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

A. Factors Controlling the Inhibition by Sulfonamides of Carboxylases. I. Antagonism Between Cocarboxylase and Sulfathiazole

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Studies previously reported on the mode of action of sulfonamides on bacteria showed that the inhibition of respiratory enzymes of the bacteria caused a proportional inhibition of the growth of *Streptococcus pyogenes* and pneumococcus, type I. This conclusion was based on simultaneous measurements, at various time intervals, of the increase in the number of bacteria (and the mg of bacterial nitrogen) and of the respiration in the presence and absence of 0.04 M sulfanilamide. On the basis of these and other observations, the "inhibition of respiration theory," as the mode of action of sulfonamides, was proposed (Sevag and Shelburne, 1942a, 1942b). This theory, in part, stated that chemotherapeutic agents which possess structural similarity to the whole or part of the coenzyme molecules may specifically combine with the protein component of the respiratory enzymes. This combination may take place as a result of the displacement of coenzymes by the drug, forming an inactive "enzyme analogue," or by a reversible union of the drug with the protein, forming an inactive "drug-protein-coenzyme complex." In this connection it was shown (Sevag, Shelburne, and Mudd, 1942) that sulfonamides exercise inhibitory affinities for bacterial and yeast carboxylases. Sulfathiazole, in comparison with other sulfonamides, being structurally most nearly related to cocarboxylase, exercised markedly greater inhibitory affinity for bacterial carboxylases.

The present report represents the results of further studies.

METHODS

As previously described (Sevag, Shelburne, and Mudd, 1942), carboxylase activities of various materials were measured in a Barcroft-Warburg setup.

Experiments with air-dried brewers' whole yeast. The reaction system contained 0.1 ml of a solution of magnesium sulfate (0.1 mg Mg), 0.7 ml of yeast suspension containing various amounts of whole yeast, 0.2 ml of sodium pyruvate (17.6 mg). The final volume was made up to 6 ml with M/150 phosphate buffer of pH 6.2. The temperature of the water bath was 37.5 C. The atmosphere of the system consisted of 95 per cent N and 5 per cent CO_2, and the experimental period was 120 minutes.

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Experiments with yeast washed with alkaline phosphate. The reaction system contained the same constituents as those given above. In experiment 1 (table 2), the yeast suspension contained 100 mg of air-dried brewers' whole yeast. In experiment 2, 100 mg of yeast were washed with alkaline phosphate, using the method of Lohmann and Schuster (1937) to remove cocarboxylase. The activity of the yeast thus treated was restored by adding pure cocarboxylase (Merck).

RESULTS

Reversal by cocarboxylase of the inhibition of the carboxylase activity of yeast cells by sulfathiazole. The results of two preliminary experiments, presented in table 1, showed that sulfathiazole exercised a 25 per cent inhibition on the carboxylase activity of 2 mg of air-dried yeast cells. This effect was reduced to 14 per cent when 2 μg of cocarboxylase was added to the system. This reduction of inhibition is 44 per cent. Two μg of cocarboxylase in a volume of 6 ml is 6.8×10^{-7} M. This concentration of cocarboxylase was found to be capable

TABLE 1

Reversal by cocarboxylase of the inhibition by sulfathiazole of the carboxylase activity of 2 mg of air-dried brewers' yeast at pH 6.2

PERIOD	CONTROLS			INHIBITION BY SULFATHIAZOLE (0.0055 M)		
	Buffer	Cocarboxylase		Buffer	Cocarboxylase	
		0.2 μg	2.0 μg		0.2 μg	2.0 μg
hour	*mm³CO₂*	*mm³CO₂*	*mm³CO₂*	*per cent*	*per cent*	*per cent*
1	497	505	618	25	21	14
2	637	690	816	24	26	14

of reducing by 44 per cent the inhibition by 0.0055 M sulfathiazole. That is, one molecule of cocarboxylase caused 44 per cent reversal of the inhibition by 8088 molecules of sulfathiazole.

On washing the whole yeast with alkaline phosphate, 97 per cent of the carboxylase activity was removed (table 2). The yeast, thus treated, contains the specific protein of carboxylase practically free from cocarboxylase. The whole yeast, treated with 3 μg, or 50 μg, of cocarboxylase, did not show increased carboxylase activity. On the other hand, the treatment of phosphate-washed yeast with 3 or 50 μg of cocarboxylase restored 56 and 71 per cent, respectively, of the original carboxylase activity. The specific protein of carboxylase, in washed yeast, treated simultaneously with cocarboxylase and sulfathiazole, should therefore be an object of competition between these two substances.

The results presented in table 2 show that 0.0055 M sulfathiazole did not exercise an inhibitory effect on 100 mg of whole yeast. In contrast, it exercised a 35 per cent inhibition on the carboxylase activity of washed cells in the presence of 3 μg of added cocarboxylase. In the presence of 50 μg of cocarboxylase the inhibition was reduced to 5 per cent, or an 86 per cent reversal of inhibition

took place. This relationship shows that one molecule of cocarboxylase is capable of preventing the union of 322 molecules of sulfathiazole with the specific carboxylase protein.

TABLE 2

Reversal by cocarboxylase of the inhibition of the carboxylase activity of yeast (washed with alkaline phosphate) by 0.0055 M sulfathiazole

ENZYME SYSTEM	CONTROL*			INHIBITION BY SULFATHIAZOLE		
	Buffer	Cocarboxylase		Buffer	Cocarboxylase	
		3 µg	50 µg		3 µg	50 µg
	mm³CO₂	*mm³CO₂*	*mm³CO₂*	*per cent*	*per cent*	*per cent*
1. Brewers' whole yeast (100 mg air-dried)	2,172	2,182	2,200	0	0	−9
2. Brewers' whole yeast† (100 mg washed with alkaline phosphate)	61	1,215	1,556	10	35	5

− = increase.

* Control system consisted of 0.1 ml of $MgSO_4$ (0.1 mg of Mg) solution + 0.7 ml of yeast suspension + 0.2 ml of sodium pyruvate (17.6 mg), made up to a volume of 6 ml with M/150 phosphate buffer of pH 6.2. Temperature = 37.5 C. In 95% N + 5% CO_2. Period = 120 minutes.

† Air-dried yeast was washed with alkaline phosphate according to Lohmann and Schuster (1937) to remove cocarboxylase.

TABLE 3

Reversal by cocarboxylase of the inhibition of the carboxylase activity of both the whole air-dried brewers' yeast (10 mg) and that of yeast treated with alkaline phosphate by 0.0055 M sulfathiazole

ENZYME SYSTEM	CONTROLS				INHIBITION BY SULFATHIAZOLE			
	Buffer	Cocarboxylase µg			Buffer	Cocarboxylase µg		
		0.3	3	25		0.3	3	25
	mm³CO₂	*mm³CO₂*	*mm³CO₂*	*mm³CO₂*	*per cent*	*per cent*	*per cent*	*per cent*
1. Brewers' whole yeast (10 mg air-dried)	1,280	1,330	1,435	1,520	20	−8	0	−1
2. Brewers' yeast washed with alkaline phosphate*	22	117	683	1,150	90	80	80	56†

− = increase.

* For experimental conditions see the first footnote table 2.

† This represents the inhibition at the end of a 2-hour period. At the end of the initial 30-minute period the inhibition was 67 per cent. The inhibitions not indicated in the preceding columns were constant throughout the 2-hour period.

Another experiment (table 3), carried out with 10 mg of whole air-dried yeast, or with the yeast washed with alkaline phosphate, gave the same results. In these experiments, 3 and 25 µg of cocarboxylase increased the carboxylase activity 12 and 19 per cent, respectively. In the absence of added cocarboxylase, 0.0055 M sulfathiazole exercised a 20 per cent inhibition. In the presence of

0.3 μg of cocarboxylase this inhibition was abolished. This means that one molecule of cocarboxylase was capable of neutralizing the inhibitory effect of 53,400 molecules of sulfathiazole.

TABLE 4

Effect of the presence and absence of glucose in culture medium on the carboxylase activity of Staphylococcus aureus

	CARBOXYLASE ACTIVITY OF WASHED SUSPENSIONS OF STAPHYLOCOCCUS AUREUS GROWN IN			
	Seeded with culture on extract agar		Seeded with culture on glucose agar	
	Extract* broth	Glucose broth	Glucose† broth	Extract broth
Carboxylase Activity at pH	7.2	7.2	7.2	7.2
Period of experiment	4 hr	5 hr	4 hr	5 hr
mm^3 CO_2 evolved	683	0	0	643
QCO_2‡	216	0	0	163

* Extract broth consisted of 10 g of peptone, 5 g of sodium chloride, and 3 g of beef extract (Difco) in 1,000 ml of tap water, boiled, filtered, adjusted to pH 7.4, and sterilized. Extract agar consisted of extract broth containing 2 per cent agar.

† Glucose broth consisted of the same materials as above except that sodium chloride was replaced by 4 g of anhydrous disodium phosphate per liter of medium and adjusted to pH 7.4. To the sterilized medium a concentrated sterile solution of glucose was added just before seeding the medium (final concentration, 0.5% glucose in the medium).

Glucose agar consisted of glucose broth containing 2 per cent agar.

‡ mm^3 CO_2 evolved per hour per mg of staphylococci.

TABLE 5

Effect of pH on the degree of inhibition exercised by sulfathiazole on the carboxylase activity of Staphylococcus aureus

PERIOD	pH 7.16			pH 6.2		
	Control* QCO_2‡	Inhibition by sulfathiazole		Control† QCO_2	Inhibition by sulfathiazole	
		0.00138 M	0.0055 M		0.00138 M	0.0055 M
minutes		*per cent*	*per cent*		*per cent*	*per cent*
30	68	36	60	104	14	31
60	90	35	61	125	16	30
90	101	31	60	134	14	29
120	114	29	58	146	13	29

* The reaction system (pH 7.16) contained 3.1 mg of *S. aureus*.

† The reaction system (pH 6.2) contained 2.1 mg of *S. aureus*.

‡ mm^3 CO_2 evolved per hour per mg of staphylococci.

The bacterial suspensions used in the two sets of experiments were prepared from the same 16-hour broth culture.

Washing 10 mg of yeast with alkaline phosphate removed 98 per cent of the carboxylase activity. The treatment of the washed yeast with 25 μg of cocarboxylase restored 76 per cent of the original activity. In the presence of 0.0055 M sulfathiazole and 0.3 or 3 μg of cocarboxylase, 80 per cent of the restorable

activity was inhibited. On the other hand, in the presence of 25 μg of cocarboxylase the inhibition was reduced from 80 to 56 per cent. This is equal to a 30 per cent reversal of inhibition. In other words, one molecule of cocarboxylase counteracted 646 molecules of sulfathiazole in bringing about this effect.

Experiments on the carboxylase activity of Staphylococcus aureus. During our daily studies, extending over a period of two years, the gradual decrease and eventual loss of carboxylase activity in *Staphylococcus aureus* was observed. It has been previously noticed that certain strains were completely devoid of carboxylase activity (Sevag and Neue-Schwander-Lemmer, 1936). Krebs (1937) also reported similar observations. The causes of these variations are not known. During our studies it has been possible to trace one of the factors responsible for the complete loss of carboxylase activity. The results presented

TABLE 6

Reversal by cocarboxylase of the inhibition by sulfathiazole (0.00138 M) of the carboxylase activity of Staphylococcus aureus

The pH of the reaction system was 6.2; the weight of staphylococci was 3 mg/6 ml reaction volume.

EXP. NO.	PERIOD	CONTROLS				INHIBITION BY SULFATHIAZOLE			
		Buffer	Cocarboxylase μg			Buffer	Cocarboxylase μg		
			5	10	25		5	10	25
	minutes	*mm³CO₂*	*mm³CO₂*	*mm³CO₂*	*mm³CO₂*	*per cent*	*per cent*	*per cent*	*per cent*
1	30	76	122	120	116	30	30	24	16
	60	167	252	250	233	25	21	18	13
	90	253	377	382	351	25	23	20	11
	120	339	489	486	458	22	18	12	10
2	30	217	249	268	222	28	18	23	14
	60	470	556	594	482	26	20	23	11
	90	735	950	900	735	27	30	23	12
	120	978	1148	1170	970	25	22	19	9

in table 4 are related to this phenomenon. It can be seen that staphylococci, when grown in glucose phosphate extract broth, were completely devoid of carboxylase activity. In contrast, when they were grown in plain extract broth, they showed carboxylase activity. This observation, though made several times, cannot, however, at present be offered as proof that growth in glucose always deprives the organism of carboxylase activity. Further studies are in progress.

In consideration of the importance of the optimal carboxylase activity, and of the optimal inhibitory effect of sulfathiazole, experiments were carried out at pH 7.16 and pH 6.2. The results presented in table 5 show that the carboxylase activity of *Staphylococcus aureus* at pH 6.2 is markedly greater than at pH 7.16. It can also be seen that the inhibitory effect of sulfathiazole at pH 6.2 is lower by 50 to 60 per cent than at pH 7.16.

In view of the fact that carboxylase activity is optimal at pH 6.2, and that its

sulfonamide-antagonizing action can best be observed at its optimal pH of activity, the experiments were carried out at this pH.

The results presented in table 6 (experiment 1) show that 5 μg (also 10 and 25 μg) of cocarboxylase increased the carboxylase activity of staphylococci 50 (one-hour period) and 44 (two-hour period) per cent. The results of experiment 2 show a similar effect. In the absence of cocarboxylase, sulfathiazole causes from 18 to 30 per cent inhibition of activity. The addition of 25 μg cocarboxylase to the system causes from 50 to 65 per cent reversal of inhibition. This indicates that one molecule of cocarboxylase is capable of counteracting 215 molecules of sulfathiazole in bringing about this effect.

SUMMARY

Sulfathiazole inhibits the carboxylase activity of whole yeast. It is shown that one molecule of cocarboxylase added to the reaction system is capable of counteracting the inhibitory effect of 8,088 to 53,400 molecules of sulfathiazole.

Washing yeast cells with alkaline phosphate removes practically all of the carboxylase activity. Addition of cocarboxylase restores from 56 to 76 per cent of the original activity. Under these conditions sulfathiazole and cocarboxylase compete for the specific carboxylase protein. This competition results in the neutralization of the inhibition by sulfathiazole. One molecule of cocarboxylase counteracts the inhibition exercised by 322 to 646 molecules of sulfathiazole.

Sulfathiazole inhibits the carboxylase activity of *Staphylococcus aureus*. One molecule of cocarboxylase counteracts the inhibitory effect of 215 molecules of sulfathiazole.

Staphylococci when grown in glucose phosphate extract broth have been found to be devoid of carboxylase activity. Staphylococci when grown in glucose-free plain extract broth have been found to manifest good carboxylase activity. The reason for this is not known.

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