invade surrounding normal tissues rather extensively and (d) do metastasize to bone. These new malignant breast tumors in mice appear closely to simulate some of the characteristics of human mammary carcinomas.

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Effect of p-Aminobenzoic Acid on Growth of Rickettsiae and Elementary Bodies, with Observations on Mode of Action

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In a previous report,[1] publication of which was delayed for reasons of military security, it was shown that the growth of typhus rickettsiae in the yolk sac of the developing chick was markedly inhibited by p-aminobenzoic acid.* We demonstrated further that the best inhibition of rickettsial growth was obtained only when maximal dosages of paba were used, which in the case of the chick embryo was 2-4 mg per egg. The lowest amount of paba which would give a certain inhibition was 0.2-0.4 mg per egg. These experiments were an outgrowth of a preliminary report by Snyder, Maier, and Anderson[2] in which data (on mice infected with murine typhus) were presented which suggested that paba might be of therapeutic value. On the basis of the fact that maximal dosages of paba are necessary for the inhibition of rickettsiae, large amounts of paba were then administered to humans infected with typhus, and good therapeutic results were obtained (Yeomans *et al.*)[3] Greiff, Pinkerton, and Moragues[4] have studied the effect of enzyme inhibitors and activators (penicillin, para-aminobenzoic acid, sodium fluoride, and vitamins) on the multiplication of typhus rickettsiae, and have also found that paba inhibits rickettsial growth.

In our first report it was mentioned that a similar inhibition of rickettsial growth was obtained when eggs that had been infected with Rocky Mountain spotted fever were treated with paba. A more detailed analysis of the action of this drug on different members of the spotted fever group showed not only that the inhibition was more striking in many cases than was obtained with typhus, but that differences in magnitude of the response of different kinds of rickettsiae to paba could be correlated with their antigenic relationships. It is the purpose of the present report to present these quantitative aspects of the growth of different kinds of rickettsiae (and some elementary bodies) in the presence of paba. Data are also included which have a bearing on the possible mode of action of paba.

Methods. The methods used in measuring the effect of paba on the growth of rickettsiae were similar to those previously described.[1] Hen's eggs which had been incubated 8 days and contained normal-appearing, vigorous embryos were selected and divided into two lots. Each egg of the first, or control, lot received an injection of 0.5 cc of sterile distilled water in the yolk sac, and each egg of the test series received a similar injection of 0.5 cc of a sterile solution of paba in distilled water. Immediately after the injection with these solutions, each egg was infected by injecting

[1] Hamilton, H. L., Plotz, H., and Smadel, J. E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 255. (Report to the Director of the U. S. A. Typhus Commission, 16 December, 1943.)

* Hereafter referred to as paba.

[2] Snyder, J. C., Maier, J., and Anderson, C. R., Report to the Division of Medical Sciences, National Research Council, Washington, D.C., 26 December, 1942.

[3] Yeomans, A., Snyder, J. C., Murray, E. S., Zarafonetis, C. J. D., and Ecke, R. S., *J. A. M. A.*, 1944, **126**, 349.

[4] Greiff, D., Pinkerton, H., and Moragues, V., *J. Exp. Med.*, 1944, **80**, 561.

TABLE I.
Influence of Paba on Time of Death of Chick Embryos Infected with Rickettsiæ of the Spotted Fever Group.

		Total No. of eggs	No. of daily deaths Day: 3	4	5	6	7	8	9	10	11	Survivors	% dead
	Controls	58	8	48	2	—	—	—	—	—	—	0	100
Rocky Mt. Spotted Fever	Paba—2 mg	56	1	1	3	3	3	2	4	6	7	26	53.6
	Controls	72	2	47	22	1	—	—	—	—	—	0	100
Brazilian Spotted Fever	Paba—2 mg	59	0	0	1	0	3	4	13	13	15	10	83.1
	Controls	75	3	56	16	—	—	—	—	—	—	0	100
Colombian Spotted Fever	Paba—2 mg	63	0	0	1	1	4	19	13	12	8	5	92.1
	Controls	51	2	6	31	10	2	—	—	—	—	0	100
South African Tick Fever	Paba—2 mg	51	0	1	2	2	4	8	8	8	10	8	84.3
	Controls	63	7	24	27	5	—	—	—	—	—	0	100
Fièvre Boutonneuse	Paba—2 mg	59	3	2	9	9	8	13	8	4	2	1	98.3

0.5 cc of a suspension of the rickettsiae to be tested. The infectious material was obtained from infected yolk sacs by grinding one part of yolk sac with alundum and diluting with 25 parts of sterile skim milk. The eggs were then sealed with wax and incubated at 37.5°C. Daily recordings were made of the deaths of embryos, and smears from each yolk sac were stained and recordings made of the average number of rickettsiae seen in each oil-immersion field. In general, we have found that the time of death is a far more reliable measuring index than the number of rickettsiae in smears, because rickettsiae vary in number according to the length of time that the egg has been dead and even according to the portion of the sac which is sampled. Therefore, the results which are presented here are based entirely on time of the embryo's death as a measure of rickettsial growth.

A uniform dosage of 2 mg of paba per egg was used in all of these experiments except when otherwise stated.

Results. Rocky Mountain Spotted Fever: Under the conditions of culture just described, the rickettsiae of Rocky Mountain spotted fever are ordinarily very toxic for chick embryos, with most of the deaths occurring between the third and fourth days after infection. Smears from the yolk sac usually contain a small number of rickettsiae (1-50 rickettsiae per oil-immersion field). The rickettsiae of typhus, on the other hand, do not ordinarily kill the embryos until 5-6 days after infection, and smears from the yolk sac are often filled with countless numbers of rickettsiae. This difference in the yield of rickettsiae may be partly explained by the lesser toxicity and consequent longer growth period of the typhus rickettsiae.

When eggs are simultaneously infected with Rocky Mountain spotted fever and injected with a 2 mg dosage of paba, then the early and complete mortality, noted above, does not occur. Table I and Fig. 1 summarize the results obtained with 58 control and 56 paba-treated eggs. All of the control embryos died early of the infection, with an average day of death of 3.9. In contrast to the sharp endpoint with the controls, embryos which had received paba showed only scattered deaths, with over 46% surviving to the time of hatching (19th day of incubation). Smears from the yolk sacs of dead control embryos contained the usual moderate number of rickettsiae (1-50 per field), but, in most cases, no rickettsiae could be found in the smears made from paba-treated eggs. The few paba-treated eggs in which rickettsiæ could be demonstrated rarely contained more than a single rickettsia per field.

The minimum amount of paba which would cause a measurable prolongation of survival time was determined in a second series of experiments. Dilutions were made of the stock paba solution (4 mg/cc) so that the 0.5 cc dosage would contain different amounts of the drug. Control embryos received 0.5 cc amounts of distilled water. By a process of bracketing, within a concentration range of

Figure 1. Effect of Paba on Time of Death (Rocky Mountain Spotted Fever)

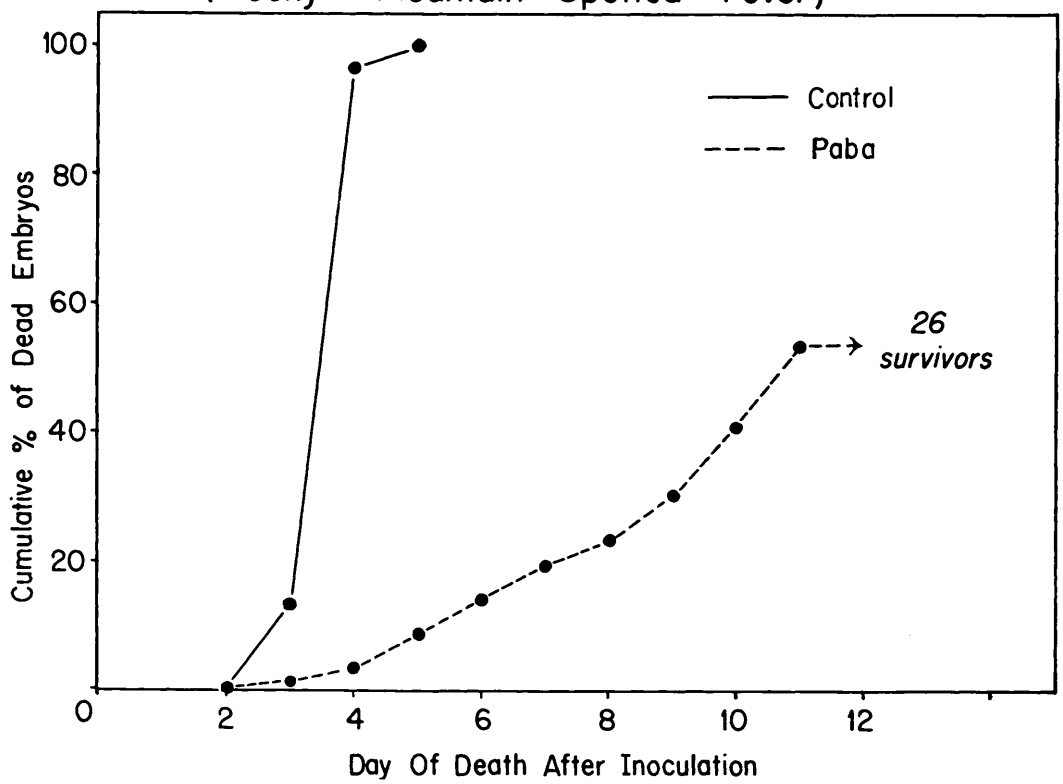
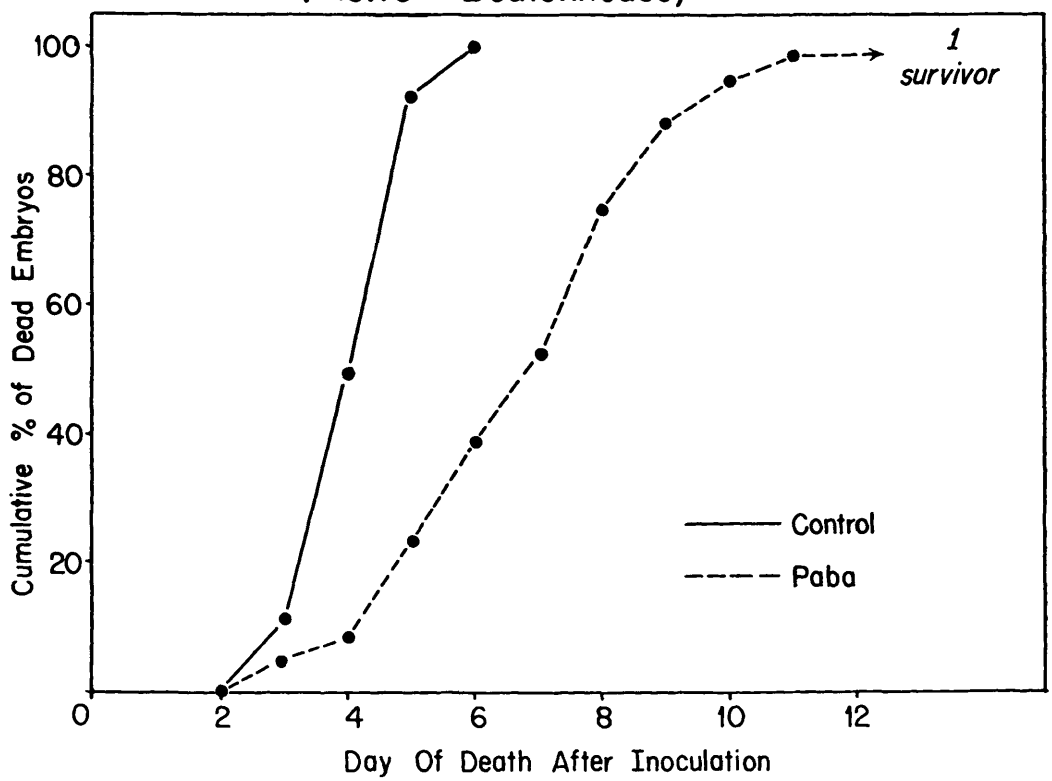


TABLE II.
Effect of Different Concentrations of Paba on Survival of Chick Embryos Infected with Rocky Mountain Spotted Fever.

	Concentration of Paba	Total No. of eggs	No. of daily deaths Day: 3	4	5	6	7	8	9	10	Avg day of death
Exp. 1	0 mg	8	1	6	1						4.0
	0.0625	8	2	4	2						4.0
	0.25	10	0	6	4						4.4
	1.0	9	1	0	0	0	0	0	7	1	8.4
Exp. 2	0 mg	9	1	6	2						4.1
	0.1	7	1	4	2						4.1
	0.25	12	0	4	8						4.7
	0.5	6	0	0	2	1	1	2			6.5
Exp. 3	0 mg	10	0	4	6						4.6
	0.1	13	1	5	7						4.5
	0.25	8	0	2	6						4.8
	0.5	9	0	0	4	3	2				5.8

0.0625-1.0 mg per egg, the minimal effectual concentration was found to be ±0.25 mg. The results of 3 separate experiments are presented in Table II. It will be noted that the average day of death was not significantly altered by a dosage of 0.1 mg or less, but that there was a consistent and appreciable increase in the average day of death with concentra-

Figure 2. Effect of Paba on Time of Death (Fièvre Boutonneuse)



tions of 0.25 mg or more. It is of interest that the number of rickettsiae seen in smears from treated eggs was inversely correlated with the amount of drug which was given. The minimal effectual dosage of paba, as determined for spotted fever (±0.25 mg), is of the same order of magnitude as that obtained with typhus rickettsiæ (0.2-0.4 mg.)[1]

Other Rickettsiae of the Spotted Fever Group: In view of the striking inhibitory action of paba on the growth of Rocky Mountain spotted fever rickettsiae, a survey was made of other members of the spotted fever group to see if differences could be detected in the magnitude of the inhibition. An identical technique was used for testing Brazilian spotted fever, Colombian spotted fever, South African tick fever, and fièvre boutonneuse, with results as shown in Table I. It will be noted that with each of these kinds of rickettsiae there was a prolongation of the survival time in the presence of paba, with some embryos surviving to hatching. Practically identical survival times were obtained with South African tick fever and Brazilian spotted fever, with 16% and 17% survivors, respectively. There were fewer survivors with Colombian spotted fever (8%) and fièvre boutonneuse (1.7%). With regard to the number of rickettsiae, there were very few in smears from treated eggs which were infected with Brazilian and Colombian spotted fevers. However, there were many rickettsiae even after paba-treatment in eggs infected with South African tick fever and fièvre boutonneuse, and, in fact, the number of rickettsiae often approached that seen in the controls. When the data in Table I are expressed graphically in terms of the cumulative percent of embryos dead per day, it is found that there has actually been more depression of the growth curve with the Rocky Mountain, Bra-

TABLE III.
Influence of Paba on Time of Death of Chick Embryos Infected with "Q" Fever, Tsutsugamushi, Lymphogranuloma Venereum, and Psittacosis.

Organism		Total No. of eggs	No. of daily deaths — Day: 2	3	4	5	6	7	8	9	10	11	% dead	Avg day of death
	Controls	51	0	2	22	24	3	—	—	—	—	—	100	4.5
"Q" Fever	Paba—2 mg	41	0	3	19	14	4	1	—	—	—	—	100	4.5
	Controls	57	0	3	0	1	2	7	14	21	9	—	100	8.2
Tsutsugamushi	Paba—2 mg	57	0	1	0	2	5	12	10	18	6	3	100	8.1
	Controls	32	2	14	3	2	9	2	—	—	—	—	100	4.3
Lymphogranuloma venereum	Paba—2 mg	32	1	13	4	4	5	5	—	—	—	—	100	4.4
	Controls	12	0	9	3	—	—	—	—	—	—	—	100	3.25
Psittacosis	Paba—4 mg	11	0	9	2	—	—	—	—	—	—	—	100	3.18

zilian, and Colombian spotted fevers than with either South African tick fever or fièvre boutonneuse. Figs. 1 and 2 illustrate the two extremes in reactivity to paba. A typical S-shaped growth curve is obtained in every case, but with paba-treatment the curve is flattened in varying amounts according to the kind of rickettsiae tested. The fact that South African tick fever had more survivors in the paba group, in spite of the lesser depression of its growth curve and the lesser inhibition of rickettsial growth, is explained by the lower toxicity of this kind of rickettsia for the chick embryo. The lower toxicity of South African tick fever rickettsiae is evident in the longer survival time of control embryos (average day of death = 5.1) than with any other member of the spotted fever group. Thus, even a small inhibition is sufficient to permit more embryos to survive to hatching. Fièvre boutonneuse rickettsiae are even less inhibited by paba (Fig. 2). This is of interest in view of the fact that Plotz, Reagan, and Wertman[5] could differentiate between Rocky Mountain spotted fever and fièvre boutonneuse by means of the complement fixation test. Other data of these same authors (to be published) indicate that there are wider antigenic differences between the African group and the American group of spotted fevers than between individual members of each group. This segregation in the spotted fever group is confirmed by our data on chemotherapy.

Organisms Not Responding to Maximal Dosages of Paba: In contrast to the striking inhibition of the growth of typhus[1] and spotted fever rickettsiae, it was found that the rickettsiae of "Q" fever and tsutsugamushi were apparently unaffected even by dosages of 2-4 mg of paba per egg. The data are presented in Table III. There was no significant difference between the controls and the embryos receiving paba, either in the average day of death or in the number of rickettsiae seen in smears from the yolk sacs.

Similarly, it was found that maximal amounts of paba had no apparent effect on the growth of elementary bodies of lymphogranuloma venereum and psittacosis (Table III).

Mode of Action of Paba: In a preceding report[1] we presented data showing that the acetyl derivative of p-aminobenzoic acid and the ortho- and meta- isomers of aminobenzoic acid were inactive on rickettsiae. It was clear from these results that the amino group could not be altered, and further that the para position was necessary in order for the aminobenzoic acid molecule to retain its inhibitory activity.

We then set out to test other compounds which were benzene ring analogs of paba, but with various modifications of the carboxyl group.† When it was found that a compound was not sufficiently soluble in water so that

[5] Plotz, H., Reagan, R. L., and Wertman, K., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 173.

† The author is indebted to Major D. A. MacFadyen, M.C., of the Army Medical School, for suggesting the use of several of these compounds, to Col. H. Plotz, M.C., of the U. S. A. Typhus Commission, for procuring them, and to Dr. W. H. Engels, of the Merck Research Laboratory, for making them available for our use.

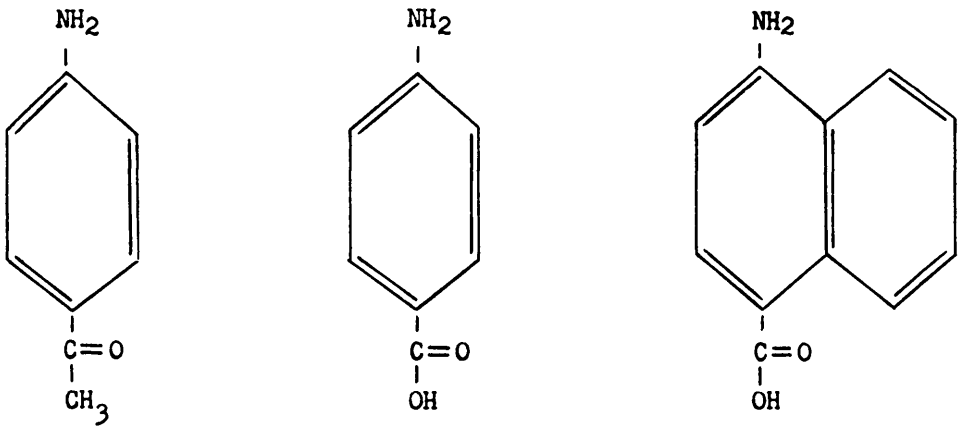


FIG. 3.

equimolecular amounts could be tested in comparison with paba, then both the paba and the compound to be tested were dissolved in sterile olive oil. It was found that chick embryos were relatively unaffected by injections of as much as 0.4 cc in the yolk sac, and the oil did not diminish the activity of paba.

The first compounds to be considered are those in which the carboxyl group is removed from its attachment to the benzene ring. Such a compound is p-aminophenylacetic acid ($NH_2 \cdot C_6H_5 \cdot CH_2 \cdot COOH$) in which the carboxyl group is separated from the ring by a single CH_2 group, but otherwise maintains its para position relative to the amino group. This compound was inactive on rickettsiae, even in amounts as large as 2 mg per egg. Other similar compounds which confirmed this result were p-aminocinnamic acid ($NH_2 \cdot C_6H_5 \cdot CH = CH \cdot COOH$) and p-aminobenzoyl-d, l-glutamic acid in which the chain is progressively longer which separates the carboxyl group from its ring position. Neither compound was effective in saturated aqueous solution, even though all three of these compounds have been reported by others to have effects similar to those of paba on the growth of bacteria.[6,7,8]

[6] Rubbo, S. D., and Gillespie, J. M., *Lancet*, 1942, **242**, 36.

[7] Johnson, O. H., Green, D. E., and Pauli, R., *J. Biol. Chem.*, 1944, **153**, 37.

[8] Auhagen, E., *Hoppe-Seyler's Z. f. physiol. Chem.*, 1942, **277**, 197.

Other compounds were tested, in which the carboxyl group was replaced by other kinds of carbonyl groups. An example of this type is p-aminoacetophenone (Fig. 3). This compound proved toxic for the chick embryo in high concentrations (2 mg/egg), but as much as 0.5 mg could be tolerated, which in terms of an equimolecular amount of paba is well above the threshold of paba-activity. No activity was observed on the time of death of infected embryos in the presence of p-aminoacetophenone, and fully as many rickettsiae were found in smears from dead eggs as in the controls. Confirmatory evidence was obtained by using 4,4′-diaminobenzophenone, which also proved to be inactive.

Since it was now clear that the integrity and relative positions of both the carboxyl and amino groups must be maintained in order to retain activity, we proceeded to test the effect of modifying the shape or size of the molecule. This can be done by the addition of extra side chains at other points on the benzene ring, without touching the 1- and 4- positions occupied by the amino and carboxyl groups. Two such compounds, 2-acetylamino-4-aminobenzoic acid, and 3-bromo-4-aminobenzoic acid, proved to be inactive. Another way of enlarging the molecule is to add an additional benzene ring so as to form a naphthalene nucleus. The compound thus formed, 4-amino-1-naphthoic acid, is homologous to p-aminobenzoic acid (Fig. 3). Equimolecular

amounts of 4-amino-1-naphthoic acid were toxic for the chick embryo even at the level of the minimal effectual dosage, but some of the embryos survived sufficiently long at levels well above the minimal dosage so that they could be examined for rickettsiae. For example, when a 0.5 mg dosage of paba was used (minimal effectual dosage = 0.25 mg), an equimolecular amount of 4-amino-1-naphthoic acid killed all of the embryos within 2-4 days. But smears from these embryos contained as many rickettsiae as the controls, whereas no rickettsiae were seen in smears from paba-treated embryos of the same age. It seems clear, therefore, that 4-amino-1-naphthoic acid does not appreciably affect the growth of rickettsiae.

These data all indicate that practically any possible modification of the paba molecule will diminish, and possibly even destroy, its inhibitory activity on rickettsiae. Such a high degree of specificity is usually associated with activity on an enzyme system, and leads one to speculate that the blocking of such a system by paba may be responsible for the inhibition of rickettsial growth.

Summary. 1. The maximal dosage of p-aminobenzoic acid for the chick embryo (2 mg) is effective in inhibiting the growth of rickettsiae of the spotted fever group in the following order of magnitude: Rocky Mountain s. f. > Brazilian s. f. > Colombian s. f. > South African tick f. > fièvre boutonneuse. There was much more inhibition of the American group than of the African spotted fevers.

2. p-Aminobenzoic acid was about twice as effective on Rocky Mountain spotted fever infection (46% survivors) as on either epidemic or murine typhus (20% and 26% survivors, respectively).

3. p-Aminobenzoic acid did not appreciably inhibit the growth of the agents of "Q" fever, tsutsugamushi, lymphogranuloma venereum, or psittacosis.

4. Studies on the mode of action of p-aminobenzoic acid indicate that not only must the amino and carboxyl groups remain intact and in the para position with respect to one another, but the total size of the molecule must not be altered, if the inhibitory properties are to be retained. Such a high degree of specificity suggests that paba may be acting on an enzyme system.

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Synaptic Transmission of the Muscles in Arthritis.*

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Thomsen, Altamirano and Luco[1] studied some aspects of the mechanism of synaptic transmission in muscles whose tendons had been cut, thus approaching the general problem of a possible synaptic perturbation by changes in the effector.

Lippmann and Selig[2] gave experimental proof to the clinical observation that muscles of diseased joints undergo a process of atrophy. These results suggested the idea that an experimentally produced arthritis might also cause changes in the neuromuscular transmission. In this paper we report some experiments that were carried out with that idea in mind.

Procedure and Methods. The experiments were performed on cats. The arthritis was produced by injecting about 0.1 cc of 3%, 5%, or 10% silver nitrate under anesthesia by ether or nembutal. The tibio-tarsal joint was

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Thomsen, P., Altamirano, M., and Luco, J. V., *Medicina* (Buenos Aires), 1942, **3**, 67.

2 Lippmann, R. K., and Selig, S., *Surg. Gynec. Obstet.*, 1928, **47**, 512.