months after operation all insulin was withdrawn for a period of 4 days. On the first day, glycosuria, previously less than 10 g per day, rose to 112 g. On the second day, the value was 143 g and ketosis appeared. On the fourth day, all food was withheld and the urine showed a D/N ratio of 2.42. Insulin was reinstituted that evening because of severe thirst and discomfort. The patient had lost 4.5 kg of weight during this period.

Six days later, with the patient's appetite failing, insulin was again withdrawn. Marked glycosuria and ketosis promptly appeared. Two days later, the urine contained 108 g of glucose and the patient stopped eating entirely. On the fourth day of this period, the blood sugar was 570 mg %, and between the fifth and sixth days the urine showed a D/N ratio of 3.59. Death occurred on the sixth day in typical diabetic coma.

*Fasting respiratory quotients* were determined daily for 5 days when the patient was in relatively good health and was receiving crystalline insulin 3 times per day, the last dose being given before supper. The values varied from 0.77 to 0.80. Fasting blood sugars on the same mornings were in the neighborhood of 100 mg per 100 cc. Determinations of the R.Q. during the complete withdrawal of insulin were unsatisfactory.

*Fat Metabolism.* The serum lipids, which before operation ranged from 800 to 1260 mg per 100 cc, slowly declined after pancreatectomy but never sank to subnormal levels, the minimum being 707 mg per 100 cc. At autopsy the liver was grossly fatty, probably owing to the patient's death in diabetic coma.

*Conclusions.* (1) The pancreas, or its hormone, insulin, is necessary to life in the human. (2) On the basis of the present case and others previously reported,[2-5] the insulin output of the normal human pancreas must be considerably smaller than has been believed and seems to lie in the range of 30 to 50 units per day. Therefore, (3) in diabetic patients requiring more than this amount, an extra-insular factor must be contributing to the diabetic syndrome. (4) The D/N ratio of from 2.42 to 3.59 indicates that a fasting, depancreatized man deprived of insulin converts protein to carbohydrate at a rate commensurate with that observed in the dog under the same conditions[6] or when treated with phloridzin.[7]

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## 14916

## Effect of p-Aminobenzoic Acid on the Growth of Typhus Rickettsiæ in the Yolk Sac of the Infected Chick Embryo.*

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The search for chemical compounds having possible therapeutic effects on typhus fever, which centered first on sulfonamide derivatives, has been extended to include all kinds of substances having effects on pathogenic bacteria and protozoa. Snyder, Maier, and Anderson[1] tested the activity of a large number of drugs (sulfonamides, anti-luetic, anti-malarial, and trypanosomicidal compounds) on the

* Report to the Director of the United States of America Typhus Commission, December 16, 1943, withheld from publication. Since this time two other papers dealing with the therapeutic effect of P.A.B.A. were published.

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TABLE I.
Effect of Paba on Survival Time of Typhus-Infected Embryos.

| | | Total No. of embryos | No. of daily deaths Day: 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Survived | % dead |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Epidemic Typhus | Controls | 43 | 3 | 10 | 14 | 13 | 3 | | | | | | 0 | 100 |
| | Paba (4 mg) | 44 | 0 | 10 | 6 | 4 | 2 | 5 | 2 | 1 | 3 | 2 | 9 | 79.5 |
| Murine Typhus | Controls | 25 | 10 | 2 | 3 | 8 | 2 | | | | | | 0 | 100 |
| | Paba (4 mg) | 23 | 3 | 5 | 4 | 2 | 2 | 1 | | | | | 6 | 73.9 |

TABLE II.
Effect of Paba on Number of Rickettsiæ.

| | | Total No. of Eggs | No. of Rickettsiæ per field: 0 No. | % | 1-10 No. | % | 100 No. | % | 1000 No. | % | Countless No. | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Epidemic | Controls | 56 | 7 | 12.5 | 29 | 51.8 | 8 | 14.3 | 8 | 14.3 | 4 | 7.1 |
| | Paba | 59 | 42 | 71.2 | 16 | 27.1 | 1 | 1.7 | 0 | 0 | 0 | 0 |
| Murine | Controls | 30 | 0 | 0 | 12 | 40 | 5 | 16.6 | 5 | 16.6 | 8 | 26.7 |
| | Paba | 27 | 14 | 51.9 | 9 | 33.3 | 2 | 7.4 | 1 | 3.7 | 1 | 3.7 |

survival time of mice and cotton rats which had been infected intraperitoneally with typhus. None of these substances had beneficial effects, but their experiments led to the trial of p-aminobenzoic acid, which gave indications that it might suppress the growth of typhus rickettsiae. More recently, Andrewes, King, and Van den Ende[2] reported that, of more than 164 compounds which were being tested, only 3 showed anti-rickettsial activity as measured by the ability of the drugs to prevent or retard the development of pulmonary lesions in intranasally-inoculated mice. A preliminary experiment by these authors[2] with p-aminobenzoic acid tended to confirm the findings of Snyder, Maier, and Anderson, in that maximal tolerable doses of the drug caused a reduction in the number and size of pulmonary lesions.

In view of the availability of p-aminobenzoic acid (hereafter referred to as paba) and its suggested therapeutic action on typhus in mice, we decided to test its effect directly on the growth of rickettsiae by inoculating it into the yolk sac of the developing chick embryo.

*Procedure.* Eggs were selected which contained normally-developing embryos after 6 days' incubation. These were divided into two equal lots. The first or control group, was inoculated into the yolk sac with 1 cc of physiological salt solution per egg, and the other group received an equal volume of salt solution containing paba. The drug was used initially in two concentrations: 4 mg/cc (in solution), and approximately 25 mg/cc (suspended crystals). On the following day each embryo, in both the control and treated series, was inoculated in the yolk sac with 0.5 cc of infective typhus material. The inoculum was prepared from either epidemic (Breinl) or murine (Wilmington strain) infected yolk sacs. These were ground with alundum and diluted 1:20 with physiological salt solution.

The data were recorded in either of two ways, according to the type of analysis which was to be made: (1) The survival time of treated eggs as compared with controls was measured by recording all deaths, beginning on the third day after inoculation with rickettsiae and extending up to hatching. Smears made from the yolk sacs of dead embryos were stained by Machiavello's method and examined for rickettsiae. Or, (2) all eggs in both treated and control series were opened after an arbitrary length of time (3-6 days) and examined for rickettsiae. Counts were made according to the following system: O, + =

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

[2] Andrewes, C. H., King, H., and Van den Ende, M., *Brit. Rep. from Nat. Inst. for Med. Res.*, Hampstead, London, 1943.

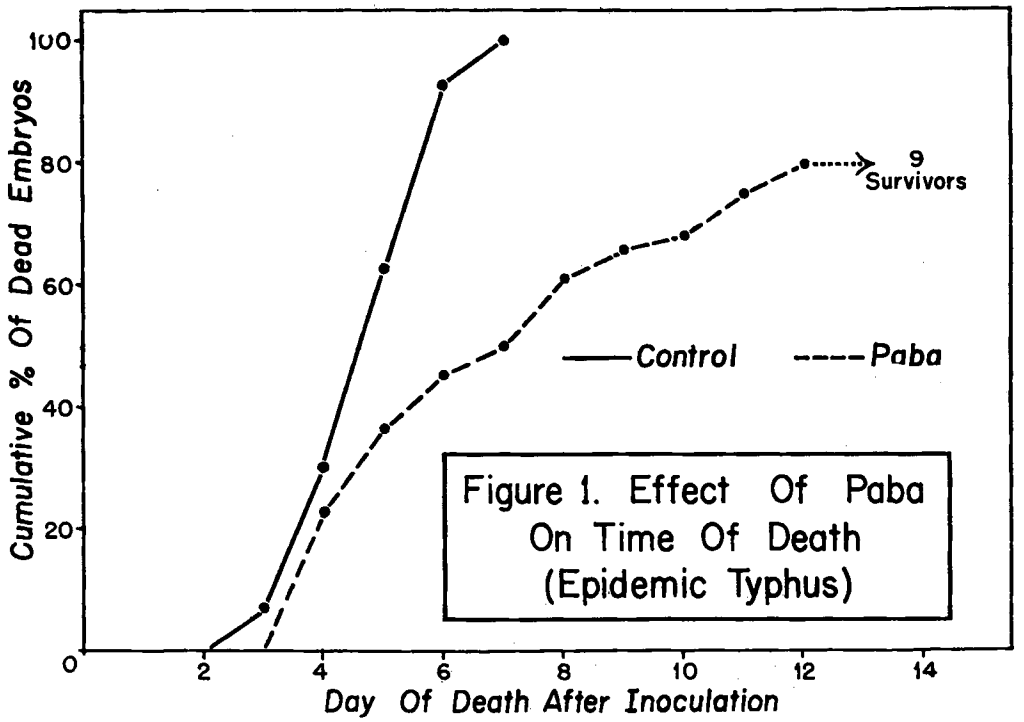



Figure 1. Effect Of Paba On Time Of Death (Epidemic Typhus)

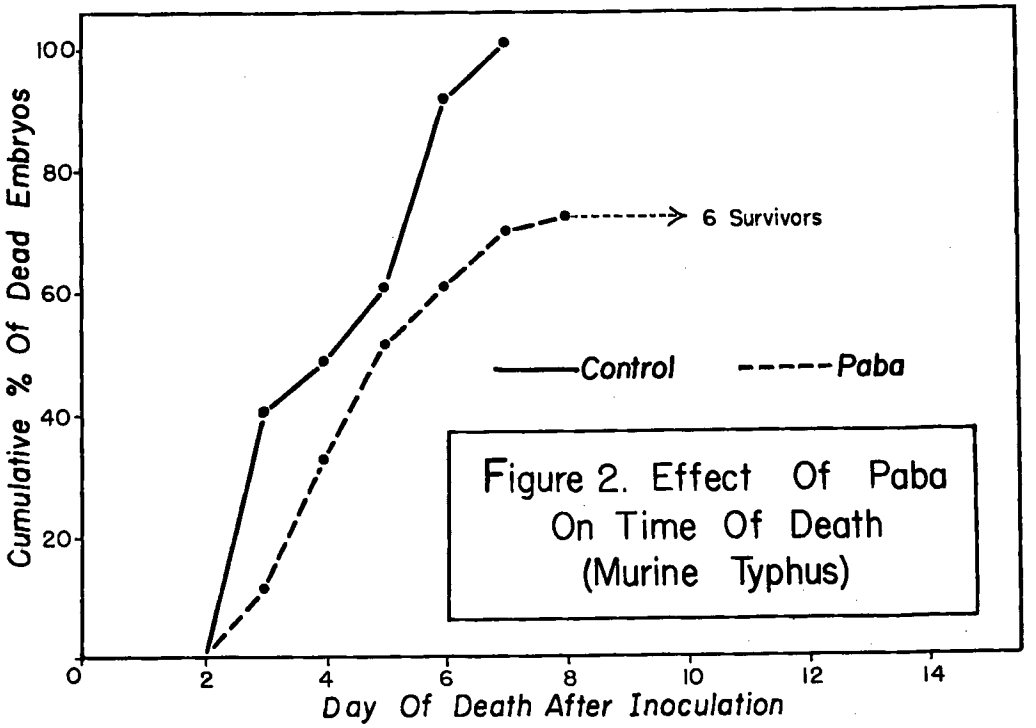
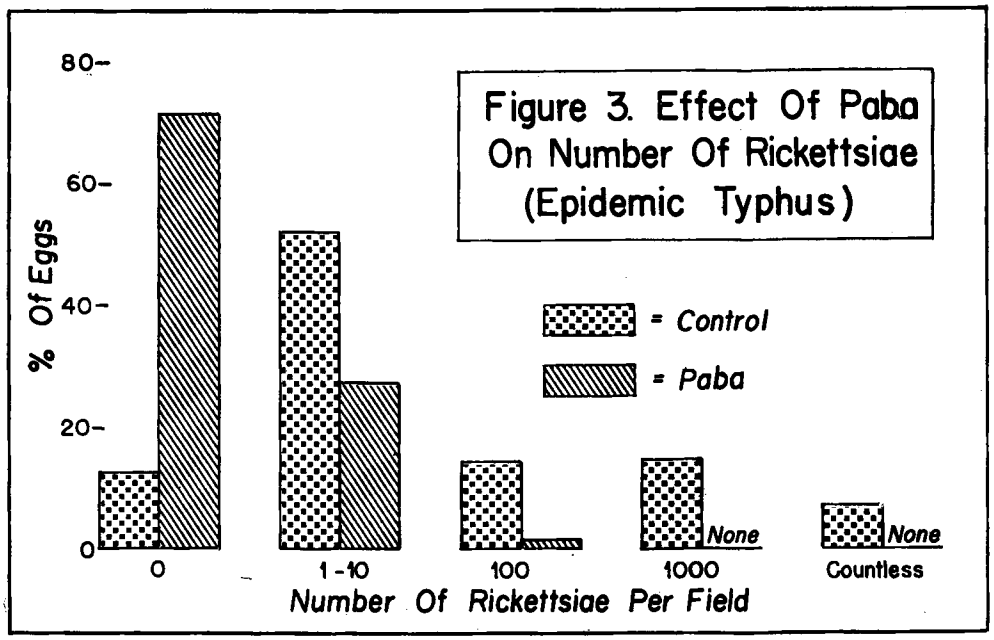
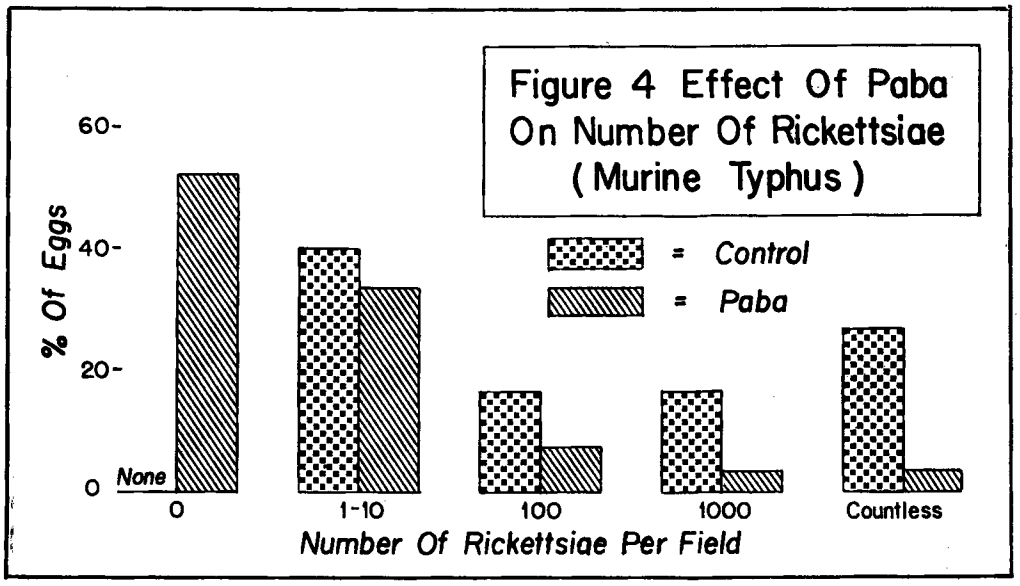



Figure 2. Effect Of Paba On Time Of Death (Murine Typhus)




1-10, ++ = 100, +++ = 1000, ++++ = countless rickettsiae per field.

This method of testing for chemotherapeutic effects on rickettsiae in the yolk sac of the developing chick embryo has several advantages: (1) it permits rather precise measurements of the action of chemicals on rickettsial growth by making rickettsial counts; (2) the action of the drugs can be tested with equal ease on either murine or epidemic strains of typhus; (3) dosages can be carefully controlled.

*Prolongation of Survival Time by Paba.* When the inoculated embryos are allowed to develop until death occurs from rickettsial infection, a rather striking difference becomes apparent between the control and paba-treated series. Table I shows the distribution of deaths in both series for a total of 87 eggs

Figure 5 Effect Of Delayed Treatment With Paba On Survival Time (Epidemic Typhus)

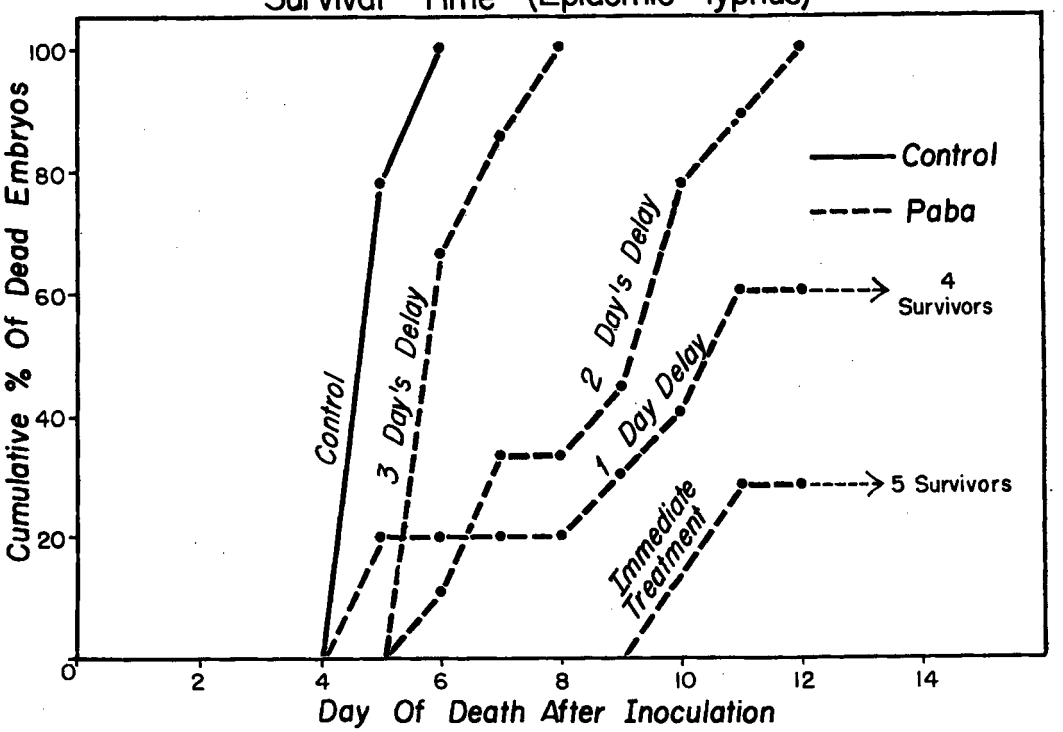



TABLE III.
Effect of Different Concentrations of Paba on Survival of Chick Embryos Infected with Epidemic Typhus.

| | Concentration of paba | Total No. of embryos | No. of daily deaths Days: 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | Avg. day of death |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Exp. No. 1 | 0 mg | 10 | 0 | 1 | 3 | 4 | 2 | | | | | | | 5.7 |
| | 0.04 | 11 | 0 | 0 | 5 | 5 | 1 | | | | | | | 5.6 |
| | 0.2 | 11 | 0 | 0 | 0 | 11 | | | | | | | | 6.0 |
| | 2.0 | 6 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 4 killed to prevent hatching | |
| Exp. No. 2 | 0 mg | 10 | 5 | 4 | 1 | | | | | | | | | 3.6 |
| | 0.2 | 12 | 0 | 12 | | | | | | | | | | 4.0 |
| | 0.4 | 12 | 2 | 5 | 3 | 1 | 1 | | | | | | | 4.5 |
| | 2.0 | 9 | 1 | 1 | 3 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 2 killed to prevent hatching | |
| Exp. No. 3 | 0 mg | 11 | 1 | 2 | 8 | | | | | | | | | 4.6 |
| | 0.4 | 14 | 0 | 3 | 2 | 5 | 3 | 0 | 1 | | | | | 5.85 |

which were infected with epidemic typhus. It will be noted that all deaths in the control series occurred between the third and seventh days following inoculation with rickettsiae. In contrast to this "bunching" of deaths in the controls, the paba-treated embryos showed only a moderate mortality on the fourth and fifth days, followed by sporadic deaths up to the thirteenth day (twentieth day of incubation). At this time there were still 9 survivors, which were killed to prevent hatching. When the cumulative % of daily deaths is plotted against time, the lag in time of death produced by paba may be shown graphically (Fig. 1).

Similar results were obtained when the eggs were infected with murine rickettsiae (Table I;

TABLE IV.
Effect of Isomers of Paba and Other Substances on Time of Death of Typhus-Infected Embryos.

| Compound | Total No. embryos | Days: 3 | 4 | No. of daily deaths 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | % dead | Avg. day of death |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| o-Aminobenzoic acid | 11 | 0 | 4 | 2 | | | | | | | | 100 | 4.2 |
| Controls | 6 | 1 | 3 | 2 | | | | | | | | 100 | 4.2 |
| m-Aminobenzoic acid | 9 | 3 | 0 | 6 | | | | | | | | 100 | 4.3 |
| Controls | 9 | 6 | 0 | 3 | | | | | | | | 100 | 3.7 |
| Acetyl p-aminobenzoic acid (Seitz filtered) | 13 | 0 | 7 | 4 | 2 | | | | | | | 100 | 4.6 |
| Controls | 11 | 0 | 4 | 7 | | | | | | | | 100 | 4.6 |
| Acetyl p-aminobenzoic acid (heat sterilized) | 9 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 3 | 1 | 2* | 77.8 | .... |
| Controls | 8 | 0 | 0 | 4 | 3 | 0 | 1 | | | | | 100 | 5.8 |
| Sulfanilamide | 10 | 3 | 0 | 6 | 1 | | | | | | | 100 | 4.5 |
| Controls | 10 | 1 | 2 | 6 | 1 | | | | | | | 100 | 4.7 |
| Sodium benzoate | 10 | 1 | 5 | 4 | | | | | | | | 100 | 4.3 |
| Controls | 13 | 2 | 3 | 7 | 1 | | | | | | | 100 | 4.5 |
| Benzoic acid | 12 | 7 | 5 | | | | | | | | | 100 | 3.4 |
| Controls | 7 | 4 | 1 | 2 | | | | | | | | 100 | 3.7 |

* Survivors.

TABLE V.
*Effect of Delayed Treatment with Paba on Survival of Typhus-Infected Embryos.*

| | Total No. embryos | Day: 1-4 | No. of daily deaths 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Survived | % dead |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Immediate treatment | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 5 | 28.6 |
| 1 day delay | 10 | 0 | 2 | 0 | 0 | 0 | 1 | 1 | 2 | 0 | 4 | 60 |
| 2 " " | 9 | 0 | 0 | 1 | 3 | 0 | 1 | 2 | 1 | 1 | 0 | 100 |
| 3 " " | 7 | 0 | 0 | 5 | 1 | 1 | | | | | 0 | 100 |
| Controls | 9 | 0 | 7 | 2 | | | | | | | 0 | 100 |

Fig. 2). Here again, all control deaths occurred between the third and seventh days. However, death was considerably delayed in the paba-treated group, with 6 embryos surviving to hatching.

An examination of smears made from these dead paba-treated eggs showed that there were usually very few rickettsiae present. However, some of the eggs which died at a later date did contain increased numbers of rickettsiae. Apparently, therefore, the drug acts by suppressing the growth rate of the rickettsiae, so that appreciable numbers develop only after a long incubation period. Further evidence for this interpretation was obtained by comparing the numbers of rickettsiae in treated and control eggs.

*Reduction in Number of Rickettsiae.* Counts were made of the number of rickettsiae per oil immersion field in yolk sac smears from 86 controls and 86 paba-treated eggs, and tabulated in 5 categories as shown in Table II. When epidemic rickettsiae were inoculated into control eggs, over 50% contained at least 1-10 rickettsiae per field when examined 3-6 days after infection. Lesser percentages contained 100, 1000, and countless rickettsiae, as shown in Fig. 3. In contrast to this distribution of controls, over 70% of the infected paba-treated eggs contained no rickettsiae when examined after the same period of incubation. Twenty-seven % showed 1-10 rickettsiae, and only 1 egg had as many as 100 per field (Fig. 3).

An exactly comparable picture was obtained when the eggs were infected with murine rickettsiae. In general, the controls were richer than when the epidemic strain was used, but the paba-treated eggs were predominantly negative or in the 1-10 group (Fig. 4). Only 1 egg was classified in each of the 2 highest categories (1000, countless).

It is clear from these data that an inhibition of rickettsial growth is produced by the use of paba. This inhibition probably accounts in part for the delay in the death of the infected

embryo.

*Specificity of the Paba-Inhibition.* The data thus far reported were obtained by using relatively enormous amounts of paba (4 mg per egg, = approximately 70γ/cc, or more) compared with the concentrations which are sufficient to affect bacterial growth (fractions of a gamma per cc[3,4]). In fact, 4 mg was the maximal dose that 6-day embryos could tolerate; larger amounts caused death in 5-8 days. When the concentration of paba was reduced to 2 mg per egg, or less, results were obtained as shown in Table III.

It is seen that the least amount of paba which will cause delay in death of the embryo is 0.4 mg per egg. The inhibition of rickettsial growth is slight, however, for a comparison of smears from the treated and control eggs shows no conclusive difference in numbers of rickettsiae. With more massive dosages, the inhibition of rickettsial growth becomes more or less complete.

The necessity for using such large quantities of paba raises the possibility that a non-specific inhibition might be involved. It is well-known that massive amounts of a chemical (particularly a growth stimulant) may produce unfavorable conditions for growth, regardless of its effect (or lack of activity) in low concentrations. Accordingly, the two isomers, ortho- and meta-aminobenzoic acid, as well as acetyl p-aminobenzoic acid (both heat-sterilized, and sterilized by Seitz-filtration), sulfanilamide, sodium benzoate, and benzoic acid were tested in concentrations equivalent to the maximal dose of paba (4 mg per egg). None of these substances had any apparent effect either on time of death (Table IV) or number of rickettsiae, with the exception of the heat-sterilized acetyl derivative. This was of particular interest, because heating of acetyl paba is known to convert a certain proportion of the compound into paba, by hydrolysis. It seems clear, therefore, that the inhibition of growth of typhus rickettsiae by paba is due to a specific action of the drug.

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*Effect of Delayed Treatment with Paba.* In connection with its possible use as a therapeutic agent, it seemed of value to know what degree of inhibition would be produced by paba if treatment were delayed beyond the time of infection. Five groups of eggs were injected with sufficient rickettsiae (Breinl strain) to cause death in 4-6 days. One group was retained as a control series, and received an additional injection of physiological salt solution immediately after infection. Another group received a similar volume (0.5 cc) of salt solution containing 2 mg of paba. The remaining 3 groups were injected with 2 mg of paba 24 hr, 48 hr, and 72 hr after infection, respectively. The results are shown in Table V and Fig. 5.

It will be noted that the efficacy of paba becomes progressively less, the longer the delay between infection and treatment, as might be expected. However, there is a surprising amount of protection when treatment is begun as late as 48 hr after infection. A similar gradient was obtained with regard to reduction in the number of rickettsiae. When treatment was delayed for 72 hr there was little, if any, reduction in number, but with 48 and 24 hr delays the smears were reduced from ++++ to +, and there was almost complete inhibition with immediate treatment. The data indicate, therefore, that good results may be obtained as long as 48 hr after infection, although earlier treatment is required for best protection.

*Discussion.* It is to be noted that the percentage of embryos protected by 2 mg of paba was, in general, higher than when 4 mg was used. For example, with 2 mg of paba, there were 22%, 66%, and 71% survivors (Tables III and V) as compared with 20% and 26% survivors with 4 mg (Table I). Since no rickettsiae were observed in a considerable number of the eggs which died in spite of the 4 mg, it seemed possible that some of these deaths might be attributed to a delayed toxic action on the part of the paba rather than to failure of the paba to inhibit rickettsial growth. While not completely studied, evidence for this interpretation was found in a later experiment with non-infected embryos which had been treated with 4 mg of paba. Although normal

development of the embryos took place during the first week, the drug apparently caused some permanent damage in that a significant number of them became moribund and died during the latter part of the incubation period. Because of this delayed toxicity of the maximal dosage, it is probable that some of the embryo deaths shown in Fig. 1 and 2 are attributable to the drug and not to the rickettsiae.

In addition to its activity against typhus rickettsiae, preliminary experiments have shown that paba also inhibits growth of the rickettsiae of Rocky Mountain spotted fever, but has no apparent effect on growth of psittacosis elementary bodies. Further experiments are now in progress on the mode of action of p-aminobenzoic acid.

*Conclusions.* I. p-Aminobenzoic acid inhibits the growth of typhus rickettsiae and prolongs the survival time of infected chick embryos. The inactivity of related compounds indicates that the inhibition is specific to paba. 2. The minimum amount of paba which will inhibit rickettsial growth is 0.4 mg per egg. In general, the inhibition is more complete with maximal dosages, which in the case of the 6-day chick embryo is 2-4 mg. These data suggest that relatively massive amounts of paba may have to be administered if beneficial effects are to be expected on human cases of typhus. 3. When treatment with paba is delayed 24, 48, and 72 hours beyond the time of infection, there is progressively less delay in the time of death and less reduction in the number of rickettsiae. However, good results are obtained with treatment as late as 48 hr after infection.

## 14917

## Penicillin as a Sporicidal Agent.

HAROLD R. CURRAN AND FRED R. EVANS. (Introduced by G. E. Holm.)

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Although Fleming's[1] first paper on penicillin furnished evidence of bactericidal as well as bacteriostatic activity, until 1941 attention was focused almost exclusively on the bacteriostatic or growth-inhibiting action of the drug.[2-4] Dawson *et al.*[5] reported that "Penicillin appears to be bactericidal against the pneumococcus *Streptococcus viridans,* staphylococcus and *Clostridium welchii, Cl. sporogenes,* and *Cl. septique*" (the latter three in the vegetative form). More recent reports have revealed strong bactericidal activity by penicillin against *Streptococcus hemolyticus,*[6-9] *S. viridans,*[10] pneumococci,[6,7] staphylococci,[7,11] and meningococci.[12]

Investigators[6,9,12,13] are essentially in agreement that penicillin activity is demonstrable only when the organisms are in an environment conducive to multiplication and it has been

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