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THE ACTIONS OF SULFONAMIDE COMPOUNDS AND *p*-AMINOBENZOIC ACID ON THE VIRUS OF LYMPHOGRANULOMA INGUINALE AND TYPHUS RICKETTSIAE IN VITRO*

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In 1940 Woods and Fildes (1) showed that *p*-aminobenzoic acid was able to inhibit the action of sulfanilamide on the growth of haemolytic streptococci in vitro. Since then the anti-sulfonamide activity of *p*-aminobenzoic acid was demonstrated in several species of bacteria both in vitro and in the animal body. For instance, Selbie (2) showed that *p*-aminobenzoic acid, administered by mouth, could inhibit the curative action of sulfanilamide in experimental infections of mice with group A haemolytic streptococci. Findlay (3) confirmed the same inhibitory action following both intracerebral and intraperitoneal inoculation of haemolytic streptococci in mice. Rubbo and Gillespie (4) showed that *p*-aminobenzoic acid inhibited the bacteriostatic action of sulfanilamide on *Clostridium acetobutylicum*. Landy and Wyeno (5) reported experiments which demonstrated that *p*-aminobenzoic acid was capable of inhibiting the effect of sulfonamide drugs on the growth of streptococci, pneumococci and staphylococci in vitro. Strauss, Dingle and Finland (6) showed that *p*-aminobenzoic acid inhibited the anti-bacterial activity of sulfathiazole on pneumococci. McCarty (7) reported that *p*-aminobenzoic acid (given subcutaneously) was found capable of nullifying the curative effect of sulfapyridine (given per os) for type I pneumococcus infection in mice. Kimming (8) showed that the effect of sulfonamide compounds on gonococci in vitro was reversed by *p*-aminobenzoic acid. Kuhn, Möller and Wend

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(9) showed that *p*-aminobenzoic acid could interfere with the bacteriostatic action of sulfonamide compounds on *streptobacterium plantarum*.

On the other hand, in the case of the sulfonamide-sensitive viruses, Findlay (3) showed for the first time that *p*-aminobenzoic acid could inhibit the chemotherapeutic action of sulfanilamide on the virus of lymphogranuloma venereum injected intracerebrally in mice. It appeared to be of interest to determine whether the chemotherapeutic action of the sulfonamide group of drugs on this virus was also inhibited in vitro growth tests by *p*-aminobenzoic acid.

In viruses and rickettsiae, however, in vitro growth tests can be undertaken only by means of tissue culture method. In former studies (10) we showed that the virus of lymphogranuloma venereum and typhus rickettsiae could be grown in deep columns of Maitland medium and their growth could be examined easily under microscope. Therefore, in the present study, the method of cultivation in deep columns of Maitland medium was used to test the actions of sulfonamide compounds and *p*-aminobenzoic acid on the virus of lymphogranuloma inguinale and typhus rickettsiae in vitro.

In the present paper the inhibition by *p*-aminobenzoic acid of the chemotherapeutic activity of sulfonamide drugs on the virus of lymphogranuloma inguinale in vitro is described. Furthermore, it is shown that *p*-aminobenzoic acid inhibits the growth of typhus rickettsiae in vitro, while sulfonamide compounds have no inhibiting effect on the growth of rickettsiae.

TECHNIQUE

The strains (H. and D.) of lymphogranuloma inguinale virus used in these experiments were tissue culture virus between the 15th and 30th passage in agar slant tissue medium with mouse embryonic tissue (11).

The strains of typhus rickettsiae (both R. mooseri and R. prowazeki) maintained also in the same medium with mouse embryonic tissue (between the 10th and 20th passage) were used.

Medium: The test medium was deep column of Maitland medium in test tube (10). The fluid consisted of 1 part of normal horse serum and 4 parts of Tyrode's solution and was sterilized by filtration through a Seitz E.K. filter. The fluid was placed in test tubes, the depth of the medium being about 4 cm; this is referred to as the standard medium. To this standard medium were added sulfonamide drugs and *p*-aminobenzoic acid in concentrations to be tested.

Inoculum: Agar slant tissue culture virus and rickettsiae were used as inoculum, that is, tissue bits from one test tube showing maximum growth were collected, mixed with 3-4 mouse embryos and minced together with scissors. Both test media, containing the substances to be tested, and standard medium were inoculated with this same inoculum (20-30 mg per tube). **Tests:** After inoculation the control and test media were incubated at 37°C for 7-10 days and then smear preparations were

made, stained by Giemsa and growth was examined under microscope.

EXPERIMENTAL RESULTS

Sulfonamide Inhibition of Growth of the Virus of Lymphogranuloma inguinale

The chemotherapeutic action of sulfonamide drugs on the virus of Lymphogranuloma inguinale in vivo is well known. The inhibitory action of sulfanilamide was also demonstrated in vitro tests by Gey and Bang (12) and Takemori (11). On the other hand it is known that so far no sulfanamide compound is found to be effective against rickettsial infections. We showed in agar slant tissue culture that neither the growth of typhus rickettsiae nor that of rickettsia orientalis could be inhibited by sulfanilamide (11).

Results of comparative studies of the action of sulfonamide drugs on the growth both of the virus of Lymphogranuloma inguinale and of typhus rickettsiae in deep columns of Maitland medium are shown in Table 1. The growth of the virus of Lymphogranuloma inguinale was completely inhibited by sulfonamide drugs at 3.03×10^{-4} M., while no inhibitory effect on the growth of typhus rickettsiae was observed in this or higher concentrations. On the contrary, multiplication of rickettsiae seemed to be more luxuriant and regular in media containing sulfonamide drugs. When minced testicular tissue of infected guinea pig was cultivated in deep columns, the growth of rickettsiae (both *R. mooseri* and *R. prowazeki*) was also better in media containing sulfonamide drugs.

The same results were also obtained in agar tissue culture.

Table 1. Sulfonamide inhibition of growth of the virus of
Lymphogranuloma inguinale.

Substance	Concentration	Growth after 7-10 days		
		Lymph. ing. virus	Rickettsia mooseri	Rickettsia prowazeki
Sulfanilic acid	3.03×10^{-4} M	—	+++	+++
Sulfanilamide	3.03×10^{-5} M	+++	+++	+++
"	$\frac{1}{3} 3.03 \times 10^{-4}$ M	—	+++	+++
"	3.03×10^{-4} M	—	+++	+++
"	3.03×10^{-3} M	—	+++	+++
Sulfapyridine	$\frac{1}{4} 3.03 \times 10^{-4}$ M	—	+++	+++
"	3.03×10^{-4} M	—	+++	+++
"	3.03×10^{-3} M	—	+++	+++
Sulfamethylthiazole	3.03×10^{-4} M	—	+++	+++

Anti-Sulfonamide Activity of *p*-Aminobenzoic Acid and Novocaine

As shown in Table 2 and 3, *p*-aminobenzoic acid and Novocaine in adequate concentrations reverse the inhibitory action of sulfonamide drugs (at 3.03×10^{-4} M) on the

growth of Lymphogranuloma inguinale virus. Various test media containing sulfonamide drugs (at 3.03×10^{-4} M) and *p*-aminobenzoic acid (or Novocaine) in different concentrations were also inoculated with typhus rickettsiae. It was found that the growth of rickettsiae became worse when the concentration of *p*-aminobenzoic acid (or Novocaine) was increased. Therefore in the next experiments we studied the effect of *p*-aminobenzoic acid and similar substances on the growth of typhus rickettsiae.

Table 2. Anti-sulfonamide activity of *p*-aminobenzoic acid.

Concentration of sulfonamide 3.03×10^{-4} M	Concentration of <i>p</i> -aminobenzoic acid (in 100 ml medium)	Growth after 7-10 days		
		Lymph. ing. virus	Rickettsia mooseri	Rickettsia prowazeki
Sulfanilamide	0.01 mg	—	+++	+++
"	0.1	+++	+++	+++
"	0.41	+++	++	++
"	4.15 (= 3.03×10^{-4} M)	+++	—	—
Sulfapyridine	0.5	+++	+++	+++
"	1.0	+++	+	++
"	4.15	+++	—	—
Sulfamethylthiazole	0.1	—	+++	+++
"	1.0	+++	+	+
"	5.0	+++	—	—

Table 3. Anti-sulfonamide activity of Novocaine.

Concentration of sulfonamide 3.03×10^{-4} M	Concentration of Novocaine (in 100 ml medium)	Growth after 7-10 days		
		Lymph. ing. virus	Rickettsia mooseri	Rickettsia prowazeki
Sufanilamide	0.01 mg	—	+++	+++
"	0.1	+++	+++	+++
Sulfapyridine	0.1	—	+++	+++
"	1.0	+++	+	+
Sulfamethylthiazole	0.1	—	+++	+++
"	1.0	+++	+	+

Effect of Substances Related to *p*-Aminobenzoic Acid on the Growth of Typhus Rickettsiae

As shown in Table 4, *p*-aminobenzoic acid and its esters (Novocaine and Anesthine) at 3.03×10^{-4} M were found to inhibit the growth of typhus rickettsiae, while other substances related to *p*-aminobenzoic acid showed no inhibitory effect. On the other hand, the growth of Lymphogranuloma inguinale virus in media containing *p*-aminobenzoic acid (or Novocaine) was usually better than in standard medium. In this connection it may be interesting to note that, in some cases of bacterial contamination, the growth of Lymphogranuloma inguinale virus was more excellent than in non-contaminated cases both in agar tissue culture and in deep columns of

Table 4. Effect of substances related to *p*-aminobenzoic acid on the growth of typhus rickettsiae.

Substance	Concentration	Growth after 7-10 days		
		Lymph. ing. virus	Rickettsia mooseri	Rickettsia prowazeki
<i>p</i> -aminobenzoic acid	3.03×10^{-5} M	+++	+++	+++
"	3.03×10^{-4} M	+++	—	—
"	3.03×10^{-3} M	+++	—	—
Novocaine	3.03×10^{-5} M	+++	+++	+++
"	3.03×10^{-4} M	+++	—	—
"	3.03×10^{-3} M	+++	—	—
Anestheseine	3.03×10^{-4} M	+++	—	—
<i>o</i> -aminobenzoic acid	"	+++	+++	+++
<i>m</i> -aminobenzoic acid	"	+++	+++	+++
<i>o</i> -nitrobenzoic acid	"	+++	+++	+++
<i>p</i> -nitrobenzoic acid	"	+++	+++	+++
benzoic acid	"	+++	+++	+++
salicylic acid	"	+++	+++	+++
nicotinic acid	"	+++	+++	+++

Maitland medium. On the contrary, we have observed no such instances in the cultures of rickettsiae. It is tempting to assume that the growth of Lymphogranuloma inguinale virus was promoted by *p*-aminobenzoic acid or related substances formed in the course of bacterial metabolism. From time to time claims have been made that vaccinia virus and typhus rickettsiae would grow in vitro in the presence of yeast cells in place of tissue cells. Findlay and MacCallum (13) think it possible that bacteria may be found which would allow of the in vitro growth of certain viruses. In our many preparations we could observe no indication that the virus of Lymphogranuloma inguinale multiplied in the associated bacteria, even in cases of bacterial contamination showing excellent growth of the virus.

Mixed Tissue Culture

For further comparative studies of the action of *p*-aminobenzoic acid (or Novocaine) and sulfonamide drugs on the virus of Lymphogranuloma inguinale and typhus rickettsiae following experiments were made.

Mouse embryonic tissue was inoculated simultaneously with both Lymphogranuloma inguinale virus and typhus rickettsiae and cultivated in deep columns of Maitland medium containing sulfonamide drugs or *p*-aminobenzoic acid (or Novocaine) or both. One of these experiments is shown in Table 5. In media (Nos. 1, 10 and 3) containing sulfonamide drugs alone (at 3.03×10^{-4} M) or plus Novocaine in insufficient concentration (0.1 mg per 100 ml medium) growth of rickettsiae occurred, but growth of the virus of Lymphogranuloma inguinale was completely inhibited. On the other hand, only the virus of Lymphogranuloma inguinale was cultivated in media (Nos. 2, 4, 7,

8 and 9) containing *p*-aminobenzoic acid in sufficient concentrations with or without sulfonamide drugs. Growth of both rickettsiae and Lymphogranuloma inguinale virus occurred in standard medium (No. 5) containing neither *p*-aminobenzoic acid (or Novocaine) nor sulfonamide drug and also in medium containing sulfonamide compound plus *p*-aminobenzoic acid in low concentration (0.1 mg per 100 ml medium) (No. 6). Both rickettsiae and Lymphogranuloma inguinale virus multiplied simultaneously in one and the same cell in the medium No. 5. Levaditi and Schoen (14) observed simultaneous appearance of Negri and Guarnieri bodies in the epithelial cells of rabbit cornea.

In our former studies on agar slant tissue culture (11), we could not find any cell containing both Lymphogranuloma inguinale virus and tsutsugamushi rickettsiae in the mixed cultures, but if the medium had been so adjusted as to be equally favorable for both, both Lymphogranuloma inguinale virus and Rickettsia orientalis would have grown simultaneously in the same cells.

It is interesting that the inhibitory action of *p*-aminobenzoic acid on the growth of typhus rickettsiae can not be reversed by sulfonamide drugs (even at 3.3×10^{-3} M). If the mode of action of *p*-aminobenzoic acid on typhus rickettsiae be similar to that of the action of the sulfonamide drugs on sulfonamide-sensitive organisms, it is suggested that an "essential metabolite" will be found for rickettsiae which reverses the inhibitory action of *p*-aminobenzoic acid on typhus rickettsiae and may be chemically related to *p*-aminobenzoic acid.

Table 5. Mixed tissue culture.

Explant material=mouse embryo + (1) agar tissue culture virus of Lymphogranuloma inguinale from one slant (2) agar tissue culture Rickettsia mooseri from one slant.

Kind of medium		Growth after 7 days	
Sulfonamide compound = 3.03×10^{-4} M	<i>p</i> -aminobenzoic acid or Novocaine per 100 ml medium	Lymph. ing. virus	Rickettsia mooseri
1. Sulfamethylthiazole	0 mg	—	+++
2. "	<i>p</i> -amin. 1.0	+++	—
3. "	Nov. 0.1	—	+++
4. "	<i>p</i> -amin. 5.0	+++	—
5. "	0	++	++
6. Sulfanilamide	<i>p</i> -amin. 0.1	++	++
7. "	<i>p</i> -amin. 0.4	++	±
8. "	<i>p</i> -amin. 4.1	+++	—
9. "	<i>p</i> -amin. 4.1	+++	—
10. Sulfapyridine	0	—	+++

DISCUSSION

For the first time in viruses, the anti-sulfonamide activity of *p*-aminobenzoic acid was confirmed in vitro growth tests. But this action, under the conditions of our

experiments, was not so striking as in the case of bacteria, namely, the ratio, molar conc. sulfonamide/molar conc. *p*-aminobenzoic acid, was smaller than in the case of bacteria (1, 4).

From the above experiments it is obvious that *p*-aminobenzoic acid which can be extracted from microorganisms (4, 15), inhibits the growth of typhus rickettsiae in vitro at 3.03×10^{-4} M. It is worthy of note that this is the same concentration of sulfonamide that is effective in inhibiting the growth of Lymphogranuloma inguinale virus in tissue culture. Although no apparent therapeutic effect against typhus infection of *p*-aminobenzoic acid was found in our few experiments, it is interesting to study the action of *p*-aminobenzoic acid on the growth of other rickettsiae in vitro. On the other hand derivatives of *p*-aminobenzoic acid should also be tested for their action on the growth of rickettsiae, as for example *p*-aminobenzoyl-*l*-glutamic acid which was reported to be 8-10 times more active than *p*-aminobenzoic acid in antagonizing sulfonamide inhibition (16).

SUMMARY

1. Sulfonamide compounds inhibit the growth of Lymphogranuloma inguinale virus, but not that of typhus rickettsiae in vitro.
2. *p*-Aminobenzoic acid and Novocaine antagonize sulfonamide inhibition of growth of Lymphogranuloma inguinale virus in vitro.
3. *p*-Aminobenzoic acid and Novocaine accelerate the growth of the virus of Lymphogranuloma inguinale.
4. *p*-Aminobenzoic acid and its esters (Novocaine and Anesthesine) were found to inhibit the growth of typhus rickettsiae in vitro.
5. In mixed tissue culture simultaneous multiplication of the virus of Lymphogranuloma inguinale and typhus rickettsiae in one and the same cell was observed.

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