TABLE 5

INCREASING AMOUNTS OF AMINOPTERIN IN THE PRESENCE OF 6000 MILLIGAMMA PGA/ML

Aminopterin (milligamma/ml)	Original No. larvae	% Larvae to pupate	Av time in days to pupation	% Pupae to become adults
0	10	100.0	11.2	90.0
3	21	100.0	11.0	100.0
100	20	95.0	11.5	100.0
1000	18	11.1	16.0	0.0

and to release it completely at 480.0 milligamma/ml.

This experiment showed that the inhibition caused by a small amount of aminopterin could be released by a slightly suboptimal amount of PGA. It was decided next to see how much aminopterin could be used without an inhibiting effect in the presence of an optimal amount of PGA. The results of these tests are presented in Table 5.

The results presented in Table 5 show that amounts of aminopterin as high as 100 milligamma/ml can be used without showing marked inhibition if the amount of PGA is also high. Even when 1000 milligamma of aminopterin/ml medium was used, complete inhibition was not achieved in the presence of 6000 milligamma PGA.

An inhibition index can be calculated which is quite consistent if one takes the concentration of inhibitor necessary to produce only half maximum growth (50% pupation), and obtains the ratio of it to the concentration of the metabolite used. In Table 4, it can be seen that 50% pupation occurs somewhere between a concentration of PGA of 14.4 and 82.0 milligamma/ml when the concentration of aminopterin is 3. Assuming that 50% pupation would occur with roughly 35 milligamma/ml PGA, the ratio of inhibitor to metabolite would be 3/35, or 0.086. If the same treatment is given to the data in Table 5, the ratio is 500/6000, or 0.083. These two values are remarkably close, indicating a constant relative inhibitive effect of aminopterin to the concentration of PGA.

Using a chemically defined medium in the absence of microorganisms, the quantitative results are of a different order of magnitude. This is strikingly illustrated by comparing the concentrations used by Goldsmith *et al.* (*1*) in a yeast medium with the concentrations found adequate in the present work on a synthetic medium. To obtain complete inhibition by aminopterin, the former workers had to use 75,000 to 200,000 milligamma/ml, whereas in the present work, 3 milligamma/ml was shown to be completely inhibitory. Nevertheless, the two sets of data agree qualitatively.

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Reversal of *p*-Aminobenzoic Acid Inhibition of Growth of Rickettsiae by *p*-Hydroxybenzoic Acid in Agar-Slant Tissue Culture

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The rickettsiostatic action of *p*-aminobenzoic acid (PABA) was first observed in mice by Snyder, Maier, and Anderson (*1*) in 1942 and also in the chick embryo by Greiff, Pinkerton, and Moragues (*2*) in 1944. Independently, Takemori (*3*) found in 1944 (published in 1949) the inhibitory action of PABA on the growth of typhus rickettsiae in tissue culture. It was argued that "if the mode of action of PABA on typhus rickettsiae be similar to that of the action of the sulfonamides on sulfonamide-sensitive organisms, it is suggested that an 'essential metabolite' will be found for rickettsiae which reverses the inhibitory action of PABA on typhus rickettsiae and may be chemically related to PABA" (*3*).

Following the announcement of *p*-hydroxybenzoic acid (POB) as a bacterial vitamin required by a mutant of *Escherichia coli* (*4*), the mechanism of inhibition of *E. coli* by PABA was shown to depend on competition with POB (*5*). The competition between POB and PABA observed with *E. coli* has led to similar reversal studies with POB of the rickettsiostatic action of PABA in chick embryos and mice (*6*).

In view of the rickettsiostatic action of PABA observed in tissue culture by Takemori (*3*), it has seemed desirable to investigate whether this action of PABA is also reversed in *in vitro* growth tests by POB. The present study shows that POB is able to reverse the rickettsiostatic action of PABA in agar-slant tissue culture.

The Wilmington strain of murine typhus (*Rickettsia typhi*), Smith strain of Rocky Mountain spotted fever (*R. rickettsii*),[1] M.K. strain of rickettsialpox (*R. akari*),[1] Nine Mile strain of Q fever (*Coxiella burnetii*),[1] and 6BC strain of psittacosis virus[1] which were employed in these experiments have been maintained on Zinsser's agar-slant culture (*7*) with dark embryonic tissue. All these agents grew quite satisfactorily on this medium, and passages were made at 8–15-day intervals.

The medium was prepared as described previously for the cultivation of rickettsiae and the viruses of lymphogranuloma venereum and tick-borne encephalitis (*8*). A 3.5% agar solution is made in twice-distilled water, dissolved at 15 psi for 15 min, cooled, and kept at about 55° C. To 75 ml of a double-strength Tyrode solution are added 50 ml of horse serum and 4 ml of a 0.04% solution of phenol red. This Tyrode-serum mixture is filtered through a Seitz filter, warmed to 55° C, and mixed with 75 ml of 3.5%

[1] Obtained through the courtesy of Rocky Mountain Laboratory, R. J. Huebner, H. R. Cox, and K. F. Meyer, respectively.

agar. The mixture is then poured into ordinary test tubes in 8–10 ml amounts. The tubes are slanted, left to harden at room temperature, then stoppered with rubber stoppers and sealed with paraffin. The pH of the medium is 7.6–7.8. Either PABA alone or PABA together with POB in various proportions was included in this medium by dissolving in Tyrode-serum mixture before filtration through a Seitz filter, previously washed thoroughly with distilled water. The agar powder employed was washed successively with 50% and 95% ethanol.

Agar-slant culture rickettsiae and virus were used as inocula. Tissue fragments (about 15 mg) from one culture tube showing maximum growth were collected with a spatula, mixed in a short test tube (15 × 80 mm) with about one g of tissue of 9–10 day-old chick embryo from which eyes and legs had been removed. The tissue was then minced well with scissors and deposited with a spatula upon the agar media, each culture tube receiving 60–70 mg of the tissue. Infective material obtained from one culture tube was usually used to inoculate fresh chick embryonic tissue for 12 new cultures, consisting of both control and test media.

The cultures were incubated at 35° C for 7–15 days, and growth was examined microscopically in Macchiavello-stained smears of cultured tissue.

TABLE 1

REVERSAL BY POB OF THE RICKETTSIOSTATIC ACTION OF PABA IN AGAR-SLANT TISSUE CULTURE

Medium		Growth after 8–15 days				
PABA (mg/100 ml)	POB (mg/100 ml)	*Rickettsia typhi*	*Rickettsia akari*	*Rickettsia rickettsii*	*Coxiella burnetii*	Psittacosis virus
0	0	+++	+++	+++	+++	+++
50	0	–	–	–	+++	+++
50	0.05	–	–	–	+++	+++
50	0.5	++	++(+++)	++(+++)	+++	+++
50	5	+++	+++	+++	+++	+++
100	0	–	–	–	+++	+++
100	0.1	–	–	–	+++	+++
100	1.0	++(+++)	++(+++)	++(+++)	+++	+++
100	10	+++	+++	+++	+++	+++
200	0	–	–	–	++·+++	+++

Degree of growth: –, none; + minimal; ++, moderate; +++, maximal.

In previous studies (*3*) it was shown that the growth of typhus rickettsiae is inhibited by PABA in deep columns of Maitland medium. The results presented in Table 1 demonstrate that the growth of three species of rickettsiae (*R. typhi, R. rickettsii,* and *R. akari*) is inhibited by PABA (0.5–1 mg/ml) in agar-slant tissue culture and that this inhibition can be reversed by POB in 1/100 the concentration of PABA. It may be noted that inhibition of *E. coli* by PABA is also reversed by POB in 1/100 the concentration of PABA (*5*). *C. burnetii,* which was reported to be least affected by PABA in the chick embryo (*9*), also is not readily susceptible to the action of PABA (1–2 mg/ml) on agar tissue culture. On a morphological, tinctorial, and cultural basis, the organism is a typical rickettsia. It requires living tissue to propagate, it stains similar to and looks like other rickettsiae. It distinctly differs from three other rickettsial organisms, however, in its susceptibility to the growth-inhibitory action of PABA, as well as in certain other biological characteristics (*10*).

Since the preservation of cell morphology of cultured tissue is, microscopically, quite satisfactory, and since both the virus of psittacosis and *C. burnettii* grow readily on agar media containing PABA (1-2 mg/ml), it is clear that the inhibitory action of PABA on the growth of the three species of rickettsiae is selective.

The agar-slant tissue culture technique has advantages and affords a procedure for studying the growth requirements of rickettsiae and viruses and for testing materials which may influence their growth.

Details of these and further studies will be published elsewhere.

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On the Presence of the Triphosphothiamine (TPT) in the Liver

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The presence in the liver of thiamine and of its diphosphoric ester (DPT) is too well known to require mention; we have obtained evidence for the existence of a triphosphoric ester of thiamine (TPT) in the liver of the albino rat by a chromatographic procedure. This is an adaptation of the methods suggested by Spadoni and Tecce (*1*) and by Viscontini and Karrer (*2*) for the separation of thiamine and of its phosphoric esters in pure solutions.

About 3–4 g of liver are homogenized in 3 vol of 0.2 *N* HCl with the Potter-Elvehjem homogenizer. The homogenate is heated in a boiling water bath for 5 min; while still warm the pH of the homogenate is