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ANALYSIS OF THE BIOCHEMICAL MECHANISM OF DRUG RESISTANCE IN CERTAIN BACTERIAL MUTANTS*

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Relatively little evidence is available on the biochemical mechanisms responsible for drug resistance (for references cf. 1, 2). An unusual opportunity to discriminate among certain possible mechanisms arose following the observation that a single compound, p-nitrobenzoic acid (PNB), could competitively interfere with the utilization of two different metabolites, p-aminobenzoic acid (PAB) and p-hydroxybenzoic acid (POB).[3] In other words, both PAB and POB, which have similar structures but separate metabolic functions, are required to reverse inhibition of growth by their common analog, PNB.

When strains resistant to each of these two inhibitions were compared with each other, and with strains resistant to inhibition by other analogs of PAB and POB, the results obtained provided evidence against a number of possible mechanisms of resistance. They were consistent, however, with another mechanism: a decrease in affinity of an enzyme for the drug. Further support for this mechanism was obtained by comparing the competitive ratio of drug to metabolite in drug-resistant mutants with the ratio in the more sensitive parent strain.

Cultural Methods.—The experiments were performed with the W strain of *E. coli* (ATCC 9637) in minimal medium A[4] supplemented with 100 μg. of DL-aspartic acid per milliliter. This supplement was added in order to prolong the inhibitory effect of the analogs of POB, and that of PNB acting as an analog of either POB or PAB; these inhibitions are otherwise quite transient.[3] Solid medium was prepared by the addition of 1.5% agar. Cultures were incubated at 35°C.

Isolation of Resistant Mutants.—Mutants of *E. coli* resistant to PNB were sought under two sets of circumstances. In the first of these, wild-

type cells were inoculated on a solid medium containing an inhibitory concentration of PNB (200 μg./ml.) plus sufficient PAB (10 μg./ml.) to reverse completely the anti-PAB action of this inhibitor. The organisms were still inhibited in this medium by the anti-POB action of PNB, but large inocula (10^8 to 10^9 cells) gave rise to a few colonies of mutant strains resistant to this inhibition. In a similar manner, mutants resistant to the anti-PAB action of PNB were isolated on a medium containing PNB (200 μg./ml.) plus POB (10 μg./ml.).

Similarly, mutants resistant to inhibition by each of four other antimetabolites were selected: sulfathiazole and 4,4'-diaminodiphenyl sulfone, which compete with PAB; and the corresponding compounds with hydroxy instead of amino groups (phenosulfazole[5] and 4,4'-dihydroxydiphenyl sulfone), which have been shown to compete with POB.[3,6] The structures of these compounds are shown in figure 1.

Specificity of Resistance.— The two types of PNB-resistant mutants were found to show essentially no cross-resistance: i.e., the type that was resistant to PNB plus PAB was not resistant to PNB plus POB, and vice versa. But decreased penetration of PNB would impede both its actions equally, and so could not produce separate resistance to each of these two actions. Increased destruction of the drug would likewise impede both its actions equally. These two possible mechanisms of resistance are therefore excluded in these strains.
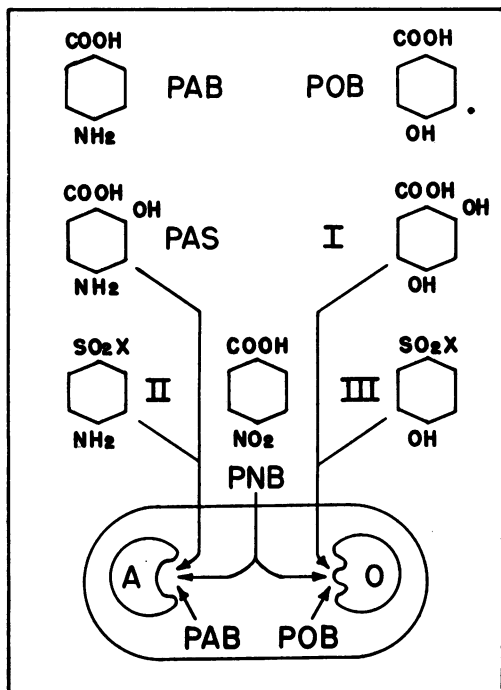

FIGURE 1

Schematic representation of competition by analogs of PAB and POB. I = β-resorcylic acid. II = sulfathiazole (X = 2-thiazolylamino) or diaminodiphenyl sulfone (X p-aminophenyl). III = phenosulfazole (X = 2-thiazolylamino) or dihydroxydiphenyl sulfone (X = p-hydroxyphenyl).

Further comparisons were made with mutants resistant to the other analogs of PAB and POB mentioned above (table 1). Extensive cross-resistance was found between the sulfonamide and the sulfone analog of

TABLE 1

RESISTANCE OF VARIOUS STRAINS TO ANALOGS OF POB AND PAB[a]

STRAIN NUMBER→		WILD TYPE	PS-4	OS-2	NA-2	NO-1	ST-1	DA-1
RESISTANCE FOR WHICH SELECTED→			PHENOSULFAZOLE	DIHYDROXYDIPHENYL SULFONE	PNB ANTI-POB	PNB ANTI-PAB	SULFATHIAZOLE	DIAMINODIPHENYL SULFONE
ADDITION TO MEDIUM	CONCENTRATION, µG./ML.				—GROWTH—			
None	–	+	+	+	+	+	+	+
Phenosulfazole	200	–	+	+	–	–	–	–
Dihydroxydiphenyl sulfone	200	–	+	+	+	–	–	–
PNB	10	–	–	–	–	–	–	–
+ PAB	200	+	–	–	–	+	–	–
PNB	10	–	–	–	–	–	–	–
+ POB	200	–	–	–	+	–	–	–
PNB	200	–	–	–	–	–	–	–
Sulfathiazole	2	–	–	–	–	–	+	+
Diaminodiphenyl sulfone	10	–	–	–	–	–	+	+

[a] The strains listed were all streaked on a single plate of each medium; these plates were then incubated for 48 hours. Solid rather than liquid media were used in order to avoid the outgrowth of any resistant mutants that might arise during the experiment. The growth recorded is therefore that of the predominant population, occasional colonies being neglected.

PAB, as would be expected, and similarly between the two corresponding analogs of POB. There was little or no cross-resistance, however, between PNB as a competitor of PAB, and the sulfonamide and sulfone competitors of PAB; similarly, there was little or no cross-resistance between PNB as a competitor of POB, and the other competitors of POB.

Since in the sulfonamide or sulfone analogs of PAB or POB it is the substituent on carbon atom 4 of the metabolite that is replaced, whereas in PNB it is the substituent on carbon atom 1, it would be of interest to determine whether specific resistance could also be obtained to an analog resulting from substitution in yet another part of the molecule. Among such compounds, an analog of POB substituted at position 2, β-resorcylic acid

TABLE 2

CROSS-RESISTANCE AMONG PAB ANALOGS SUBSTITUTED AT CARBON 1[a]

STRAIN →		W		ST-1		DDS-1		PAP-1	
ADDITION	μG./ML.	←———		—GROWTH AT 1 AND 2 DAYS—				———→	
None	..	2	4	2	4	$^{1}/_{2}$	4	2	4
Sulfathiazole	0.1	1	4	2	4	$^{1}/_{2}$	4	1	4
	0.2	0	4	2	4	$^{1}/_{2}$	4	0	4
	0.5	0	0	2	4	m	4	0	0
	1.0	0	0	$^{1}/_{2}$	4	0	m	0	0
	2.0	0	0	0	0	0	0	0	0
Diaminodiphenyl sulfone	2	2	4	2	4	$^{1}/_{2}$	4	..	..
	5	0	4	2	4	$^{1}/_{2}$	4	..	..
	10	0	0	2	4	$^{1}/_{2}$	4	..	..
	20	0	0	$^{1}/_{2}$	4	m	4	..	..
	40	0	0	0	0	0	3	..	..
p-Aminophenylphosphonous acid	20	2	4	2	4	..	..	2	4
	50	1	4	2	4	..	..	2	4
	100	0	$^{1}/_{2}$	1	4	..	..	2	4
	200	0	0	0	4	..	..	1	4

[a] From the wild type (W), one-step mutants were selected for resistance to sulfathiazole (ST-1), diaminodiphenyl sulfone (DDS-1), and p-aminophenyl-phosphonous acid (PAP-1). About 100 cells of each strain, in 5 ml. of solid medium supplemented as indicated, were poured in small Petri dishes (5 cm. diam.). Growth was recorded after incubation: m = microscopic; 1 = barely visible; 4 = full growth.

(Fig. 1), was found at concentrations of 200–600 μg./ml. to be an effective competitor of POB in *E. coli*. Mutants resistant to β-resorcylic acid were accordingly isolated, and were found to show little cross-resistance to the other analogs of POB under investigation.

The corresponding analog of PAB, p-aminosalicylic acid (PAS) (Fig. 1), is known to compete with PAB in tubercle bacilli. We were unable to utilize PAS in the present study because this compound, even in high concentrations, failed to inhibit *E. coli*. However, while this work was in

progress Yegian and Long[7] showed that in tubercle bacilli there was no cross-resistance between PAS and sulfathiazole.

Attention was next directed to a comparison of analogs substituted at · the same position rather than at different ones.   It is well known that sulfonamide and sulfone analogs of PAB, which are substituted at position 1, show cross-resistance, as has been seen in table 1 with sulfathiazole and diaminodiphenyl sulfone.   In table 2, however, which employs a more refined test, it is seen that cross-resistance between these two inhibitors is not complete; strain ST-1 is approximately twice as resistant as strain DDS-1 to sulfathiazole, and half as resistant to diaminodiphenyl sulfone.

A mutant resistant tò another 1-substituted competitive analog of PAB, *p*-aminophenyl-phosphonous acid[8] (PAP) was also obtained.   As is seen in table 2, the mutant resistant to PAP showed no cross-resistance to sulfathiazole, though the sulfathiazole-resistant mutant did show cross-resistance to PAP.   Incomplete cross-resistance was also obtained between sulfathizole and another analog substituted at the same position, *p*-aminophenylarsonic acid (*p*-arsanilic acid, Atoxyl), but these results are not presented in detail.

Similar results have been obtained' with two analogs of POB that are substituted at position 4.   One of these, PNB, has been described above; the other, PAB, though required as a metabolite at low concentrations, is also at much higher concentrations a competitor of POB.[3]   Mutants resistant to PAB, and those resistant to the anti-POB action of PNB, showed only partial cross-resistance.

Specificity of resistance to various inhibitors was originally observed with one-step mutants that were two to eight times as resistant as the parent strain; the differences persisted even after higher degrees of resistance had been obtained by successive selections.

It is clear that the mechanism of drug resistance can discriminate between different analogs even when the substitutent groups are as similar as a sulfonamide and a sulfone.   The important feature of the above observations, for the present analysis, is this specificity, rather than the fact that in some cases some degree of cross-resistance does exist.

This specificity of resistance excludes a number of possible mechanisms, to be discussed below, which could not discriminate between different analogs of the same metabolite.   It is compatible, however, with the hypothesis that these resistant mutants possess an enzyme with decreased affinity for the drug compared with its affinity for the competing metabolite.

Competitive Ratio of Analog to Metabolite (Inhibition Index).—This hypothesis of an altered enzyme was tested further on the basis of the expectation that a shift in the relative affinity of the enzyme for the drug and for the metabolite should be associated with a corresponding shift in the competitive ratio of these two substances for growing cells.

In this study, to insure that the endogenous metabolite formed would have no significant influence on the competitive ratio, two concentrations of the drug, both in excess, were used.   Table 3 shows that at both these concentrations the competitive ratio of sulfathiazole to PAB was about 25 in the parent strain, 200 in a one-step sulfathiazole-resistant mutant, and 1000 in a multi-step resistant mutant;   furthermore, these competitive ratios were proportionate to the levels of drug resistance of these three

TABLE 3

SULFATHIAZOLE SENSITIVITY, AND SULFATHIAZOLE/PAB COMPETITIVE RATIO, IN WILD TYPE AND IN RESISTANT MUTANTS[a]

STRAIN → SULFATHIAZOLE, µG./ML.	PAB, µG./ML.	W	ST-1	ST-2
			LIGHT TRANSMISSION, %	
0	0	50	50	56
0.2	0	51	...	...
0.3	0	68	...	...
0.5	0	99	...	...
2.0	0	...	52	...
3.0	0	...	94	...
5.0	0	...	100	...
10.0	0	...	...	58
15.0	0	...	...	72
20	0	...	...	92
40	.0	100	100	100
40	0.015	...	...	95
40	0.02	...	...	92
40	0.03	...	...	83
40	0.05	...	...	67
40	0.15	...	85	...
40	0.2	...	63	...
40	0.3	...	53	...
40	1.0	92	...	...
40	1.5	71	...	...
40	2.0	52	...	...
120	0.05	...	...	99
120	0.10	...	...	88
120	0.15	...	...	80
120	0.2	...	...	81
120	0.3	...	99	78
120	0.5	...	80	...
120	0.7	...	51	...
120	3.0	90	...	...
120	5.0	65	...	...
120	7.0	52	...	...
Sulfathiazole sensitivity		0.3	2.5	15
ST/PAB ratio (ST = 40)		40/1.5 (27)	40/0.18 (210)	40/0.04 (1000)
ST/PAB ratio (ST = 120)		120/4.5 (27)	120/0.6 (200)	120/0.1 (1200)

[a] Strain ST-1 was derived from the wild type (W) by one selective exposure to sulfa-
thiazole, and strain ST-2 in turn was similarly derived from ST-1. With each strain
10^{-4} ml. of a fresh 18-hr. culture, resuspended in water, was inoculated into 10 ml. of
minimal medium A, supplemented as indicated, in tubes 22 mm. in diameter. After
24 hrs. of incubation, turbidity was measured with an Evelyn photoelectric colorimeter,
filter 620 mμ.

Fifty per cent inhibition was taken as the end point for the interpolations at the bot-
tom of the table. This value was equivalent to 70% transmission, except with strain
ST-2 at 120 μg./ml. sulfathiazole, where it was equivalent to 88% transmission. (In
this last case the very high drug concentration produces partial inhibition that was not
reversed by PAB; only the PAB-reversible inhibition was considered.)

Essentially identical results were obtained with the pour-plate technique described
in table 2, except that the partially inhibitory concentrations observed were about one-
half those obtained in tubes.

strains. Likewise, in strains resistant to a POB competitor (phenosulfazole)
a similar shift was observed in the competitive ratio of drug to metabolite.
These results support the inference of an enzyme of altered affinity.

Sulfonamide Resistance in a PAB Auxotroph.—Further evidence against
certain possible mechanisms was obtained by observing the effect of sul-
fonamide resistance on the PAB requirement of a mutant of *E. coli*. Start-
ing with PAB auxotroph 48A-33, a resistant derivative was obtained by
two successive selections with sulfathiazole. These selections were carried

TABLE 4

EFFECT OF SULFATHIAZOLE RESISTANCE ON PAB REQUIREMENT[a]

PAB, MμG./ML.	STRAIN 48A-33 (SENSITIVE)			STRAIN 48A-33ST2 (RESISTANT)		
	18 HRS.	24 HRS.	48 HRS.	18 HRS.	24 HRS.	48 HRS.
			LIGHT TRANSMISSION, %			
0	100	100	100	100	100	100
0.1	98.5	97	96	100	100	99
0.2	96	94.5	90.	99	99	99
0.4	91	83	80	98.5	96.5	95
0.8	83	73.5	74	95	83	75
1.6	73.5	64	66	82	66	68
3.2	62.5	60	55	73	60	57
6.4	56.5	56	50	60	56.5	50
12.8	53	53.5	46	53	53.5	46

[a] Strain 48A-33, a stable PAB auxotroph, and strain 48A-33ST2, a sulfonamide-re-
sistant strain derived from it, were each inoculated (5×10^{-3} ml.) into colorimeter
tubes, incubated, and measured turbidimetrically as in table 3.

Essentially identical results were obtained with streaks on solid medium.

out in a medium containing a PAB concentration (0.01 μg./ml.) that could
satisfy the growth requirement of the bacteria but would not reverse the
inhibitory effect of the drug at the concentrations used. The derivative
strain was found to be 15 times as sulfathiazole-resistant as its parent strain.

Table 4 shows that this resistant derivative not only still requires PAB,
but actually has an increased threshold of response. With an excess of

PAB it responds as well as its parent, but with decreasing concentrations of PAB it grows more slowly than its parent, and at very low concentrations (0.1–0.4 mμg./ml.) the resistant strain not only grows more slowly, but also yields much less final growth.

The significance of these results will be discussed below.

It has similarly been observed that β-alanine auxotrophs retain their requirement following mutation to resistance to an inhibitory competitor of β-alanine (D-serine).[9]

Discussion.—The following possible changes in resistant mutants suggest themselves as mechanisms that might account for the resistance.

1. Decreased penetration of the drug.
2. Increased destruction of the drug (or decreased conversion of an inactive to an active compound).
3. Increased concentration of a metabolite antagonizing the drug.
4. Increased concentration of an enzyme utilizing this metabolite.
5. Decreased quantitative requirement for a product of the metabolite.
6. Alternative metabolic pathway by-passing the metabolite.
7. Enzyme with decreased relative affinity for the drug compared with the metabolite.

As has been pointed out above, both mechanisms 1 and 2 (decreased penetration and increased destruction of the drug) have been excluded as explanations of PNB resistance in the mutants studied, since these mutants show that resistance to each of the two inhibitory actions of PNB (competition with PAB and with POB) develops independently.

In interpreting further observations, it is assumed that PAB is the substrate of only one enzyme and POB that of another, and that the several analogs of each substrate compete with it at the active site of its enzyme (Fig. 1). If this assumption is correct, the specific resistances observed with different competitors of the same metabolite eliminate mechanisms 3 to 5; for these mechanisms would involve only quantitative alterations in the substrate-enzyme-product system, and such changes could not discriminate between two different competitors of the same metabolite. Mechanism 6 (an alternative metabolic pathway) would also be unable to discriminate between these competitors, and would in addition be difficult to reconcile with the observation that resistance is graded rather than all-or-none. Finally, the persistence of a PAB requirement in a sulfonamide-resistant derivative of a PAB auxotroph likewise excludes mechanisms 3, 5 and 6.

All these observations, however, are compatible with mechanism 7: production of an enzyme with decreased affinity for the inhibitor compared with its affinity for the substrate. But the inference of this mechanism would require not only the assumption given in the preceding paragraph,

but also two further assumptions: (*a*) that the exclusion of mechanisms 1 and 2 can be extended from PNB to the other, structurally similar analogs; and (*b*) that no other possible mechanisms have been overlooked.   Though these three assumptions seem reasonable, they cannot be experimentally tested at this time.   Mechanism 7 therefore lacks rigorous proof.

Further, more direct evidence for this mechanism is provided by the demonstration that resistant strains have an altered competitive ratio of analog to metabolite, proportionate to their degree of resistance.   A similar shift in competitive ratio was previously observed by Ivanovics in sulfonamide-resistant strains of staphylococci.[10]   But even this evidence is not conclusive since it would also be compatible with mechanisms 1, 2, 4 or 5. However, the value of this evidence is strengthened by the existence of reasons for excluding these mechanisms.

The shift in competitive ratio was interpreted by Ivanovics as evidence for an enzyme with increased affinity for PAB.[11]   This mechanism, however, could not distinguish between various analogs of PAB, and hence could not account for the specificity of resistance that we have observed. Moreover, the threshold PAB requirement of a PAB auxotroph was found to increase rather than decrease following development of sulfathiazole resistance.   These observations suggest that in the resistant mutants there is a decrease in affinity of the enzyme for the metabolite, together with an even greater decrease in affinity for the drug.

This apparent decrease in affinity for the metabolite suggests that in this species optimal PAB utilization might be necessarily associated with sensitivity to sulfonamides.   On this basis it would be easy to understand why a sulfonamide-sensitive wild type should have greater evolutionary survival value, in the ordinary sulfonamide-free surroundings, than the more sulfonamide-resistant mutants to which it frequently gives rise.

We have been led to postulate that mutations can lead to a variety of changes in the affinity of an enzyme for different drugs; this picture would imply the production of a variety of changes in the configuration of the binding site of the enzyme.   This possibility is more readily visualized when it is noted that the competitors with the most strikingly differing resistance are those that differ in the positions of their substituent groups: PAB or POB analogs substituted at position 1 (carboxyl) of the ring, compared with respective analogs substituted at positions 2 or 4.   It is of interest to note, however, that some specificity of resistance was also observed with different analogs substituted at the same position.

That enzymes of identical function in different species can differ in their relative affinity for various compounds is well known from observations of comparative biochemistry.   For example, such enzymes have been shown, after isolation in the crystalline state, to differ in immunological specificity[12] and in susceptibility to various inhibitors;[13] and hemoglobins

found in the animal kingdom exhibit a 200-fold range in the ratio of their affinities for oxygen and for carbon monoxide.[14]   The results described in this paper strongly suggest that such a change in affinity can result from a single mutation.   This inference is further strengthed by the direct demonstration with extracted enzymes, reported elsewhere,[15] that a mutation can cause bacteria to produce an enzyme altered in another quality, temperature sensitivity.

A more rigorous test for an enzyme of altered affinity would be possible if this enzyme could also be studied in extracts of the cells, but the enzymes involved in the present study have not seemed promising for such investigation.   Evidence for altered dehydrogenase systems in extracts has been reported by Sevag and Gots,[16] who studied pneumococci resistant to a number of drugs that appear to interfere with riboflavin metabolism; but it has not been established that inhibition of these dehydrogenases is the mechanism by which these drugs interfere with growth.

It is not implied that the mechanism inferred here is the only one responsible for drug resistance.   Indeed, an example of mechanism 2 is the formation of penicillinase by many penicillin-resistant organisms; and an example of mechanism 3 is increased PAB production, which has been reported for certain sulfonamide-reistant bacteria.   However, even in these sulfonamide-resistant strains other mechanisms may also be involved, since the degree of resistance is not always paralleled by the amount of PAB excreted.[17]

Summary.—*p*-Nitrobenzoic acid is an inhibitory competitor of both *p*-aminobenzoic acid and *p*-hydroxybenzoic acid in *E. coli*.   Mutants resistant to either of these inhibitions are not resistant to the other.   These resistances could therefore not be due to decreased penetration or increased destruction of the drug.

Resistance to various analogs of a single metabolite has also been observed to be partly or completely specific.   This finding excludes a number of other possible mechanisms of drug resistance.

These results are all compatible with the presence of an altered enzyme, with decreased relative affinity for the inhibitor compared with the metabolite.   This hypothesis is supported by the observation that increased resistance is accompanied by a proportionate increase in the competitive ratio of analog to metabolite.   In addition, evidence is presented that in some resistant strains the PAB-utilizing enzyme has a decreased affinity for PAB, as well as an even more decreased affinity for the sulfonamide analog.   .

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PRODUCTION OF AN ALTERED PANTOTHENATE-SYNTHESIZING ENZYME BY A TEMPERATURE-SENSITIVE MUTANT OF ESCHERICHIA COLI

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Studies on the growth requirements of many microbial mutants have shown that in each of these strains a single biosynthetic reaction is blocked. To account for these blocks it has been proposed that a mutant either (a) fails to produce any enzyme protein corresponding to the blocked reaction;[1] or (b) produces an altered, inactive protein;[1] or (c) produces an inhibitor of a normal enzyme.[2]

A number of attempts have been made to test these hypotheses, mainly with mutants of Neurospora,[2–5] including a temperature-sensitive one.[6] In several of these studies one of these mechanisms was ruled out but no definitive evidence for either of the remaining mechanisms was obtained. In the present study, however, it was possible to demonstrate one of the three mechanisms in a temperature-sensitive auxotroph of *Escherichia coli*.

This mutant requires pantothenate only above 30°C.; below this temperature it grows, like the wild type, without pantothenate.   It was de-