor their antigenicity were affected when the phages were injected intravenously into mice at doses of 5×10^8 and 6×10^7 (T7), or 2×10^8 (T6) plaque-forming units per mouse.

The time course of antibody production is presented in experiment 1 (Fig. 1). In recipients which received cells incubated without actinomycin, the sharp increase in the rate of antibody formation caused by T7 phage, the first antigen (inducing determination), occurred between day 4 and day 15. From then onward, the titers had values between 30,000 and 40,000 until day 35. Antibody against the second antigen, T6, used here to test competence, appears more slowly, and the titers scatter more widely. For this reason values for individual recipient mice are given. Antibody against the second antigen also did not reach the titers of those against the first antigen. A late appearance of antibody has also been noted, when rats were immunized with very small quantities of bacterial flagellar antigen (*15*). On days 1 and 4, the second antigen was still present in the sera as live phage in this and other experiments.

In the group of 5 mice that had received peritoneal fluid cells treated with actinomycin D for 40 minutes at 37°C in vitro during transfer, at a concentration of $1.4 \times 10^{-6} M$, no antibody against T7, the first antigen, was produced in four cases, and a titer of 14 to 62 was produced in one case (still lower by three orders of magnitude than in the untreated group). By contrast, the actinomycin affected the titers against T6, the second antigen, only slightly. Antibody to T6, however, did appear somewhat later in the actinomycin group than in the group receiving untreated cells (see, however, experiment 4).

The titers of three similar experiments are given in Table 1. In experiment 2, T7 was the first antigen, inducing determination, and T6 was the second antigen, given after the actinomycin treatment. In experiments 3 and 4 the order of antigens was reversed. When T7 was used as the first antigen, higher titers were obtained than with T6 as the first antigen.

It is evident from these data that actinomycin D in concentrations ranging from 0.8 through 3.6 μM abolishes determination completely or almost completely. By contrast, competence appeared to be affected only slightly by these actinomycin concentrations in experiment 1 (Fig. 1) and not at all in experiments 2, 3, and 4. The somewhat later production of competence antibody by actinomycin-treated cells, as compared to untreated cells (experiment 1, Fig. 1) was not found in experiment 4, when the serums were assayed on day 16 (data not shown). Actinomycin in the 0.6 μM concentration depressed determination titers partially (experiment 3), and it had no significant effect at 0.2 μM (data not shown). In an additional experiment, not included in the table, 4.2 μM actinomycin resulted in an inhibition of competence-antibody production also, while 1.4 μM actinomycin had the same differential effect as in the other experiments.

One possible criticism of these experiments follows. In contrast to the determined cells, the competent cells prepare for the production of antibody in an environment in which antigen is present. Antigen is known to stimulate proliferation of competent cells (*12*). A possible interpretation of the actinomycin effect would be that actinomycin destroys both determined and competent cells to the same degree, but that the latter, under the influence of antigen, compensate for their loss by extensive proliferation. This possibility is remote, since, in the experiments where T7 is the first antigen (experiments 1 and 2), the titers due to determination were depressed by the actinomycin D (1.4 and 3.6 μM) by a factor of more than 4000 in 11 out of 13 mice; the compensation by proliferation would have to be a very efficient process indeed. It is also quite incompatible with extensive data on the relationship between the number of transferred cells

and the resulting antibody titers, obtained by Makinodan *et al.* in a system very similar to the present one (*14*). Also against this possibility are the results obtained in groups of mice that received a reduced number of untreated cells. Injecting only one-fourth (experiment 2), or one-fifth (experiment 1, Fig. 1), the number of cells as compared to the other groups, leads to an approximately equal reduction in both determination and competence titers. If there were compensation by proliferation on the part of the competent cells, only the determination titers should have been reduced.

The statistical significance of the actinomycin effect can be assessed simply. Within each experiment (that is, for each preparation of peritoneal fluid cells), the ratio of antibody titer due to determination as opposed to antibody titer due to competence, is smaller in every mouse that received actinomycin-treated (0.8 to 3.6 μM) cells, than in every mouse that received untreated cells. Since there are 27 mice in the former group, and 24 mice in the latter (including those which received reduced cell numbers) the probability for this result to occur by chance is $P << 0.0001$.

Two conclusions can be derived from the experiments presented.

1) The significantly higher sensitivity towards actinomycin D of antibody formation due to cellular determination establishes the immunologically determined cellular state as something different in character from the competent state.

2) Both determined cells and competent cells have to prepare for antibody formation (differentiate), and then produce antibody after the time when the actinomycin treatment takes place. But determined cells have had the necessary interaction with antigen before, and competent cells have it after the actinomycin treatment. It appears that whatever is retained in the cells from this interaction with antigen is more sensitive toward actinomycin than the capacity of these or other cells in the population to produce antibody in general. Since actinomycin interacts with DNA, and inhibits the transcription of information from DNA to RNA, this process appears to take part in determining the immunologically specific structure of antibody. For such a conclusion one possibility, however, has to be considered as a reservation. It is still conceivable that actinomycin, in these experiments, acts simply by destroying cellular functions in an immunologically nonspecific way. One would then have to assume, that by interacting with antigen and becoming determined, cells also change in physiological aspects—for example, their membrane permeability for actinomycin D. The differential effect might then just be due to a difference in the effective actinomycin concentration that reaches the chromosomes.

EBERHARDT WEILER
Institute for Cancer Research, Philadelphia 11, Pennsylvania

References and Notes

1. E. Reich, *Cancer Res.* **23**, 1428 (1963).
2. E. Harbers, W. Müller, R. Bachmann, *Biochem. Z.* **337**, 224 (1963).
3. S. H. Stone, *Science* **119**, 100 (1954).
4. M. H. Adams, *Bacteriophages* (Interscience, New York, 1959).
5. W. Vielmetter and C. W. Wieder, *Z. Naturforsch.* **14B**, 312 (1959).
6. J. L. Barlow, H. van Vunakis, L. Levine, *J. Immunol.* **80**, 349 (1958).
7. E. Weiler, *Immunology*, in press.
8. S. Harris and T. N. Harris, *J. Exptl. Med.* **100**, 269 (1954); N. A. Mitchison, *J. Cell. Comp. Phys.* Suppl. 1, 247 (1957); E. Weiler, *Immunology*, in press.
9. I. J. Weiler, in preparation.
10. C. G. Cochrane and F. J. Dixon, in *Advanc. Immunol.* **2**, W. H. Taliaferro and J. H. Humphrey, Eds. (Academic Press, New York, 1962), pp. 206–235.
11. E. H. Perkins, M. A. Robinson, T. Makinodan, *J. Immunol.* **86**, 533 (1961).
12. P. Urso and T. Makinodan, *ibid.* **90**, 897 (1963).
13. T. N. Harris, S. Dray, B. Ellsworth, S. Harris, *Immunology* **6**, 169 (1963).
14. T. Makinodan, M. A. Kastenbaum, W. Petersen, *J. Immunol.* **88**, 31 (1962).
15. G. L. Ada, G. J. V. Nossal, J. Pye, A. Abbot, *Nature* **199**, 1257 (1963).
16. Supported by USPHS grants CA 04946-04 and CA 06927-02. I thank Elsa P. Wright for assistance, and Dr. J. Schultz for advice in preparing this manuscript.

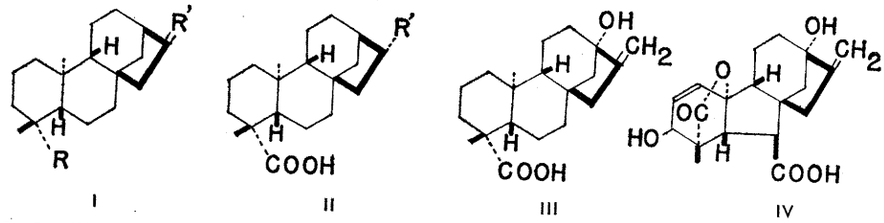
10 February 1964

Growth Response of the d-5 and an-1 Mutants of Maize to Some Kaurene Derivatives

Abstract. *(−)-Kaur-16-en-19-oic acid and (−)-kaur-16-en-19-ol oxygenated derivatives of (−)-kaurene, stimulated seedling elongation for the two nonallelic dwarf mutants of maize,* d-5 *and* an-1. *Replacement of the exocyclic methylene group attached to ring D by a keto-, methyl-, hydroxymethyl-, carboxy-, or methylcarboxy group resulted in compounds which were biologically inactive. These kaurene derivatives are structurally related to the gibberellins which produce a similar type of elongation for the* d-5 *and* an-1 *mutants.*

Several neutral and acidic diterpenoid metabolites have been isolated and characterized from the flowering plant, *Ricinocarpus stylosus* Diels; in addition, a number of derivatives of these compounds have been prepared (*1*). The possible metabolic relationship of some of these compounds to the gibberellins and to the kaurene derivative, steviol, has led us to test their biological activity as gibberellin-like substances, that is, their stimulation of organ elongation when applied to seedlings of certain flowering plants (*2*).

Thus far eight new compounds (Fig. 1, compounds 2–9) have been tested



I

1. R : CH_3, R′ : CH_2 (Kaurene)
2. R : CH_2OH, R′ : CH_2 (Kaurenol)
3. R : COOH, R′ : CH_2 (Kaurenoic acid)
4. R : CH_2OH, R′ : O
5. R : COOH, R′ : O

II

6. R′ : CH_3
7. R′ : CH_2OH
8. R′ : COOH
9. R′ : $COOCH_3$

III

10. (Steviol)

IV

11. (Gibberellic acid) (GA-3)

Fig. 1. Structure of compounds tested for biological activity. Gibberellic acid (IV-11), steviol (III-10), the kaurenoic acid (I-3), and the kaurenol (I-2) were active on *d-5* and *an-1* mutants of maize. The kaurene derivatives, (I-4), (I-5), (II-6), (II-7), (II-8), and (II-9), were biologically inactive.