FURTHER STUDIES OF DRUG-RESISTANT STRAINS OF *RICKETTSIA PROWAZEKII*[1]

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The selection of a strain of *Rickettsia prowazekii* of increased resistance to *p*-aminobenzoic acid has been described (Weiss *et al.*, 1957). The amount of *p*-aminobenzoic acid required to inhibit the growth of this strain in the yolk sac of the chick embryo appeared to be approximately 20 times greater than that required for the parent strain. However, inhibition could still be demonstrated with large doses of *p*-aminobenzoic acid, such as 10 mg per egg.

Following the isolation of this strain, attempts were made to isolate other drug-resistant strains, as well as to exploit the new strains for genetic and physiological investigations. This paper deals primarily with the selection of three new strains and with the description of some of their basic properties.

MATERIALS AND METHODS

Rickettsiae. The strains of *R. prowazekii* used in this work have been derived from the Madrid E strain (Perez Gallardo and Fox, 1948). The previously described parent and *p*-aminobenzoic acid-resistant, limit-dilution strains (Weiss *et al.*, 1957), will be called parent and PAB^r strains, respectively. The three new strains will be called PAB^{rr}, Q^r, and $PAB^{rr}Q^r$. The symbols indicate a further step in *p*-aminobenzoic acid resistance, resistance to the quinoxaline compound described below, and resistance to both drugs, respectively. Except for some simplifications, the procedures for the preparation of pools, serial passages, and purification of rickettsial suspensions were the same as those described by Weiss *et al.* (1957).

Drugs. Stock solutions containing 10 g of *p*-aminobenzoic acid per 100 ml were prepared in distilled water containing 0.002 per cent phenol red. The drug was neutralized to pH 7.4 with $NaHCO_3$, sterilized by filtration, and further diluted, as needed, in Hanks' (Hanks and Wallace, 1949) balanced salt solution.

The second compound used for the preparation of resistant strains was 2,3-dimethyl quinoxaline-1,4-dioxide. The first batch of this compound was kindly sent to us by Dr. J. W. Moulder, who originally received it from Dr. E. W. Hurst. A second batch was prepared by Dr. S. L. Friess at the Naval Medical Research Institute. Stock solutions containing 1 g per 100 ml were prepared in distilled water, sterilized by filtration, and further diluted, as needed, in the balanced salt solution. The high degree of toxicity of this compound for the chick embryo did not preclude the use of large doses, because most of the toxic deaths occurred on or after the 8th day following inoculation and inhibition of rickettsial growth could be adequately demonstrated.

In previous experiments (Weiss *et al.*, 1957) *p*-aminobenzoic acid was injected into the embryos via the air sac 24 hr before rickettsial inoculation. In some of this work, the procedure was simplified and the drugs were injected via the yolk sac, immediately prior to rickettsial inoculation. In the case of *p*-aminobenzoic acid, there is some evidence that a slightly greater inhibition of rickettsial growth was obtained by the former procedure than by the latter. Volumes injected in most cases were 0.1 ml per embryo.

Chick embryos. The eggs used were White Leghorn from a flock maintained on an antibiotic-free diet. The age of the embryos was 7 days, with some exceptions in the serial passages. Rickettsial suspensions were injected into the yolk sac in volumes of 0.5 ml per embryo in titration experiments, and 0.4 ml when following injection of drug or diluent control. The temperature of incubation was 35 C and the period of observation 13 days.

Preliminary determinations of the effect of drugs were made by comparing mean survival time of control and treated groups of 12 to 15 embryos, each. A difference of 1 day, or greater,

[1] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

was considered to indicate significant protection. Detailed studies were carried out by comparing the infectivity titers of yolk sac pools prepared from untreated and treated groups at various intervals of time following rickettsial inoculation. Duplicate pools of 4 yolk sacs each were usually prepared for such tests. Rickettsial titers were determined from the mean survival time of 12 to 15 embryos inoculated with a single dilution of rickettsiae. This method has been previously described (Weiss *et al.*, 1957) and is further discussed in a later section.

Animal inoculations and miscellaneous procedures. Mouse toxicity was determined as in previous experiments (Weiss *et al.*, 1957) except that 0.25-ml volumes, instead of 0.5 ml, were injected. Repeated titrations of pools yielded almost identical results and differences in infectivity titers greater than 2-fold can be considered significant.

Cotton rats were obtained from Tumblebrook Farm, Brant, New York, and from the Division of Laboratories, Michigan Department of Health, Lansing. Immunizing injections were given intraperitoneally. For the determination of LD_{50} titers or challenge, the suspensions were injected intracardially. The volume for either type of injection was 0.25 ml per animal. Four to 6 cotton rats were used for each dilution, but these numbers were occasionally reduced by nonspecific deaths. LD_{50} titers were determined by 2-fold dilutions of the rickettsial suspensions with a degree of precision approaching that of mouse toxicity. ID_{50} titers were determined by challenge with 2 to 4 LD_{50} of cotton rats immunized 21 days previously with serial 10-fold dilutions of rickettsiae. Differences in ID_{50} titers of 1 logarithm or greater were considered significant.

Guinea pigs were inoculated intraperitoneally with 0.5-ml volumes of concentrated, purified rickettsial suspensions. Rectal temperatures were taken daily and classified as fever if exceeding 103.8 F. The guinea pigs were bled 28 days after inoculation and complement fixation tests were performed with their sera using soluble epidemic typhus antigen, kindly furnished by Dr. F. S. Markham, of the Lederle Laboratories.

Hemolysin titrations were carried out with rabbit cells by the methods developed by Snyder *et al.* (1954) as in previous experiments (Weiss *et al.*, 1957). The precision of this test is probably the same or greater than that of mouse toxicity, provided the yolk sac suspensions can be diluted at least 1:10.

RESULTS

Selection of drug-resistant strains. (1) PAB^{rr} strain:—The PAB^{rr} strain was obtained by serial passage of the PAB^{r} strain in chick embryos that had received 10 mg of *p*-aminobenzoic acid per embryo. Preliminary tests indicated that the rickettsiae had changed between the 14th and 16th passage and were no longer inhibited by 10 mg of *p*-aminobenzoic acid. The high level of drug resistance was maintained through subsequent passages. Limit-dilution isolations, without drug, were carried out with a rickettsial pool obtained from the 21st passage, or the 58th drug passage of the parent strain. The PAB^{rr} strain was obtained from a single infected egg of a group having an incidence of infection of 18 per cent.

A comparison of the levels of *p*-aminobenzoic acid resistance of PAB^{r} and PAB^{rr} is shown in figure 1. The test was carried out by procedures identical to those already described and the results obtained with PAB^{r} were approximately the same as those which were previously reported (Weiss *et al.*, 1957). The inhibition produced by 10 mg of *p*-aminobenzoic acid, although relatively small, appeared to be consistent. With PAB^{rr}, on the other hand, the titers of the pools from the two groups were approximately the same in most

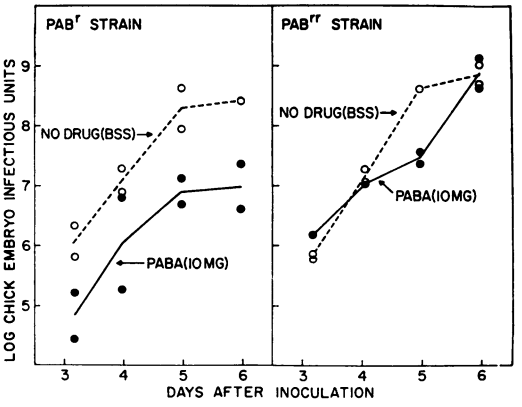

Figure 1. Infectivity titrations during growth of the PAB^{r} and PAB^{rr} strains in eggs which received either balanced salt solution (BSS) or 10 mg of *p*-aminobenzoic acid (PABA). Each circle represents the titer of a pool of 4 yolk sacs, expressed as the logarithm of the number of infectious rickettsiae per yolk sac. The lines connect the means of duplicate titrations.

cases. Whatever differences were found, for example, on the 5th day, could not be reproduced with consistency. Several other experiments, in which the pools were titrated either in eggs or mice, have similarly failed to reveal any consistent inhibition of PAB^{rr} by 10 mg of the drug.

(2) Q^r strain:—Hurst *et al.* (1953) have shown that 2,3-dimethyl quinoxaline-1,4-dioxide although too toxic for human use, was as effective as the most efficacious antibiotics against psittacosis and lymphogranuloma venereum viruses. They also showed that resistant strains of virus appeared after a few drug passages. For these reasons this drug was selected to produce a genetic marker in rickettsiae.[2]

Table 1 illustrates several experiments in which rickettsial inhibition was measured by increased mean survival times of the chick embryos. It is obvious that doses of quinoxaline as low as 0.1 mg per egg produced an appreciable inhibition of the parent strain. The effect of larger doses, 0.25 and 0.5 mg, although highly inhibitory, cannot be accurately estimated. Many of the yolk sacs, when smeared, revealed no or very few rickettsiae and embryo deaths must be attributed to a combination of rickettsial infection and drug toxicity.

The rickettsiae were passed serially in the presence of quinoxaline 20 times. In the first few passages, the dose was 0.2 mg per egg, it was then slowly increased to 1 mg, and then reduced to 0.5 mg. Changes in resistance were noted during the 4th and 14th passages. As shown in table 1, rickettsiae from the 10th passage were inhibited by 0.5, but possibly not by 0.25 mg per egg, whereas the rickettsiae from the 20th passage had acquired an appreciable amount of resistance, even to the larger amount of drug. It should be noted that the smears of yolk sacs of treated embryos inoculated with the resistant rickettsiae usually did contain numerous rickettsiae.

The Q^r strain is a limit-dilution, single egg isolate, obtained following the 20th drug passage, from a group having an incidence of infection of 24 per cent. As seen in table 1, with this strain embryo survival time was not appreciably increased by 0.25 or 0.5 mg of quinoxaline per egg. However, a small increase in mean survival time, usually less than 1 day, has been obtained in almost all experiments with the Q^r strain. Therefore, it can be assumed that this strain is still affected by quinoxaline to a small degree.

[2] Greenland and Moulder (1958) have reported the development of a strain of feline pneumonitis virus resistant to this compound.

TABLE 1

Effect of quinoxaline compound on rickettsial strains

Strain	Mean Chick Embryo Survival Time			
	Days	Increase (days)		
	Untreated	Treated (mg/eggs)		
		0.10	0.25	0.50
Parent	6.0	2.8	—	—
	5.5	—	3.2	5.5
	5.6	—	—	6.0
10th drug passage	5.8	0.1	0.8	2.2
20th drug passage	6.9	—	—	0.9
Q^r	6.2	—	0.8	0.5
	5.0	—	—	0.8

Figures 2 and 3 illustrate detailed studies of the effect of quinoxaline on the growth of the parent and Q^r strains, carried out in identical fashion to those previously described with the *p*-aminobenzoic acid resistant strains. The inhibition of the parent strain by 0.25 mg of drug and the more pronounced inhibition by 0.5 mg are quite obvious (figure 2). However, if the drop in rickettsial titer which follows inoculation (see Weiss *et al.*, 1957) is taken into account, it is also apparent that an appreciable increase in rickettsial titer took place during the first 4 or 5 days after inoculation. Following this increase, the titers of the rickettsiae, in the group which received 0.25 mg, declined slightly or remained the same at a level approximately 2 logarithms lower than the peak control titers. With 0.5 mg the rickettsial titers declined after 4 days, and although there are great variations between some of the duplicate pools, the titers tended to remain at a level approximately 4 logarithms lower than the peak control titers.

With Q^r (figure 3) differences among pools prepared from the three groups, control, 0.25 mg, and 0.5 mg, were relatively small. Only one pool, 5th day, 0.25 mg group, appeared to have a titer significantly lower than the other of the same time interval. Peak titers were attained at the same time in the three groups, at about the 7th day. Although differences were small, the experi-

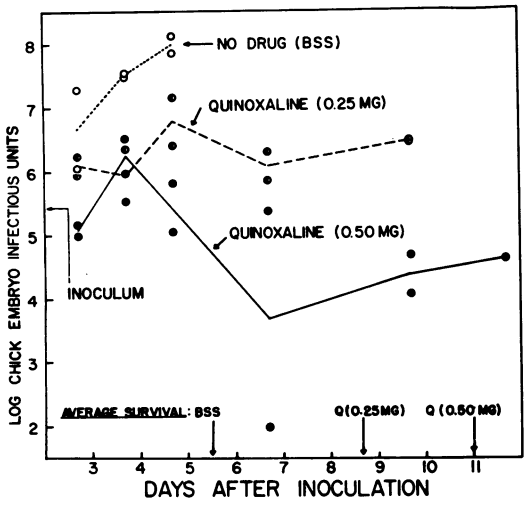

Figure 2. Infectivity titrations during growth of the parent strain in eggs which received either balanced salt solution (BSS) or 0.25 or 0.5 mg of the quinoxaline compound. The mean survival times of the embryos in the three groups are indicated by arrows. Circles and lines as in figure 1.

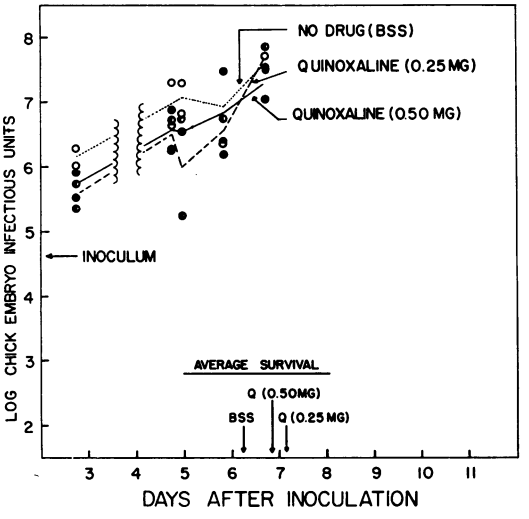

Figure 3. Same as figure 2, except that the Q^r strain was used. The wavy lines separate two experiments carried out in identical fashion, in which the mean survival times of the embryos were almost exactly the same. BSS, balanced salt solution.

ment illustrated in figure 3 suggests, as well as the previous experiments, that the Q^r strain retained some of its susceptibility for quinoxaline.

(3) $PAB^{rr}Q^r$ strain:—Attempts were made to obtain a strain which would be resistant to the simultaneous administration of *p*-aminobenzoic acid and quinoxaline, by passing a mixture of PAB^{rr} and Q^r in eggs in the presence of heavy concentrations of both drugs. These preliminary experiments yielded only one $PAB^{rr}Q^r$ strain, which was studied in detail.

The procedure which yielded $PAB^{rr}Q^r$ is shown in table 2. On the basis of more recent experiments, it appears that of the 4 passages (2 with drugs), at least 1 egg passage in the presence of both drugs, immediately followed by a limit-dilution isolation, was essential for the selection of $PAB^{rr}Q^r$. The limit-dilution experiment yielded only 1 obviously infected egg of a group of 35.

Figures 4 and 5 illustrate tests with both drugs used simultaneously. A control experiment (figure 4) was carried out with a suspension of approximately equal numbers of infectious PAB^{rr} and Q^r rickettsiae. Preliminary experiments had already clearly shown that resistance to one drug did not extend to the other. In the experiment shown in figure 4, with both strains and both drugs (*p*-aminobenzoic acid, 5 mg; quinoxaline, 0.5 mg), inhibition was very marked. A titer of approximately 4 logarithms per egg was reached on the 4th day. This slight rise was followed by a decline to about 1.5 logarithms on the 6th day. The rickettsial titers then slowly rose to approximately 4 logarithms on the 12th day. It is quite obvious from figure 4 that PAB^{rr} was inhibited by quinoxaline and Q^r by *p*-aminobenzoic acid.

Figure 5 offers a marked contrast with figure 4. $PAB^{rr}Q^r$ produced approximately the same titers in the absence of drugs, as with either drug alone,

TABLE 2

Isolation of the $PAB^{rr}Q^r$ strain from a mixture of PAB^{rr} and Q^r

Passage No.	Dilution of Yolk Sac	Drugs (mg)		Days in Eggs
		p-Aminobenzoic acid	Quinoxaline	
1*	1.3×10^{-2}	—	—	3
2	2.5×10^{-3}	5	0.5	13
3	1.3×10^{-1}	5	0.5	11
4	Limit-dilution	—	—	13

* Inoculum consisted of approximately equal numbers of PAB^{rr} and Q^r rickettsiae, as determined by preliminary titration in mice.

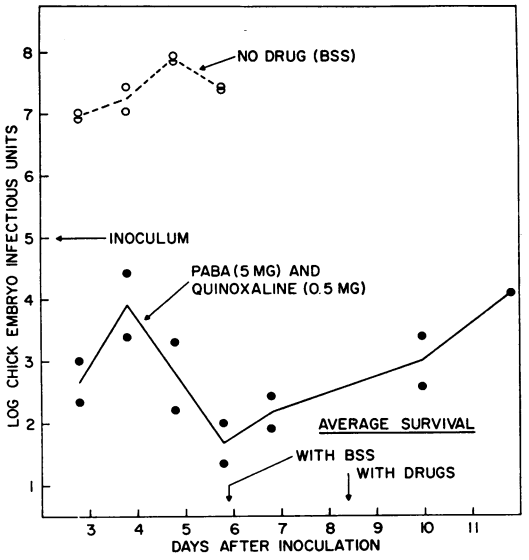

Figure 4. Infectivity titrations during growth of a mixture of the PAB^{rr} and Q^{r} strains in eggs which received either balanced salt solution (BSS) or both drugs. Circles, lines, and arrows as in figure 2, except that the last pool of the group receiving drugs was prepared from the only surviving embryo.

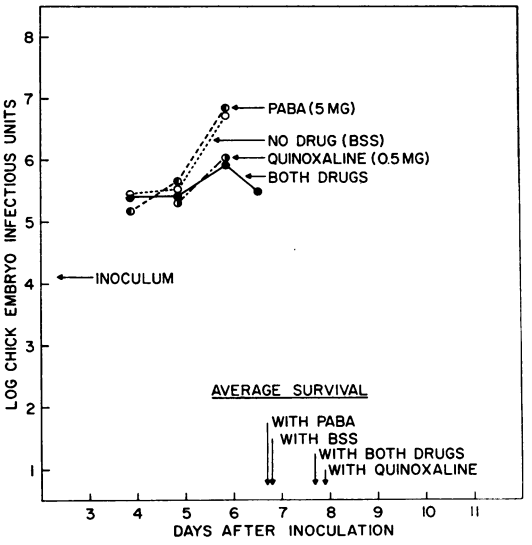

Figure 5. Similar to figure 4 with the PAB^{rr}Q^{r} strain, tested against each drug alone and in combination. Pools were not prepared in duplicate. BSS, balanced salt solution.

or with the combination of the two drugs. Differences among the various pools were no greater than those encountered with Q^{r} grown in the presence of quinoxaline. The pool collected on the 6th day from the group of eggs which had received *p*-aminobenzoic acid was tested against quinoxaline and it was found that full resistance to quinoxaline was retained. Similarly, the pool collected from the quinoxaline group was fully resistant to *p*-aminobenzoic acid. The experiment shown in figure 5 was performed in various forms four times and identical results were obtained.

Characteristics of the drug-resistant strains. (1) Infectivity and virulence for chick embryos:— The rickettsial titrations illustrated in figures 1 to 5 were carried out in eggs by the single dilution method discussed in a previous publication (Weiss *et al.*, 1957). This method was based upon the results of several ID_{50} determinations carried out with parent and PAB^{r} strains which indicated that a linear relationship exists between the mean number of rickettsiae injected and the mean number of days of chick embryo survival (mean survival time). Both numbers are generally expressed as their logarithms. The log mean survival time produced by 1 infectious rickettsia was estimated by extrapolation from ID_{50} titrations to be 1.12 (or 13.2 days). Each 10-fold increase in the number of infectious rickettsiae reduces this number by 0.07. For example, a specimen killing embryos in 8.1 days (logarithm = 0.91), according to these calculations, contains $(1.12 - 0.91)/0.07$ logarithms or 10^3 infectious rickettsiae.

The same constants were used in all experiments described above. The validity of using the same constants for all strains was tested in the experiment illustrated in figure 6. The four strains, parent, PAB^{rr}, Q^{r}, and PAB^{rr}Q^{r} were titrated by the routine 10-fold dilution method. The infectious unit was calculated from the group containing approximately equal numbers of infected and noninfected embryos from the formula,

$$e^{-x} = P_0,$$

where x = mean number of infectious rickettsiae per inoculum, and P_0 = fraction of noninfected embryos. The numbers of rickettsiae injected at each dilution were plotted against the mean survival time which they produced (both expressed in logarithms) and compared to the theoretical values (figure 6, solid line) obtained from the constants used in previous experiments.

The agreement between theoretical and experi-

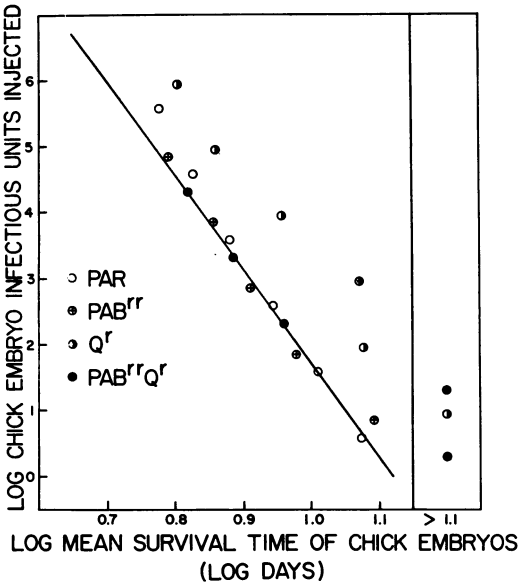

Figure 6. Relationship of numbers of rickettsiae injected into eggs to survival time of the embryos. (See text.) PAR, parent strain.

mental values is very good for parent strain and PAB^{rr} (figure 6). The two strains appear to grow equally well in the yolk sac and to be equally virulent for the chick embryo. In general, the titers of pools of PAB^{rr} have been lower than those of the parent strain, but this difference has been small and of doubtful significance.

The results obtained with Q^r do not agree with the theoretical values. It appears that in each case the deaths of the embryos were delayed by approximately 0.1 logarithm day, and many of the embryos surviving at 13 days were infected. The results can best be interpreted by assuming that this strain has lost some of its ability to kill the embryos (i. e., virulence) but not the ability to grow in the yolk sac. In fact, most of the Q^r pools, possibly because of the delay in embryo deaths, have had higher infectivity titers than those of the parent strain.

The results presented in figure 3 were computed by the constants of the parent strain. The data, just discussed, indicate that the rickettsial titers were possibly 1 to 1.5 logarithms higher than those shown.

The behavior of PAB^{rr}Q^r in chick embryos appeared to differ, too, from that of the other strains. In high concentration, it behaved in a manner remarkably similar to the parent strain (figure 6). However, when the pools were diluted, they failed to kill embryos. With low concentrations, such as 2 infectious units per egg, in contrast to the other strains, infection could not be detected by the microscopic examination of yolk sac smears, but only by the subinoculation of yolk sac suspensions into eggs. Infectivity titers of PAB^{rr}Q^r pools have always been considerably lower than those of the other strains, even though microscopic examination of smears of yolk sacs used to prepare these pools revealed at least as many rickettsiae as with the other strains. These results can be interpreted by assuming that both virulence and infectivity have been appreciably reduced and a considerably greater number of PAB^{rr}Q^r rickettsiae are required to produce an effect comparable to that of the other strains.

The results presented in figure 5 were also computed by the constants of the parent strain. Since all the titers were relatively high, no correction is required. It can be noted that the peak titers are considerably lower than those obtained in experiments with other strains.

Virulence and infectivity for the chick embryo of the four rickettsial strains are presented in table 3. Mouse toxicity titers are included as standards of comparison. Table 3 further illustrates the above-described differences among the strains and shows that the same pools, which displayed pronounced differences when injected into eggs, appeared to contain comparable rickettsial titers, when injected into mice.

(2) Infectivity and virulence for cotton rats:—The results of a cross-protection test between parent and PAB^r strains are given in table 4. It is quite apparent that the two strains are about equally virulent and infectious for cotton rats.

TABLE 3

Comparison of chick embryo virulence and infectivity to mouse toxicity of rickettsial pools

Strain	Mouse Toxicity*	Chick Embryo*	
		LD_{50}†	ID_{50}‡
Parent	1.4	7.6	7.7
PAB^{rr}	0.9	6.8	7.0
Q^r	1.4	6.5	8.1
PAB^{rr}Q^r	0.8	3.1	5.5

* Expressed as the inverse of the logarithms of the dilutions of the pools.

† Determined by survival at 13 days.

‡ Determined by smear and subpassage into eggs.

TABLE 4

Cross protection between parent and PAB^r strains in cotton rats

Strain	LD_{50}*	ID_{50}* (by Challenge)	
		Homologous	Heterologous
Parent	1.5	7.8	7.4
PAB^r	1.6	7.7	7.8

* Inverse logarithms of the dilutions.

Homologous and heterologous challenges appeared to have the same effects, indicating that an antigenic change has not taken place in PAB^r.

When these studies were extended to the other strains, differences were noted as shown in tables 5 and 6. In table 5, virulence for the cotton rat is compared to mouse toxicity. Cotton rats were killed by parent, PAB^{rr}, Q^r, and a mixture of PAB^{rr} and Q^r, but not by PAB^{rr}Q^r, despite the fact that rickettsial titers of this strain, as measured by mouse toxicity, were comparable to those of the other strains. The difference between the PAB^{rr}Q^r and the other strains is possibly greater than that which is indicated in table 5. During the 10-day period of observation, none of the cotton rats inoculated with PAB^{rr}Q^r appeared to be ill, whereas many of the surviving animals which had received 0.5 or 0.25 LD_{50} of any of the other strains lay prostrate for 1 or 2 days. A slight drop in cotton rat virulence of Q^r with respect to mouse toxicity is also suggested by the results shown in table 5.

Table 6 illustrates the results of a partial cross-protection test in cotton rats in which the drug-resistant strains were used as immunizing agents and the parent strain as the challenge. The results with PAB^{rr} and Q^r appeared to be entirely analogous to those obtained with PAB^r (table 4). On the other hand, the immunizing capacity of PAB^{rr}Q^r was reduced by 4 to 5 logarithms, although clearly demonstrated in this and other experiments not shown in table 6. Because of the previously demonstrated loss of virulence for the cotton rat, the reduction in immunizing capacity can plausibly be attributed to a loss of infectivity.

(3) Antibody response in the guinea pig:—To minimize antigenic stimulation of the guinea pigs with yolk sac, partially purified rickettsial suspensions of the four strains were used. By precipitin tests with rabbit anti-yolk sac material, the injected suspensions were found to contain approximately as much tissue material as a crude yolk sac preparation diluted to 10^{-3}. Five guinea pigs, inoculated with a crude, normal yolk sac suspension diluted to 10^{-2}, did not develop demonstrable complement fixing antibodies against rickettsial antigen.

TABLE 5

Comparison of LD_{50} in mice and cotton rats of rickettsial pools

Strain	LD_{50} (Inverse Dilution of Yolk Sac)		Ratio LD_{50} Mice/Cotton Rats
	Mice	Cotton rats	
Parent	28	16	1.8
PAB^{rr}	7	4	1.8
Q^r	24	7	3.4
Mixture of PAB^{rr} and Q^r	20	5	4.0
PAB^{rr}Q^r	24	<2	>12.0
	14	<2	>7.0
	14	<2	>7.0

TABLE 6

Comparison of cotton rat infectivity and mouse toxicity of rickettsial pools*

Strain	Mouse LD_{50}†	Cotton Rat ID_{50}†
PAB^{rr}	0.9	6.7
Q^r	1.4	7.3
PAB^{rr}Q^r	0.8	2.3

* Determined by challenge with PAR strain.
† Inverse logarithms of the dilutions.

Approximately the same results were obtained with the four strains. The guinea pigs developed fever for only 1 day, the day after inoculation. The antibody response, tested against a commercial antigen of *R. prowazekii*, is shown in table 7. The results obtained in a previous study of PAB^r (Weiss *et al.*, 1957) are included. The complement fixing antibody titers ranged from 1:20 to 1:320, but the geometric means tended to be proportional to the numbers of mouse LD_{50} injected. These results are in agreement with those reported by Perez Gallardo and Fox (1948).

(4) Hemolysin and mouse toxicity:—It was assumed, in the previously described tests, that mouse toxicity was the one property of the four strains which had not appreciably changed. This assumption was tested by comparing mouse

TABLE 7

Complement fixing antibody titers of guinea pig sera

Strain	Mouse LD_{50} Injected (per Guinea Pig)	No. of Guinea Pigs					Geometric Means of Titers
		Inverse dilution of guinea pig serum					
		20	40	80	160	320	
Parent	23		1	1	2	5	190
PAB^{r}*	—†			5	4	1	120
PAB^{rr}	11		3	5	2		75
Q^{r}	5		7	3			50
$PAB^{rr}Q^{r}$	20	1		3	2		80

* From previous publication (Weiss *et al.*, 1957).

† Titrated in eggs: approximately 10^{7} infectious rickettsiae.

TABLE 8

Comparison of hemolysin and mouse toxicity titers of rickettsial pools

Strain	Hemolysin Units*	Mouse Toxicity Units*	Ratio Hemolysin/ Mouse Toxicity	Mean Ratio
Parent	250	90	2.8	2.9
	300	98	3.1	
	750	256	2.9	
PAB^{rr}	83	32	2.6	3.0
	255	90	2.8	
	200	54	3.7	
Q^{r}	500	104	4.8	4.4
	1550	320	4.8	
	825	226	3.6	
$PAB^{rr}Q^{r}$	167	28	6.0	10.1
	1600	114	14.0	
	825	80	10.3	

* Per g of yolk sac.

toxicity to hemolysin titers of three pools of each of the four strains. The results are shown in table 8. As in previous tests, the properties of parent and PAB^{rr} strains appeared to be the same. A reduction in mouse toxicity, with respect to hemolysin, was noted in the case of Q^{r} and $PAB^{rr}Q^{r}$. The differences were small and that of Q^{r} was not necessarily significant. The variation in the ratios of hemolysin to mouse toxicity titers noted among the three pools of $PAB^{rr}Q^{r}$ suggested that one of the two properties was less stable than the other. Experiments not shown in table 8 indicated that mouse toxicity was destroyed at a more rapid rate than hemolysin during freezing and thawing and other routine procedures. The more labile property, mouse toxicity, is, possibly, the better indicator of the activities of the rickettsial pools.

Summary of the characteristics of the drug-resistant strains. A summary of the characteristics of the four rickettsial strains is presented in table 9. Mouse toxicity was used as the standard of measurement and each value was calculated on the basis of 1 mouse LD_{50}. It is obvious that the three tests based on "resting" rickettsiae, that is, mouse toxicity, hemolysin, and antigenic stimulation of the guinea pig, revealed only minor differences. On the other hand, tests based on rickettsial multiplication, virulence and infectivity for chick embryos and cotton rats, revealed major changes in $PAB^{rr}Q^{r}$ and one significant change, virulence for the chick embryo, in Q^{r}.

DISCUSSION

The change of the parent strain to PAB^{r} has been attributed to a two-step mutation, and has been defined as a 20-fold increase in the level of *p*-aminobenzoic acid required to inhibit the

TABLE 9

Summary of characteristics of rickettsial strains

Strain	Mouse LD_{50}	One Mouse LD_{50} Contains or Elicits					
		Chick embryo		Cotton rat		CF titer in guinea pig*	Hemolysin Units
		LD_{50}	ID_{50}	LD_{50}	ID_{50}		
Parent	1	16×10^{5}	2×10^{6}	0.6	11×10^{5}	8.3	2.9
PAB^{rr}	1	8×10^{5}	1×10^{6}	0.6	6.3×10^{5}	6.8	3.0
Q^{r}	1	1.3×10^{5}	5×10^{6}	0.3	8×10^{5}	10.0	4.4
$PAB^{rr}Q^{r}$	1	2×10^{2}	5×10^{4}	0.0	3.2×10^{1}	4.0	10.1

* Inverse of the dilution of guinea pig serum.

rickettsiae (Weiss *et al.*, 1957). The change from PAB^r to PAB^{rr} can be regarded as a third step in the acquisition of resistance. Since it has not been possible to demonstrate the inhibition of PAB^{rr} with *p*-aminobenzoic acid, this change cannot be expressed quantitatively. The second and third steps were demonstrated only after relatively large numbers of drug passages. The difficulty in selecting drug-resistant mutants can be attributed either to a low rate of mutation or to limited growth of the rickettsiae during many of the serial drug passages. In either event, PAB^{rr} appears to have a genetic label which is not likely to be acquired by the parent strain during the course of experiments involving few drug passages.

The change of the parent strain to Q^r can also be attributed to a two-step mutation, but a quantitative definition of the increase in resistance cannot be formulated. From the experiments presented in a previous section and limited experience with 1.0 mg of quinoxaline per egg, it seems that the slight inhibitory effect on Q^r is not dependent upon the concentration of the drug. Quinoxaline resistance was obtained after fewer passages than with *p*-aminobenzoic acid. The extensive growth of the parent strain in the presence of small doses of quinoxaline, possibly, was a contributory factor. Loss of virulence of Q^r for the chick embryo, possibly associated with increased growth, favored its survival in the presence of the drug, in competition with the parent strain. The genetic label of Q^r cannot be considered as reliable as that of PAB^{rr}, and the possibility cannot be discounted that the parent strain may acquire quinoxaline resistance, under favorable conditions, in the course of few drug passages.

The $PAB^{rr}Q^r$ strain was obtained by procedures which were quite different from those used for the other strains. In the first place, the concentrations of the drugs, presumably, were sufficiently high to inhibit first-step mutants. Secondly, the rickettsiae have been maintained in the presence of the two drugs for only 2 egg passages, for a total length of time no longer than 4 passages of previous series. Thirdly, the concentrations of the yolk sac suspensions were not as high as in the previously described serial egg passages. Therefore, it is likely that $PAB^{rr}Q^r$ originated from PAB^{rr} and Q^r by a process of genetic transfer, such as recombination. The view that genetic transfer was responsible for $PAB^{rr}Q^r$ is strengthened by the fact that this strain appeared to have acquired the complete drug-resistance of the PAB^{rr} and Q^r strains, rather than the partial resistance of first-step mutants. However, other explanations, such as mutation of one drug-resistant strain to resistance to the other drug, cannot be ruled out. A further analysis of the mechanism by which $PAB^{rr}Q^r$ was formed must be delayed until strains with the two genetic labels can be obtained in a predictable manner.

A study of the drug resistant strains revealed changes of some of the characteristics, not obviously related to drug-resistance. However, a connection between drug-resistance and change in other properties is suggested by the fact that the most important changes were encountered in $PAB^{rr}Q^r$, which was least exposed to selective factors. This connection cannot be further analyzed until additional strains with the dual drug resistance can be studied.

The extensive changes of $PAB^{rr}Q^r$ in virulence and infectivity for the chick embryo and cotton rat can best be explained by assuming that each change in drug resistance was accompanied by some change in the other properties of the rickettsiae. These changes were small and, possibly, in some cases too small to be demonstrable by the procedures used in this investigation. However, the combination of small changes resulted in the emergence of new conspicuous characteristics.

It is obvious that the chief contribution of quinoxaline resistance was loss of virulence, defined by ability to cause death. In the case of the chick embryo, this loss is significant. The evidence for a loss of virulence for the cotton rat or loss of toxicity for the mouse is suggestive, but not conclusive. These losses were not accompanied by loss of infectivity, as evidenced by the high ID_{50} titers which were obtained in chick embryos and cotton rats. The contribution of *p*-aminobenzoic acid resistance is not clear, but there is some suggestion that it was accompanied by loss of infectivity. However, there is no indication that infectious rickettsiae of PAB^{rr} were less virulent than those of the parent strain. The changes of $PAB^{rr}Q^r$ cannot be regarded simply as the addition of the changes in virulence and infectivity encountered in the two strains resistant to single drugs, but, rather, as enhance-

ments of each characteristic by the presence of the other.

In conclusion, these studies have provided basic tools and information for an analysis of genetic events which may take place in rickettsiae. These events cannot yet be interpreted, but genetic changes were demonstrated following simpler screening procedures than those used in previous investigations.

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SUMMARY

Three drug-resistant Madrid E strains of *Rickettsia prowazekii* were selected and some of their characteristics have been studied. The three strains, reisolated by limit-dilution techniques, were called (1) PAB^{rr}, denoting complete resistance to 10 mg of *p*-aminobenzoic acid per egg, (2) Q^r, indicating resistance to 0.5 mg of a quinoxaline compound which inhibits the parent strain in concentrations of 0.1 mg per egg, and (3) $PAB^{rr}Q^r$, resistant to both drugs. The PAB^{rr} strain was obtained from PAB^r, resistant to 3 but not to 10 mg of *p*-aminobenzoic acid per egg, following 21 egg passages in the presence of 10 mg of *p*-aminobenzoic acid. The Q^r strain was obtained from the parent strain, following 20 egg passages in the presence of increasing concentrations of quinoxaline. The $PAB^{rr}Q^r$ strain was obtained, possibly through a mechanism of genetic recombination, from a mixture of PAB^{rr} and Q^r following 2 egg passages in high concentrations of both drugs. $PAB^{rr}Q^r$, in contrast to a mixture of PAB^{rr} and Q^r, seemed to be fully resistant to a combination of the two drugs.

The three drug-resistant strains produced amounts of mouse toxin, rabbit-cell hemolysin, and elicited complement fixing antibody responses in guinea pigs comparable to those of the parent strain. Differences were noted, however, when the strains were studied in chick embryos and cotton rats. Q^r, although highly infectious when injected into the yolk sac, failed to kill the embryos as rapidly as the parent strain and PAB^{rr}. $PAB^{rr}Q^r$ appeared to have reduced virulence and infectivity for the chick embryos, failed to kill cotton rats, and immunized them only when injected in high concentration. A connection between drug-resistance and change in other properties is suggested by the fact that the most important changes were encountered in $PAB^{rr}Q^r$, which was least exposed to selective factors.

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