INHIBITION BY ACETYLSALICYLIC ACID OF RICKETTSIAL STRAINS RESISTANT TO *p*-AMINOBENZOIC ACID

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Two *p*-aminobenzoic acid-resistant mutants of the Madrid E strain of *Rickettsia prowazekii*, P^r and P^{rr}, have been described in previous publications (Weiss *et al.*, 1957; 1959). It has been shown that certain properties, such as antigenic specificity, infectivity, and virulence for the chick embryo and cotton rat, have not changed in these strains. However, when the study was extended to susceptibility to compounds chemically related to *p*-aminobenzoic acid, new characteristics were detected. These characteristics are described in this paper.[1]

MATERIALS AND METHODS

Rickettsiae. The Madrid E strain (Perez Gallardo and Fox, 1948) of *R. prowazekii* and its following substrains were used: P^{sr}, P^r, and P^{rr}, which can be considered as first, second, and third-step mutants, respectively, to *p*-aminobenzoic acid resistance; A^r, resistant to acetylsalicylic acid; Q^r, resistant to 2,3-dimethylquinoxaline-1,4-dioxide; and PQ^r resistant to both *p*-aminobenzoic acid and the quinoxaline compound. The selection of these strains was described in previous publications (Weiss *et al.*, 1957; 1959), except for P^{sr} and A^r which are described in later sections of this paper.[2] Rickettsial pools were prepared from the infected yolk sacs of chick embryos and diluted in Bovarnick's isotonic solution (Bovarnick *et al.*, 1950).

Chemical compounds. The chemical compounds were obtained from several commercial sources. Solutions were prepared immediately before use, except for leucovorin and sulfadiazine which were received as solutions. The compounds were dissolved in distilled water with sufficient sodium bicarbonate to bring the pH to between 7.0 and 7.4, sterilized by filtration, and further diluted, as needed, in Hanks' balanced salt solution (Hanks and Wallace, 1949). When two or more compounds were to be used, their solutions were mixed just before injection. The solutions were injected into eggs via the yolk sac, immediately prior to rickettsial inoculation, in a volume of 0.1 ml per egg. Untreated embryos were given the diluent.

Chick embryos. The eggs used were White Leghorn from a flock maintained on an antibiotic-free diet. The age of the embryos was 7 or 8 days, the temperature of incubation 35 C, and the period of observation 13 days. Rickettsial suspensions were injected into the yolk sac in volumes of 0.4 ml per egg.

Determinations of the effect of chemicals were made by comparing mean survival times of control and treated groups of embryos. Most groups were composed of approximately 20 embryos, exclusive of those which died because of injection trauma. Yolk sacs from approximately one fourth of the embryos were smeared and checked for the presence of rickettsiae. The few embryos surviving at 13 days were arbitrarily given 14 as the day of death. Some of the compounds were toxic in high concentration and tended to reduce embryo survival time. However, interference with rickettsial growth could be demonstrated, even though in some instances it was not possible to separate death due to infection from death due to toxicity.

A statistical analysis of the data obtained in the course of this work indicated that the reliability of the tests was of the same order as that reported by other investigators who have studied chemotherapeutic effects on rickettsial infection in eggs (Ormsbee *et al.*, 1955; Robbins *et al.*, 1951). On the basis of these analyses, the following guiding criteria can be formulated for the significance of differences in single experiments.

[1] Some of this work was presented at the Seventh International Congress for Microbiology in Stockholm, Sweden, August 4 to 9, 1958.

[2] The *p*-aminobenzoic acid-resistant strains were designated PAB^r, PAB^{rr}, and $PAB^{rr}Q^r$ in a previous publication (Weiss *et al.*, 1959).

With concentrations of rickettsiae sufficient to kill untreated embryos in approximately 6 days, the least increase in survival time of a group of drug-treated embryos that can be considered as indicating significant protection is 1.0 day. The least difference between two protected groups that can be considered significant is 1.5 days. However, differences are not significant when both protected groups survive at least 4.0 days longer than the control group.

Most of the data presented in this paper are based on experiments repeated two or more times.

EXPERIMENTAL PROCEDURES AND RESULTS

Selection of the P^{sr} strain. The series of egg passages of the parent strain in the presence of *p*-aminobenzoic acid which had led to the isolation of P^{r} (Weiss *et al.*, 1957) was subsequently utilized for the selection of P^{sr}. A pool prepared from the 15th passage, which had been stored at −50 C for over 3 years, was passed once more in the presence of 22 μmoles of *p*-aminobenzoic acid per egg. From this 16th passage P^{sr} was obtained by limit-dilution isolation (see Weiss *et al.*, 1957, for procedure). As shown in table 1, the susceptibility of this strain to *p*-aminobenzoic acid appeared to be intermediate between the parent strain and P^{r}.

Reversal of p-aminobenzoic acid inhibition by p-hydroxybenzoic acid. Snyder and Davis (1951) and Takemori and Kitaoka (1952) have shown that the rickettsiostatic effect of *p*-aminobenzoic acid can be competitively reversed by *p*-hydroxybenzoic acid. Ratios of at least 1:10 of *p*-hydroxy- to *p*-aminobenzoic acid were required for complete reversal.

As shown in table 2, the above described results were reproducible with the present parent strain. Complete or almost complete reversal was obtained with a ratio of 1:8, but only partial reversal with 1:16. Furthermore, table 2 shows that the slight inhibitory effect of *p*-aminobenzoic acid on P^{sr} was also reversed by *p*-hydroxybenzoic acid. The same is possibly true of P^{r}, although differences in chick embryo survival times were too small to be significant. The same type of test could not be extended to P^{rr}, because of its apparently complete resistance to *p*-aminobenzoic acid. But it is obvious from the results shown in table 2, that even large amounts of *p*-hydroxybenzoic acid, such as 36 or 72 μmoles per egg, did not inhibit this strain. The results presented in table 2 thus indicate that *p*-hydroxybenzoic acid has similar effects on the *p*-aminobenzoic acid-resistant as on the susceptible strains.

The specificity of the reversing effect of *p*-hydroxybenzoic acid on the parent strain is illustrated in table 3, which lists a number of compounds that did not inhibit the parent strain or reverse the inhibition by *p*-aminobenzoic acid.

TABLE 1

Effect of p-aminobenzoic acid on rickettsial strains

Strain	Chick Embryos	
	Untreated Survival (mean days)	Treated (22 μmoles/egg) Increased survival (mean days)
Parent	5.8	4.6
P^{sr}	6.1	1.4
P^{r}	6.2	0.3

TABLE 2

Reversal of p-aminobenzoic acid inhibition by p-hydroxybenzoic acid

Strain	Micromoles/Egg		Chick Embryos	
	p-Amino-benzoic acid	*p*-Hydroxy-benzoic acid	Untreated Survival (mean days)	Treated Increased survival (mean days)
Parent	0	0	6.0	
	8	0		5.5
	8	0.25		2.4
	8	0.5		1.5
	8	1		0.8
	8	2		0.5
	8	4		0.5
	8	8		0.1
	0	8		0.5
P^{sr}	0	0	6.3	
	32	0		1.5
	32	8		0.2
	0	8		−0.1
P^{r}	0	0	6.7	
	22	0		0.6
	22	7		−0.3
	0	7		−0.2
P^{rr}	0	0	6.3	
	0	36		0.0
	0	72		0.5

This list includes compounds either chemically related to *p*-aminobenzoic acid or involved in its metabolism, as demonstrated in other microorganisms.

Effect of salicylic acid and acetylsalicylic acid. Robbins *et al.* (1951) have shown that salicylic acid inhibits the Wilmington strain of *Rickettsia typhi* to a moderate degree. In the present studies, inhibition of the parent Madrid E strain with this compound could be demonstrated only with great difficulty, but P^r and P^{rr} were inhibited to an appreciable extent by concentrations which did not affect the parent strain. Typical results are illustrated in table 4. Some of the experiments suggested that the susceptibility of P^{rr} to salicylic acid was slightly greater than that of P^r.

As shown in table 5, the three *p*-aminobenzoic acid-resistant strains were also inhibited by concentrations of acetylsalicylic acid which did not affect the parent strain. This effect appeared to be more marked than that obtained with salicylic acid and the evidence that the susceptibility to acetylsalicylic acid increases with the level of *p*-aminobenzoic acid resistance of the strain is more convincing than in the case of salicylic acid. Because of the higher inhibitory effect of acetylsalicylic acid and its lower toxicity for the chick embryo, it was used for most of the subsequent experiments in preference to salicylic acid.

TABLE 3

Effects of various compounds on rickettsial strains

Compound	Effect			
	p-Aminobenzoic acid-sensitive strains		*p*-Aminobenzoic acid-resistant strains	
	Inhibition	Reversal of *p*-aminobenzoic acid inhibition	Inhibition	Reversal of acetylsalicylic acid inhibition
p-Aminobenzoic acid	+	—	—	+
p-Hydroxybenzoic acid	—	+	—	—
Salicylic acid	—*	—	+	—
Acetylsalicylic acid	—*	—	+	—
Anthranilic acid	—	—	—	—
p-Aminosalicylic acid	—	—	—	—
p-Nitrobenzoic acid	—*	—	—*	—
Shikimic acid	—	—	—	—
Nicotinic acid	—	—	—	—
Pantothenic acid	—	—	—	—
Glutamic acid	—	—	—	—
Pteroylglutamic acid	—	—	—	—
Leucovorin	—	—	—	—
Sulfadiazine	—	—	—	

* Very slight inhibition.

TABLE 4

Effect of salicylic acid on rickettsial strains

Strain	Chick Embryos			
	Survival (mean days) Untreated	Increased survival (mean days) Treated (μmoles/egg)		
		29	43	58
Parent	5.8–6.6	0.0	0.0	−0.1
P^r	5.9–6.6	0.6	1.3	2.7
P^{rr}	5.7–6.6	1.0	1.5	2.3

TABLE 5

Effect of acetylsalicylic acid on rickettsial strains

Strain	Chick Embryos		
	Survival (mean days) Untreated	Increased survival (mean days) Treated (μmoles/egg)	
		33	67
Parent	5.8–6.0	0.0	0.7
P^{sr}	6.1		1.5
P^r	5.8–6.6	1.6	2.5–3.7
P^{rr}	5.5–6.1	2.0	4.1

The *p*-aminobenzoic acid-resistant strains did not acquire a susceptibility to a number of other related compounds, listed in table 3. However, *p*-nitrobenzoic acid inhibited all strains tested, including the parent, to a very slight degree.

Strains Q^r and PQ^r were also tested for the susceptibility to acetylsalicylic acid. The former appeared to be resistant, the latter susceptible, providing further confirmation that acetylsalicylic acid susceptibility was associated with the *p*-aminobenzoic acid marker.

Reversal of acetylsalicylic acid inhibition by p-aminobenzoic acid. The effect of various compounds on inhibition of the rickettsial strains by acetylsalicylic acid was investigated. The surprising results which were obtained are shown in table 3, figure 1, and table 6. Of the compounds tested only *p*-aminobenzoic acid reversed the inhibition by acetylsalicylic acid. Figure 1 depicts

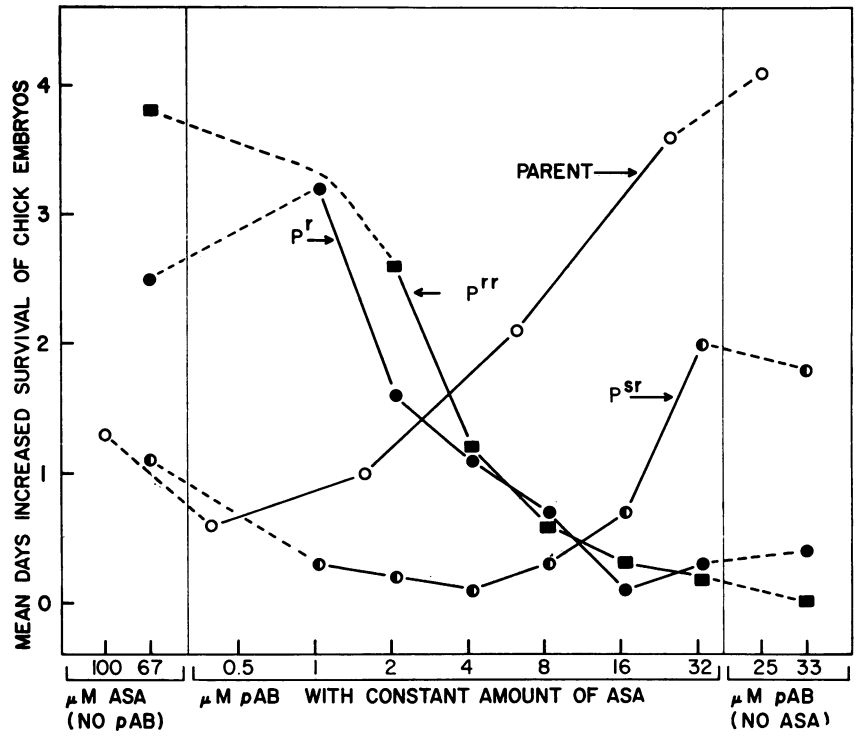

Figure 1. Reversal of acetylsalicylic acid (ASA) inhibition by *p*-aminobenzoic acid (*p*AB). The effect of acetylsalicylic acid alone is shown at the left, of the maximum amount of *p*-aminobenzoic acid used is shown at the right, and of the mixture of the two drugs is shown at the center. Note that in the experiments with the three resistant strains, the following amounts were used: acetylsalicylic acid, 67 μmoles; *p*-aminobenzoic acid, 2-fold increases from approximately 1 or 2 μmoles (as shown) to 33 μmoles. With the parent strain the amounts were: acetylsalicylic acid, 100 μmoles; *p*-aminobenzoic acid, 4-fold increases from approximately 0.4 to 25 μmoles. See text.

experiments with four strains in which a constant amount of acetylsalicylic acid was tested against varying concentrations of *p*-aminobenzoic acid. The inverse relationship between acetylsalicylic and *p*-aminobenzoic acid susceptibility of the four strains is quite apparent. *p*-Aminobenzoic acid, added in increasing concentrations, reduced and finally eliminated the inhibition of P^{rr} by acetylsalicylic acid. The effect on P^{r} is very similar, except that the highest concentration of *p*-aminobenzoic acid used alone or in combination with acetylsalicylic acid is, possibly, slightly inhibitory. P^{sr} is inhibited to a small, but significant, extent by both acetylsalicylic and *p*-aminobenzoic acid and reversal can be shown when small amounts of the latter drug are used in combination with large concentrations of the former. With large concentrations of both drugs this strain is inhibited, but apparently there is no summation of the inhibitory effects of acetylsalicylic and *p*-aminobenzoic acid. The parent strain is not generally inhibited by the amount of acetylsalicylic acid (67 μmoles) used with the other strains, but an appreciable inhibition was obtained with 100 μmoles. The results shown in figure 1 suggest that this inhibition is reversed by *p*-aminobenzoic acid within a very narrow range of concentration. As the concentration of this drug is increased it rapidly becomes inhibitory. When the four experiments are compared, they indicate that the amount of *p*-aminobenzoic acid required for reversal increases with the degree of susceptibility of the strain to acetylsalicylic acid.

Table 6 illustrates several attempts to determine whether the reversal of acetylsalicylic acid inhibition of P^{rr} by *p*-aminobenzoic acid is competitive or noncompetitive. The results include

TABLE 6

Reversal of acetylsalicylic acid inhibition by p-aminobenzoic acid (P^{rr} strain)

p-Aminobenzoic Acid (μmoles/egg)	Chick Embryos					
	Increased survival (mean days*)					
	Acetylsalicylic acid (μmoles/egg)					
	25	33	50	50	67	100
0	0.9	1.8	3.2	3.0	3.8	5.5
0.5	*0.5*†	1.2	1.8	1.2		4.5
1	**0.0**†	*0.7*	4.0	2.9		5.3
2	0.2	**0.5**	*1.5*	*1.1*	2.6	3.9
4	−0.1	0.6	1.4	1.0	*1.2*	4.0
8	−0.2	0.6	**0.4**	**0.1**	0.6	*1.7*
16		0.5	0.8		**0.3**	
33					0.2	

* Untreated embryos died 5.6 to 6.0 days after rickettsial inoculation.

† Results which seem to indicate partial or complete reversal of acetylsalicylic acid inhibition with smallest amounts of p-aminobenzoic acid are indicated in *italics* or **boldface** type, respectively.

discrepancies which cannot be satisfactorily explained. For example, in two separate experiments with 50 μmoles of acetylsalicylic acid it appeared that inhibition was partially reversed by 0.5 but not 1 μmole of p-aminobenzoic acid. If these results are disregarded, a competitive relationship is suggested. The reaction seems to fit the kinetics of a second order reaction. For example, the effect of 0.5 μmoles of p-aminobenzoic acid on 25 μmoles of acetylsalicylic acid can best be compared to the effect of 2 on 50 μmoles or 8 on 100 μmoles.

Salicylic acid inhibition of P^r and P^{rr} was also reversed by p-aminobenzoic acid.

Antagonism between p-hydroxybenzoic and p-aminobenzoic acid in the reversal of acetylsalicylic acid inhibition. Table 7 illustrates findings which have been most surprising. The first three lines show results which are entirely analogous to those obtained in previous experiments: P^{rr} was inhibited by acetylsalicylic acid and its inhibition was reversed by an adequate amount of p-aminobenzoic acid. The other lines show the effect of adding increasing amounts of p-hydroxybenzoic acid to the mixture of the two compounds. As the concentration of p-hydroxybenzoic acid was increased, rickettsial inhibition reappeared. Since p-hydroxybenzoic acid has no direct effect on P^{rr} (table 2), it must be assumed that it antagonized the reversing effect of p-aminobenzoic acid. The results shown in table 7 indicate that partial and nearly complete antagonisms were obtained with ratios (p-hydroxybenzoic to p-aminobenzoic acid) of 1:8 and 1:4, respectively. These ratios do not differ substantially from those required for the reversal of the inhibitory effect of p-aminobenzoic acid of the parent strain.

The specificity of the reaction is attested by the fact that anthranilic acid and sulfadiazine failed to replace p-hydroxybenzoic acid in the restoration of acetylsalicylic acid inhibition.

Similar results were obtained with P^r.

Isolation of strain A^r. P^{rr} was passed serially in eggs in the presence of increasing concentrations of acetylsalicylic acid. Resistance to this compound rapidly developed and a limit-dilution isolate was obtained following 9 drug passages. This strain, called A^r, was not appreciably affected by as much as 100 μmoles of acetylsalicylic acid per egg. A test of the susceptibility of this strain to p-aminobenzoic and p-hydroxybenzoic acid is shown in table 8. It is clear from this test, and several others which are not shown, that the resistance of A^r to p-aminobenzoic acid was somewhat reduced. The results presented in table 8 furthermore suggest that the slight susceptibility of A^r to p-aminobenzoic acid was reversed by p-hydroxybenzoic acid.

TABLE 7

Effect of p-hydroxybenzoic acid on the reversal of acetylsalicylic acid inhibition by p-aminobenzoic acid (P^{rr} strain)

Micromoles/Egg			Chick Embryos
Acetylsalicylic acid	p-Amino-benzoic acid	p-Hydroxy-benzoic acid	Treated Increased survival (mean days)
0	0	0	—*
67	0	0	4.7
67	16	0	0.3
67	16	0.5	0.6
67	16	1	0.5
67	16	2	1.0
67	16	4	2.7
67	16	8	2.3
67	16	16	3.8

* The mean survival time of untreated embryos was 6.1 days.

TABLE 8

Effect of p-aminobenzoic and p-hydroxybenzoic acid on the A^r strain

Micromoles/Egg		Chick Embryos
p-Aminobenzoic acid	*p*-Hydroxybenzoic acid	Treated Increased survival (mean days)
0	0	—*
16	0	0.9
32	0	1.3
0	16	0.2
0	32	0.3
16	16	0.3
32	16	0.4

* The mean survival time of untreated embryos was 5.2 days.

TABLE 9

*Comparison of p-aminobenzoic acid susceptibility of rickettsial strains**

Strain	Chick Embryos	
	Untreated survival (mean days)	Treated (73 μmoles/egg) increased survival (mean days)
A^r	6.7	1.3
	6.2	1.2
P^r	5.9	1.2
	5.9	1.8
P^{rr} isolate 1	6.1	0.5
	5.9	0.6
P^{rr} isolate 2	6.6	1.1
	6.5	1.1
P^{rr} isolate 3	5.7	0.1
	6.1	0.8
P^{rr} isolate 4	6.0	0.8
	6.1	0.8

* Two independent determinations were made in each case.

It was assumed that the small loss of *p*-aminobenzoic acid resistance occurring during the change from P^{rr} to A^r was due to the acquisition of acetylsalicylic acid resistance. An experiment originally intended for a different purpose served as a check for this assumption. A mixture of P^{rr} and Q^r was passed serially in eggs 10 times. All limit-dilution isolates obtained after the tenth passage appeared to be of the P^{rr} type. Determinations were made of *p*-aminobenzoic acid susceptibility of 4 isolates along with similar tests on P^r and A^r. The results are shown in table 9. They are in agreement with those shown in table 8 and indicate that the levels of *p*-aminobenzoic acid susceptibility of A^r and P^r were comparable. The tests with the isolates, however, cast some doubt on the stability of the third step (from P^r to P^{rr}) of *p*-aminobenzoic acid resistance or its phenotypic expression. Therefore, the development of acetylsalicylic acid resistance cannot be held entirely responsible for the reduction in *p*-aminobenzoic acid resistance of A^r.

DISCUSSION

Table 3 lists the effects of compounds that were tested in attempts to link the observations described in this paper with metabolic steps that have been found to be involved, directly or indirectly, with the action of *p*-aminobenzoic acid on other microorganisms. Although superficial similarities were found, the experiments suggest some unique features in the metabolism of rickettsiae. A few illustrations of the differences encountered will be given.

Emerson and Cushing (1946) and Emerson (1947) isolated a strain of Neurospora, in which the roles of sulfanilamide and *p*-aminobenzoic acid were reversed, the former compound being the one required and the latter the competitive inhibitor. Zalokar's (1948) experiments indicated that this strain produced more than the tolerated amount of *p*-aminobenzoic acid and required sulfanilamide as a detoxicant. Table 3 offers evidence that such a mechanism did not operate in our rickettsial strains. Sulfadiazine did not inhibit either strain or reverse the inhibition of the parent strain by *p*-aminobenzoic acid. It should be noted, however, that the sulfonamide to *p*-aminobenzoic acid molar ratio of 100:1 required for the reversal of either inhibition in Neurospora was not quite achieved in our tests.

The discovery of the tuberculostatic activity of *p*-aminosalicylic acid (Lehman, 1946) was aided by the report by Bernheim (1941) that sodium salicylate increased the oxygen uptake of tubercle bacilli. However, Youmans *et al.* (1947) found that *p*-aminobenzoic acid, and not salicylate, reversed the effect of *p*-aminosalicylic acid. As shown in table 3, *p*-aminosalicylic acid had no effect on the rickettsial strains, despite its close structural resemblance to the two active compounds, salicylic and *p*-aminobenzoic acids.

The inhibition of microorganisms by salicylic and acetylsalicylic acids has been attributed to interference in a step in pantothenic acid metabolism (Ivánovics, 1942*a*, *b*; Maas, 1952). Our finding that pantothenic acid did not reverse acetylsalicylic acid inhibition was not surprising in view of the report by Kleinschmidt *et al.* (1956) that purified rickettsiae do not contain significant amounts of pantothenic acid.

Davis (1951*a*) obtained a strain of *Escherichia coli* with a multiple requirement for tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid, and partial requirement for *p*-hydroxybenzoic acid. Shikimic acid substituted for the quintuple requirement of this strain, apparently functioning as an early metabolic intermediate. In our studies, as shown in table 3, shikimic acid did not replace either *p*-hydroxy- or *p*-aminobenzoic acid in their respective roles as reversers of inhibition.

The work of Morgan (1948, 1952) offers some interesting points of comparison, because his methods were almost identical to ours. Morgan showed that inhibition of psittacosis virus by sulfadiazine was reversed competitively by *p*-aminobenzoic acid and noncompetitively by pteroylglutamic acid or citrovorum factor. Colón and Moulder (1958) obtained conclusive evidence that viruses of the psittacosis group contain folic acid. They have also demonstrated a parallelism between loss of infectivity and loss of folic acid in purified virus suspensions and a markedly slower decline in infectivity in the presence of pteroylglutamic acid in the suspending medium. In our experiments with rickettsiae, pteroylglutamic acid and leucovorin did not replace *p*-aminobenzoic acid in the reversal of acetylsalicylic acid inhibition. This would indicate that *p*-aminobenzoic acid is not involved in folic acid metabolism of rickettsiae or that a folic acid different from pteroylglutamic acid or leucovorin is produced. The results of Kleinschmidt *et al.* (1956) which indicate that rickettsiae do not contain significant amounts of folic acid tend to support the first explanation.

Davis (1951*b*) and Davis and Maas (1952) furnished the most satisfactory bacterial model which can be used as the starting point for an explanation of competitive inhibition in rickettsiae. They isolated a strain of *E. coli* which required both *p*-amino- and *p*-hydroxybenzoic acids. Large concentrations of *p*-aminobenzoic acid interfered with the utilization of *p*-hydroxybenzoic acid, but the competition between the two metabolites was not symmetrical. Even large

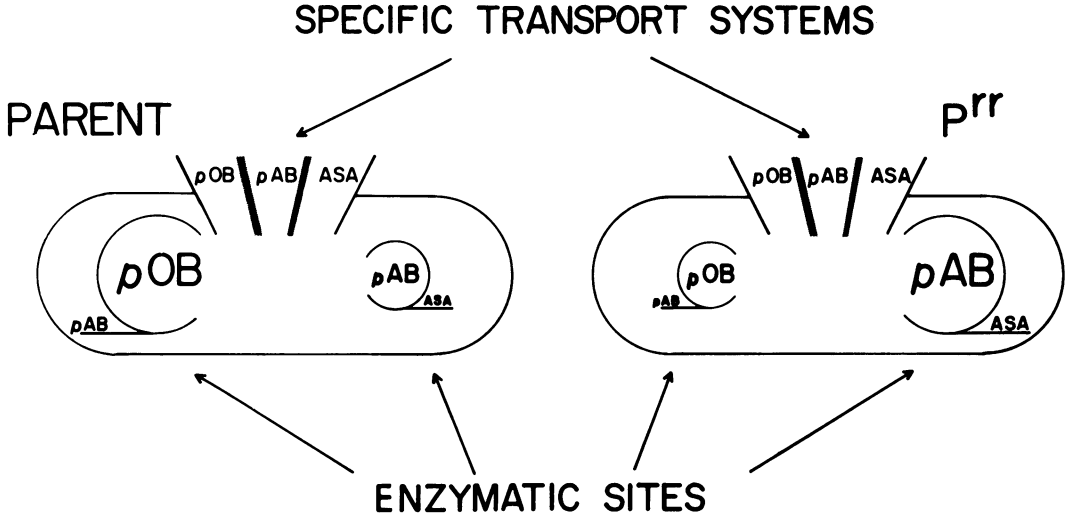

Figure 2. Attempt to represent the action of drugs on rickettsiae and the changes involved in mutations. This model is based on the assumption that the rickettsiae have specific transport systems for *p*-hydroxybenzoic (*p*OB), *p*-aminobenzoic (*p*AB), and acetylsalicylic acid (ASA) and enzymatic sites for the first two compounds. Drug competition may occur at the site of specific transport, indicated by indefinite borders between contiguous systems, and at the enzymatic sites, indicated by the symbols below the sites. Strain characteristics are influenced by the relative strengths of the two enzymatic sites. See text.

concentrations of *p*-hydroxybenzoic acid were not inhibitory. *p*-Nitrobenzoic acid interfered with the utilization of both compounds, being reversed by *p*-hydroxybenzoic acid plus *p*-aminobenzoic acid, but not by either compound alone. There are several points of similarity between these results and ours with rickettsiae, including resistance to *p*-hydroxybenzoic acid and very slight susceptibility to *p*-nitrobenzoic acid of both parent and P^{rr} strains.

Figure 2 is an attempt to represent our results based on the above described bacterial model. It takes into consideration the findings of Mathieson and Catcheside (1955), who have shown that competition may occur not only at the enzymatic site, but also at the site of specific transport. Parent as well as drug-resistant strains are assumed to have (a) an enzymatic site for the attachment and utilization of *p*-hydroxybenzoic acid, which site also has some affinity for competing *p*-aminobenzoic acid; (b) a similar site for *p*-aminobenzoic acid, with affinity for competing acetylsalicylic acid; (c) specific transport systems for *p*-hydroxybenzoic, *p*-aminobenzoic, and acetylsalicylic acid.

Strain characteristics are influenced by the relative strengths of the two enzymatic sites. As in the case of the experiments of Mathieson and Catcheside (1955) with *Neurospora crassa*, it is not necessary to assume that a change has taken place at the sites of specific transport. According to this schema, the enzymatic *p*-hydroxybenzoic acid site of the parent strain has high affinity for its metabolite and relatively high affinity for competing *p*-aminobenzoic acid. As the strain mutates to *p*-aminobenzoic acid resistance, this site loses affinity for its metabolite and competitive inhibitor. This change is accompanied by an increase in affinity of the *p*-aminobenzoic acid enzymatic site for its metabolite and a corresponding increase for its competitive inhibitor, acetylsalicylic acid. A^r can be visualized as a strain which has lost some affinity at both enzymatic sites.

Drug inhibition is produced by two types of competitions: *p*-hydroxybenzoic- *p*-aminobenzoic acid and *p*-aminobenzoic-acetylsalicylic acid. Attempts to show a direct interaction between *p*-hydroxybenzoic and acetylsalicylic acid have not been successful. However, an indirect action was shown in the presence of *p*-aminobenzoic acid (see table 7). It is necessary to assume that competition may occur at both enzymatic and specific transport sites. Thus, a high concentration of *p*-aminobenzoic acid interferes with *p*-hydroxybenzoic acid utilization by the parent strain. Similarly, a high concentration of acetylsalicylic acid interferes with the utilization of *p*-aminobenzoic acid by P^{rr}. *p*-Hydroxybenzoic acid, although very effectively eliminating *p*-aminobenzoic inhibition of the parent strain at its own site, has no affinity for the *p*-aminobenzoic acid site and cannot directly inhibit P^{rr}. However, it can compete with *p*-aminobenzoic acid at the specific transport site, and restore acetylsalicylic acid inhibition when *p*-aminobenzoic acid acts as a reverser of this inhibition.

The above representation of our results is the simplest of several that we have attempted. Its adequacy must be tested in further experiments.

SUMMARY

Changes associated with the *p*-aminobenzoic acid resistance of three Madrid E strains of *Rickettsia prowazekii*, P^{sr}, P^r, and P^{rr}, representing first-, second-, and third-step mutants, respectively, were analyzed. Tests were carried out with 7- to 8-day-old chick embryos and inhibition of rickettsial growth was measured by the increase in survival time of the embryos. Previous findings that the parent strain is highly susceptible to *p*-aminobenzoic acid and that the inhibition by this drug is competitively reversed by *p*-hydroxybenzoic acid were confirmed. *p*-Hydroxybenzoic acid, which by itself did not inhibit any of the strains, also reversed the slight inhibition by *p*-aminobenzoic acid of P^{sr} and P^r. The drug-resistant strains were inhibited by salicylic acid and acetylsalicylic acid to a larger extent than the parent strain and this drug susceptibility appeared to increase with resistance to *p*-aminobenzoic acid. Inhibition by acetylsalicylic acid was competitively reversed by *p*-aminobenzoic acid. The addition of *p*-hydroxybenzoic acid to a mixture of *p*-aminobenzoic acid and acetylsalicylic acid reestablished the inhibitory effect of acetylsalicylic acid. By 9 serial egg passages of P^{rr} in the presence of acetylsalicylic acid, a strain resistant to this compound (A^r) was obtained, which retained most, but not all, of its resistance to *p*-aminobenzoic acid. Ten additional compounds, chemically related to *p*-aminobenzoic acid or which have been involved in the *p*-aminobenzoic acid metabolism of other microorganisms, were tested for their effects on the rickettsial strains. They were found to be ineffective when

used either alone or in combination with *p*-aminobenzoic acid or acetylsalicylic acid. An interpretation of the changes involved in the mutant strains, based on specifically balanced *p*-hydroxybenzoic and *p*-aminobenzoic acid requirements, and on competitions occurring at the enzymatic and specific transport sites, has been offered.

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