STUDIES ON THE ACTION OF SULFONAMIDES ON THE RESPIRATION AND GROWTH OF BACTERIA[1]

A. Factors Controlling the Inhibition by Sulfonamides of Carboxylases. II. Antagonism between *p*-Aminobenzoic Acid and Sulfathiazole

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Received for publication August 15, 1944

In a preceding report it was shown that cocarboxylase antagonizes the inhibitory effect exercised by sulfathiazole on the carboxylase activities of (a) whole yeast cells, (b) yeast cells washed with alkaline phosphate, and (c) *Staphylococcus aureus*. The results showed that one molecule of cocarboxylase counteracted the inhibitory effect of 322 to 53,400 molecules of sulfathiazole on yeast carboxylase, and the inhibitory effect of 215 molecules of sulfathiazole on *Staphylococcus aureus*. The present report deals with experiments in which the antagonism between sulfathiazole and *p*-aminobenzoic acid is determined.

RESULTS

Competition between sodium pyruvate and sulfathiazole for the carboxylase of Escherichia coli. To determine the exact mechanism of the action of sulfathiazole on the carboxylase activity of bacteria, the following experiments were conducted. In one experiment, using two-armed Warburg flasks, sulfathiazole was first mixed with the bacteria, and then the substrate, sodium pyruvate, was added. In another experiment, sulfathiazole was added to the system immediately after sodium pyruvate and the bacteria were mixed. The results of these two simultaneous experiments are given in table 1. It can be seen that when sodium pyruvate was added to the mixture of sulfathiazole and bacteria, the carboxylase activity was inhibited from 35 to 37 per cent. In contrast, when sulfathiazole was added to the mixture of sodium pyruvate and bacteria, the inhibition was only from 4 to 7 per cent. This shows that both the substrate and sulfathiazole are attracted to the same active sites in the enzyme carboxylase.

Failure of p-aminobenzoic acid to reverse the inhibition by acetaldehyde of the carboxylase activity of E. coli. It is known that acetaldehyde, the decarboxylation product of sodium pyruvate, exercises a strong inhibitory effect on the carboxylase activity of cells. This inhibitor evidently combines specifically with the active site of the enzyme and prevents its activity. This could be compared with the inhibition of carboxylase activity by sulfathiazole if both inhibitions could be counteracted by *p*-aminobenzoic acid. It was therefore of interest

[1] This investigation has been aided by a grant from the Josiah Macy, Jr., Foundation.

to determine whether or not the inhibition by acetaldehyde is reversible by *p*-aminobenzoic acid. The results of experiments are presented in table 2.

It can be seen that 4.06×10^{-2} M of acetaldehyde alone caused 83 per cent inhibition of the carboxylase activity of *E. coli*; 1×10^{-2} M *p*-aminobenzoic acid caused a 30 per cent, and 2.5×10^{-3} M, a 17 per cent inhibition. Also 1×10^{-2} M, 2.5×10^{-3} M, and 6.25×10^{-4} M *p*-aminobenzoic acid failed to

TABLE 1

Competition between sodium pyruvate (5×10^{-2}M) and sulfathiazole (4.14×10^{-3}M) for the carboxylase of E. coli (1.238 mg)

The pH of the reaction medium was 7.2

E. COLI	SODIUM PYRUVATE ADDED LAST				SULFATHIAZOLE ADDED LAST			
	Control		Sulfathiazole		Control		Sulfathiazole	
	a*	b*	a	b	a	b	a	b
mm³ CO_2 evolved (2 hr)	488	520	318	324	406	413	390	380
QCO_2†	197	210	128	131	164	166	157	153
Per cent inhibition	—	—	35	37	—	—	4	7

* a and b represent the results of two different experiments.

† QCO_2 = mm³ CO_2/hour/mg *E. coli*.

TABLE 2

Failure of p-amino benzoic acid to reverse the inhibition of the carboxylase activity of E. coli (1.088 mg) by acetaldehyde (4.06×10^{-2}M) and vice versa

E. COLI	CONTROL		*p*-AMINOBENZOIC ACID					
			1×10^{-2}M		2.5×10^{-3}M		6.25×10^{-4}M	
	Buffer	Acetaldehyde	Buffer	Acetaldehyde	Buffer	Acetaldehyde	Buffer	Acetaldehyde
mm³ CO_2 evolved (3 hr)	882	132	593	51	706	92	781	131
QCO_2*	261	44	182	15	216	27	240	36
Per cent inhibition (when compared with absolute control)	—	83	30	94	17	90	8	87
Per cent inhibition (when compared with the respective controls)	—	83	30	92	17	87	8	85

* QCO_2 = mm³ of CO_2/hour/mg *E. coli*.

exercise any antagonistic action on the inhibition by 4.06×10^{-2} M acetaldehyde. In the presence of both acetaldehyde and *p*-aminobenzoic acid the inhibition was the same as that exercised by acetaldehyde alone. That is, the inhibition in the presence of both substances was not additive.

Reversal by p-aminobenzoic acid of the inhibition by sulfathiazole of the carboxylase activity of E. coli. It was shown above that *p*-aminobenzoic acid was incapable of counteracting the inhibition exercised on the carboxylase activity of *E. coli* by acetaldehyde. In the following experiments acetaldehyde was replaced by sulfathiazole. The results are presented in table 3. It can be seen

TABLE 3

Reversal by p-aminobenzoic acid (p-ABA) of the inhibition by 2.8 × 10⁻³ M sulfathiazole of the carboxylase activity of E. coli

PERIOD	CONTROL*	INHIBITION BY SULFATHIAZOLE	INHIBITION BY		REVERSAL BY *p*-ABA (6.7 × 10⁻⁴ M) OF THE INHIBITION BY SULFATHIAZOLE	INHIBITION BY		REVERSAL BY *p*-ABA (6.7 × 10⁻³ M) OF THE INHIBITION BY SULFATHIAZOLE
			p-ABA (6.7 × 10⁻⁴ M) alone	*p*-ABA + sulfathiazole		*p*-ABA (6.7 × 10⁻³ M) alone	*p*-ABA + sulfathiazole	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
minutes	*mm³CO₂*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
30	94	26	6	0	100†	22	26	85‡
60	317	19	6	5	100	21	20	100
120	810	20	6	10	80	21	19	100

* The reaction system consisted of 0.2 ml of *E. coli* (measured with a Kahn pipette) suspension in M/15 phosphate buffer of pH 7.16 + 5.4 ml of M/120 phosphate buffer of pH 7.16 + 0.1 ml of $MgSO_4$ solution (0.1 mg Mg) + 0.3 ml of sodium pyruvate of pH 7.16. Temperature = 37.5 C. Atmosphere = 95% N + 5% CO_2.

† The percentage of reversal was calculated by comparing the differences between columns (5) and (4) with that given under column (3), or column (3) − [column (5) − (4)]/(3) × 100 = % reversal.

‡ The values were obtained by identical treatment as described under the preceding note.

TABLE 4

Reversal by p-aminobenzoic acid (p-ABA) of the inhibition of the carboxylase activity of Escherichia coli (2.016 mg) by 2.8 × 10⁻³ M sulfathiazole at pH 7.2

E. COLI	CONTROL (BUFFER)	SULFATHIAZOLE	*p*-AMINOBENZOIC ACID			
			6.2 × 10⁻⁵ M		2.5 × 10⁻⁴ M	
			Buffer	Sulfathiazole	Buffer	Sulfathiazole
	a	b	a′	b′	a′	b′
mm^3 CO_2 evolved (3 hr)	1205	735	950	694	844	765
QCO_2	200	121	157	114	138	126
Inhibition, per cent						
a. When compared with absolute control	—	40	21	45	31	37
b. When compared with respective controls	—	40	21	27	31	8
Reversal by *p*-ABA of the inhibition by sulfathiazole, in per cent	—	—	—	32*	—	80

* [a−b/a] × 100 = A, per cent inhibition by sulfathiazole.
[a′b′/a′] × 100 = B, per cent inhibition by sulfathiazole in the presence of *p*-ABA. (This is on the assumption that the inhibition by *p*-ABA is unchanged by addition of sulfathiazole.)
A−B/A × 100 = per cent reversal by *p*-ABA of the inhibition by sulfathiazole.

that 6.7 × 10⁻⁴ M *p*-aminobenzoic acid alone caused only a 6 per cent inhibition, and 6.7 × 10⁻³ M caused 22 per cent inhibition of carboxylase activity. On the other hand, 2.8 × 10⁻³ M sulfathiazole alone caused from 19 to 26 per cent

inhibition. This inhibition is reversed from 80 to 100 per cent by 6.7×10^{-4} M, or by 6.7×10^{-3} M *p*-aminobenzoic acid. In the simultaneous presence of 6.7×10^{-3} M *p*-ABA and 2.8×10^{-3} M ST the inhibition of carboxylase activity was equal to that caused by either alone. This indicates that the inhibition is not additive, and that both substances compete for the same active group in the enzyme. This may be interpreted to indicate that *p*-ABA antagonizes by maintaining its own inhibitory effect (when a higher concentration is used) on the enzyme and thus preventing sulfathiazole from exercising its inhibitory effect. In a lower concentration (6.7×10^{-4} M), *p*-ABA maintains its own union with the enzyme, without exercising inhibitory effect, and thus also prevents sulfathiazole from exercising an inhibitory effect. In the former case one molecule of *p*-ABA antagonized about 2 molecules of ST. In the latter case one molecule of *p*-ABA antagonized about 20 molecules of ST.

TABLE 5

Reversal by p-aminobenzoic acid of the inhibition by sulfathiazole of the carboxylase activity of Staphylococcus aureus

PERIOD	QCO_2—CONTROL			INHIBITION BY SULFATHIAZOLE 1.38×10^{-3} M		
	Buffer	*p*-Aminobenzoic acid		Buffer	*p*-Aminobenzoic acid	
		1×10^{-4} M	1.2×10^{-3} M		1.4×10^{-4} M	1.2×10^{-3} M
hr				*per cent*	*per cent*	*per cent*
1	55	52	75	40	32	26
2	71	69	84	45	32	24
3	78	74	85	42	27	21
4	80	80	86	38	28	18
5	80	80	85	33	25	17

The reaction system consisted of 0.2 ml of staphylococcal (2 mg) suspension + 0.1 ml of $MgSO_4$ sol. (0.1 mg of Mg) + 5.4 ml of M/150 phosphate buffer of pH 7.16 + 0.3 ml of sodium pyruvate (25 mg) in an atmosphere of 95 per cent N and 5 per cent CO_2, at a temperature of 37.5 C. QCO_2 = mm^3 CO_2/hour/mg staphylococci.

In a similar experiment (table 4), 6.2×10^{-5} M and 2.5×10^{-4} M *p*-ABA alone caused, respectively, 21 and 31 per cent inhibition of carboxylase activity; 2.8×10^{-3} M ST alone caused 40 per cent inhibition. In the simultaneous presence of *p*-ABA and ST, the inhibition was not only not additive but with 2.5×10^{-4} M *p*-ABA the 40 per cent inhibition by ST was reduced to 8 per cent, and with 6.2×10^{-5} M *p*-ABA it was reduced to 27 per cent. These amount to from 32 to 80 per cent reversal of inhibition. In bringing about an 80 per cent reversal of inhibition, one molecule of *p*-ABA was capable of antagonizing 10 molecules of ST.

Reversal by p-aminobenzoic acid of the inhibition by sulfathiazole of the carboxylase activity of Staphylococcus aureus. The results of an experiment with staphylococci are presented in table 5. In this experiment the hourly course of the antagonism between *p*-ABA and ST was determined for a period of five hours. It can be seen that in the presence of 1.4×10^{-4} M *p*-ABA the 33 to 45 per cent

inhibition by 1.38 $\times$ 10^{-3} M ST was reduced to 25 to 32 per cent, and with 1.2 $\times$ 10^{-3} M *p*-ABA the inhibitions were reduced to from 17 to 26 per cent. These amount to a reversal of inhibition of from 35 to 53 per cent by ST. That is, one molecule of *p*-ABA antagonized, respectively, 10 and 1 molecules of ST.

DISCUSSION

p-Aminobenzoic acid was found to be incapable of reversing the inhibition, exercised on the carboxylase activity of *E. coli*, by acetaldehyde. The inhibition in the simultaneous presence of *p*-aminobenzoic acid and acetaldehyde was found to be nonadditive despite the fact that each alone caused, respectively, 83 and 30 per cent inhibition. In the presence of both, the observed inhibition corresponded to that exercised by acetaldehyde alone. Evidently the inhibitory combination between acetaldehyde and carboxylase prevents *p*-aminobenzoic acid from also combining with the active site of the enzyme. In contrast, *p*-aminobenzoic acid counteracted the inhibition exercised on carboxylase by sulfathiazole. One molecule of the former was capable of counteracting from 1 to 20 molecules of the latter. In a preceding report it was demonstrated that one molecule of cocarboxylase was capable of counteracting the inhibitory action of from 200 to 600 molecules of sulfathiazole on the activity of carboxylase. These show that the coenzyme cocarboxylase is from 10- to 20-fold more effective as a sulfathiazole antagonist than is *p*-aminobenzoic acid.

In this connection a reference to previous observations may be of interest. In experiments extending over a 4-hour period, Sevag and Shelburne (1942) found that one molecule of *p*-aminobenzoic acid neutralized the inhibitory effect exercised on the aerobic and anaerobic respiration of *Streptococcus pyogenes* by 6 to 66 molecules of sulfanilamide. Clifton and Loewinger (1943) reported that the inhibitory effect of sulfanilamide on the anaerobic respiration of *E. coli* is prevented by *p*-aminobenzoic acid. Their results show that one molecule of *p*-aminobenzoic acid neutralized 125 molecules of sulfanilamide.

In connection with the results on the inhibition of carboxylase by sulfathiazole, it may also be of interest to refer to the fact that one molecule of *p*-aminobenzoic acid was found by Wyss *et al.* (1942) to neutralize the inhibition of the half-maximal growth (16-hour period) of *Staphylococcus aureus* by 53, and that of *E. coli* by 27 molecules of sulfathiazole. These ratios compare favorably with those found in our experiments on the carboxylase activity of *S. aureus* and *E. coli*.

In all of these instances, there is no evidence that *p*-aminobenzoic acid participates actively in the metabolic activities of the organisms studied to account for its sulfonamide-antagonizing property. This is particularly true in those cases in which resting cells, and isolated enzyme systems, have been tested. Kohn and Harris (1941) postulated that *p*-aminobenzoic acid acts as a catalyst. As will be discussed later, this is contrary to the known facts. It is known that *p*-aminobenzoic acid inhibits the growth of certain bacteria and the activity of certain enzymes. This will be evident also from the discussions which follow. Considering, however, the postulate of Kohn and Harris in the light of the

criteria of catalysis, one must hold in view the fact that a catalyst accelerates a thermodynamically possible reaction, to attain an equilibrium in a shorter period of time. It is, therefore, inconsistent to attribute a catalytic role to a substance which inhibits the attainment of states of reaction equilibria, and likewise inhibits thermodynamically possible reactions. It can only do these things when it functions not as a catalyst but as an inhibitor of catalysts. This is evident also from the following observations. *Para*-aminobenzoic acid exercised its antagonistic action to sulfathiazole by maintaining a certain degree of inhibitory action of its own on the activity of carboxylase. In contrast, cocarboxylase counteracted the inhibitory action of sulfathiazole without exercising any inhibitory action on carboxylase. That is, unlike cocarboxylase, *p*-aminobenzoic acid functioned as an inhibitor, as well as an anti-inhibitor.

It is to be noted that *p*-aminobenzoic acid exercised sulfonamide-antagonizing action in a system where the respiring material was incapable of multiplication. Since it did not accelerate the activity of the enzyme, it could not have functioned as a coenzyme or growth factor. These facts do not offer any support to the postulate that *p*-aminobenzoic acid acts as a catalyst. That *p*-aminobenzoic acid acts as an anti-inhibitor, first suggested by Sevag and Shelburne (1942), was also shown by Eyster (1943). He found that a sufficient concentration of *p*-aminobenzoic acid antagonized the inhibitory effect of sulfanilamide on the hydrolysis of starch by diastase. It also antagonized the inhibition by sulfanilamide of the adsorption of methylene blue on charcoal.

In none of these instances does *p*-aminobenzoic acid set a new pattern in enzyme reactions. Its action is in accordance with well-known facts. One or two examples suffice to explain this point. The antisulfonamide action of *p*-aminobenzoic acid may be compared to the protective action of malonate against oxidized glutathione which inactivates succinoxidase (Hopkins *et al.*, 1938). Malonate, structurally related to succinic acid, competes with it and inhibits succinoxidase. In the capacity of anti-inhibitor, however, malonate prevents the inactivation of succinoxidase by oxidized glutathione. A second example of this type is provided in a recent study by Potter and DuBois (1943). They showed that quinone and *p*-phenylenediamine inhibit succinoxidase. Malonate, as stated above, in the capacity of an inhibitor neutralized any additive inhibition by quinone or *p*-phenylenediamine. These facts are in agreement with the properties manifested by *p*-aminobenzoic acid in connection with our studies on carboxylase.

SUMMARY

Sulfathiazole and sodium pyruvate compete for the active site of carboxylase. Presence or absence of inhibition depends on which of the two substances gets to the active site of the enzyme first.

Para-aminobenzoic acid failed to reverse the strong inhibition exercised by acetaldehyde on carboxylase. In contrast, it reversed the inhibition exercised by sulfathiazole on the carboxylase activities of *Escherichia coli* and *Staphylococcus aureus*. In experiments on the inhibition of growth and carboxylase

activities of *E. coli* and *S. aureus*, practically the same number of sulfathiazole molecules were found to be neutralized by one molecule of *p*-aminobenzoic acid. The sulfonamide-antagonizing property of *p*-aminobenzoic acid has been shown to compare with the protective action of other well-known enzyme inhibitors. From the standpoint of the criteria of catalysis, it has been pointed out that *p*-aminobenzoic acid behaves like an inhibitor of catalysis, and not like a catalyst.

REFERENCES

CLIFTON, C. E., AND LOEWINGER, I. E. 1942 Sulfanilamide activity against *Escherichia coli* under anaerobic conditions. Proc. Soc. Exptl. Biol. Med., **52**, 225–227.

EYSTER, H. C. 1943 Mechanism of sulfanilamide action and its interaction with *p*-aminobenzoic acid. J. Cellular Comp. Physiol., **21**, 191–198.

EYSTER, H. C. 1943 Auxin action. Science, **97**, 358–359.

HARRIS, J. S., AND KOHN, H. I. 1941 On the mode of action of the sulfonamides. II. The specific antagonism between methionine and the sulfonamides in *Escherichia coli*. J. Pharmacol., **73**, 383–400.

HOPKINS, F. G., MORGAN, E. J., AND LUTWAK-MANN, C. 1938 The influence of thiol groups in the activity of dehydrogenases. II. With an addendum on the location of dehydrogenases in muscle. Biochem. J., **32**, 1829–1848.

KOHN, H. I., AND HARRIS, J. S. 1941 On the mode of action of the sulfonamides. I. Action on *Escherichia coli*. J. Pharmacol., **73**, 343–361.

POTTER, V. R., AND DUBOIS, K. P. 1943 Studies on the mechanism of hydrogen transport in animal tissues. J. Gen. Physiol., **26**, 391–404.

SEVAG, M. G., AND SHELBURNE, MYRTLE. 1942 The respiration of *Streptococcus pyogenes* and pneumococcus type I. III. Bearing of respiration on existing theories of the mechanism of the action of the chemotherapeutic agents. J. Bact., **43**, 447–462.

WYSS, O., GRUBAUGH, K. K., AND SCHMELKES, F. G. 1942 Nonspecificity of sulfonamides. Proc. Soc. Exptl. Biol. Med., **49**, 618–621.