THE RESPIRATION OF STREPTOCOCCUS PYOGENES

II. The Inhibition of Respiration and Growth by Sulfanilamide; the Inhibition of Respiration by Hydroxylamine and Its Sulfonamide and Other Derivatives[1]

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INTRODUCTION

The therapeutic properties and growth-inhibiting effects of sulfonamide drugs on bacteria have been studied extensively and are well known. The type and number of reactions as well as other conditions involved in the processes of bacterial multiplication in animal systems and *in vitro* are so numerous and complex that it is difficult to gain a clear insight as to the mechanism of the action of these drugs. In contrast, a study of the drug effect on relatively simple biological processes, known to be essential for bacterial and other cell multiplication, can be analyzed and understood. The measurement of the oxygen uptake and carbon dioxide evolution in the presence of foodstuffs of high energy content through the intermediation of bacterial enzymes is suited for such a study. Furthermore, as these processes provide the cells with energy requisite for growth, measurable inhibition of these processes by these drugs supplies us with clues regarding certain of the possible modes of their action.

Barron and Jacobs (1937) studied the effect of 0.01 M sulfanilamide on the oxidation of glucose by washed suspensions of hemolytic streptococci, *Escherichia coli*, Friedländer bacilli, and gonococci. They reported 7.9 per cent inhibition with streptococci, none with *E. coli* and gonococci, and 12.1 per cent inhibition with Friedländer bacilli. The maximum error being 5 per cent, a slight inhibiting effect was stated to have been demonstrated. Chu and Hastings (1938) studied the effect of 0.66

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gram per cent sulfanilamide on the oxidation of glucose, (O_2 uptake), by washed suspensions of hemolytic streptococci, gonococci, pneumococci Type I and III, and meningococci. The experiments with streptococci were unsuccessful because the oxygen uptake was negligible under the experimental conditions they used. Gonococcus (24–29 cmm. O_2/hr./mgm.) and meningococcus (13–24 cmm. O_2/hr./mgm.) were poor respirers. Three experiments each with type I and III pneumococci (127–147 cmm. O_2/hr./mgm.) were reported. With type I in one experiment there was no inhibition, in the second and third experiments the inhibitions were 13 and 23 per cent. With type III two experiments showed no inhibition and in the third 27 per cent inhibition was demonstrated.

Whereas the above investigators experimented with washed suspensions of bacteria, we have tried to simulate the respiratory conditions *in vivo* by the addition of yeast extract, with or without serum, or defibrinated whole blood.

Bliss and Long (1939) reported that sulfanilamide and sulfapyridine inhibited the anaerobic growth of streptococcus C203. Broh-Kahn (1939) working with *E. coli*, confirmed their findings. Kalmanson (1940) reported that sulfanilamide in a concentration of 20 mgm. per cent was able to sterilize small inocula of streptococcus, strain C203 in 48 hours aerobically and anaerobically. The inhibiting effect of sulfanilamide on the anaerobic respiration of streptococcus to our knowledge has not been studied previously. In the present study this aspect of the problem has been investigated in parallel with the aerobic respiration.[2]

In parallel with p-aminobenzenesulfonamide the effect of p-hydroxylaminobenzenesulfonamide, p-aminobenzenesulfonhydroxyamide, benzenesulfonhydroxamide, benzhydroxamic acid, and hydroxylamine on the aerobic and anerobic respiration of streptococci was studied. This comparative study was made in the hope that valuable data might be forthcoming as to what particular

[2] Coggeshall (1940) reported that the oxygen consumption of *Plasmodium knowlesi* (100 million parasites) was markedly reduced in a system containing 20 mg. of sulfanilamide. There was no observable effect on the *P. inui* parasites. The anaerobic CO_2 production of both parasites was unaffected by sulfanilamide.

structural characteristics exercise the highest antistreptococcal activity.

Since it has been assumed that some of the drugs undergo changes both in the animal system and *in vitro* in order to manifest their therapeutic properties, the behavior of the above-mentioned drugs *per se* was also studied under conditions employed for the measurement of respiration. Certain of these drugs were found to consume oxygen and certain others to liberate a gas not absorbable by potassium hydroxide. These changes were measured accurately and corrected for in evaluating our findings. The results presented in tables 1 and 2 and the percentage inhibitions cited in the text represent the corrected values.

INHIBITION EXPERIMENTS

1. p-Aminobenzenesulfonamide

The results of the inhibition experiments on the respiration of streptococci with this drug are given in tables 1 and 2. These show that sulfanilamide inhibits the aerobic and anaerobic respiration of streptococci. Mayer (Mayer and Oechsin, 1937; Mayer, 1937 a and b; Mayer, 1939) advanced the hypothesis that this substance is oxidized in the animal body and *in vitro* to p-hydroxylaminobenzenesulfonamide which is responsible for the chemotherapeutic and growth-inhibiting property. In our experiments, sulfanilamide *per se* does not consume oxygen in aerobic systems and does not evolve any gas in anaerobic systems. The possible oxidation of this drug in defibrinated whole blood in the presence and absence of glucose was also investigated. In no case did we observe oxygen uptake by the drug itself. Our experimental findings under these conditions do not support the above assumption as applied to *in vitro* conditions.

Experiments correlating the effect of sulfanilamide on respiration and growth. Whether the inhibition of the aerobic and anaerobic respiration by sulfanilamide and similar drugs has a direct bearing on the growth-inhibiting effect and therapeutic property of these drugs is a natural question. We have tried to obtain the answer to this question experimentally. After a period of three hours of

TABLE 1

Inhibition of the aerobic respiration of Streptococcus pyogenes

Cmm. O_2/hr./mgm. Streptococci, strain C203M

NUMBER	AEROBIC SYSTEMS		H_2N—⬡—SO_2NH_2* *p*-Aminobenzenesulfonamide					HONH—⬡—SO_2NH_2† *p*-Hydroxylaminobenzenesulfonamide			H_2N—⬡—SO_2NHOH* *p*-Aminobenzenesulfonhydroxamide			⬡—SO_2NHOH* Benzenesulfonhydroxamide			⬡—$CONHOH$† Benzhydroxamic acid			H_2N—OH‡ Hydroxylamine		
			None	0.02 M	0.04 M	Per cent inhibition		None	0.02 M	Per cent inhibition	None	0.02 M	Per cent inhibition	None	0.02 M	Per cent inhibition	None	0.02 M	Per cent inhibition	None	0.02 M	Per cent§ inhibition
						0.02 M	0.04 M															
		ml.																				
1	Streptococci	0.4	24	25		0					26	12	54	10	1	90						
	Saline	2.0	10	10		0					10	7	30	26	3	88						
	Glucose 0.1 M	0.4																				
2	Streptococci	0.4	63	59		6																
	Yeast extract	1.0	141	122	85	13	40				63	37	41	63	13	75				51	4	92
	Saline	1.0	163		83		49				132	44	66	132	32	79				81	14	82
	Glucose 0.1 M	0.4	163		87		46															
3	Streptococci	0.4	101	93		8					101	42	58	101	39	61				65	12	81
	Rabbit serum¶	1.0	109	103		5					109	39	64	109	42	61				90	31	65
	Saline	1.0																				
	Glucose	0.4																				
4	Streptococci	0.4	135	122		10					206	113	45†				122	89	27			
	Yeast extract	0.5	270	265	219	2	19	54	0	100	208	92	55	208	85	59	141	94	23	125	24	80
	Rabbit serum¶	0.5	155		109		29	91	0	100	146	96	34	176	8	95	197	136	31	84	13	84
	Saline	4.0	91		55		40	104	0	100	176	96	47	146	50	65	212	164	23	107	36	66
	Glucose 0.1 M	0.4																				

5	Streptococci..... 0.4	133	129	108	3	19	104	0	100	146	97	33†									
	Yeast extract..... 0.5	133		118		11	93	0	100												
	Rabbit serum¶..... 0.5						109	0	100												
	Catalase..... 0.2																				
	Saline..... 3.8																				
	Glucose 0.1 M..... 0.4																				
6	Streptococci..... 0.4	104	95		8																
	Saline..... 4.0	91		58		36	133	8	94	121	76	37†									
	Rabbit whole blood.... 1.0	129		80		38	121	0	100	138	93	32†									
	Glucose 0.1 M..... 0.4	164		135		23															
		134		131		2															

* Solutions containing calculated amount of drugs were used.

† Drugs were introduced into the system in dry form just before the measurements were started.

‡ Solutions containing calculated amounts of salt adjusted to pH 7.0 with glass electrode were used.

§ 0.02 M, 0.004 M, and 0.001 M hydroxylamine exercised respectively 67, 55, and 55 per cent inhibition.

¶ Serum inactivated for 30 minutes at 56 C.

TABLE 2

Inhibition of the anaerobic respiration of Streptococcus pyogenes

Cmm. CO_2/hr./mgm. streptococci, strain C203M

NUMBER	ANAEROBIC SYSTEMS		H_2N—⬡—SO_2NH_2* *p*-Aminobenzenesulfonamide					HONH—⬡—SO_2NH_2† *p*-Hydroxylaminobenzenesulfonamide					H_2N—⬡—SO_2NHOH* *p*-Aminobenzenesulfonhydroxamide			⬡—SO_2NHOH* Benzenesulfonhydroxamide			⬡CONHOH† Benzhydroxamic acid			H_2N—OH‡ Hydroxylamine		
			None	0.02 M	0.04 M	Per cent inhibition 0.02 M	Per cent inhibition 0.04 M	None	0.02 M	0.04 M	Per cent inhibition 0.02 M	Per cent inhibition 0.04 M	None	0.02 M	Per cent inhibition	None	0.02 M	Per cent inhibition	None	0.02 M	Per cent inhibition	None	0.02 M	Per cent§ inhibition
		ml.																						
1	Streptococci	0.4	204		182		17						18	0	100	20	0	100						
	Saline	5.0	51	4		92							20	0	100	18	0	100						
	Glucose 0.2 M	0.2	44	11		40																		
	$NaHCO_3$ 1 M	0.2	66	53		20																		
2	Streptococci	0.4	231	208	164	10	29						280	20	92	314	19	94				194	6	96
	Yeast extract	1.0	220		192		12						338	236	30	309	26	87				270	4	98
	Saline	4.0											386	0	100	386	30	92						
	Glucose 0.2 M	0.2																						
	$NaHCO_3$ 1 M	0.2																						
3	Streptococci	0.4	160		106		33						206	20	90	291	0	100				204	45	77
	Rabbit serum	1.0	408		303		26						291	39	80	302	0	100				270	68	74
	Saline	4.0	353	319		9							302	0	100	206	24	87						
	Glucose 0.2 M	0.2	421	358		15																		
	$NaHCO_3$ 1 M	0.2																						

4	Streptococci.....	0.4	512	452	382	11	25	318	272		14[c]		336	113	66	396	0	100	283	103	63*	159	0	100
	Yeast extract.....	0.5	487	392		20		335	359		0[c]		396	216	45	336	0	100	291	193	34*	267	0	100
	Rabbit serum.....	0.5	351	144		59		286	192	149	33[a]	47[a]	658	513	22				216	127	42	205	48	76
	Saline.....	4.0	396	276		30		301	263		12[a]		193	114	40				243	150	38	330	65	80
	Glucose 0.2 M.....	0.2	227		134		41	310	235		24[a]		167	158	6¶				283	152	46*			
	$NaHCO_3$ 1 M.....	0.2	291		197		32																	
5	Streptococci.....	0.4	165		152		8	192		0		100	319	225	30									
	Rabbit whole blood.	1.0	328		247		24	247		53		79	233	173	26									
	Saline.....	4.0	523	520		0																		
	Glucose 0.2 M.....	0.2	215		145		32																	
	$NaHCO_3$ 1 M.....	0.2																						

* Solutions containing calculated amount of drugs were used.

† Drugs were introduced into the system in dry form just before the measurements were started.

‡ Solutions containing calculated amounts of hydroxylamine hydrochloride adjusted to pH 7.0 were used.

§ 0.02 M, 0.004 M, and 0.001 M hydroxylamine exercised respectively 86, 55, and 48 per cent inhibition.

¶ Same as 2 and the system contained 3 mgm. of streptococci.

[c] The hydroxylaminobenzene melting at 139–140 C., and [a] melting at 160–161 C. were used.

TABLE 3

Inhibition of the respiration of Streptococcus pyogenes (strain C203M) in the absence of growth in comparison to the inhibition of respiration during growth

(133 × 10^{-3} mgm. nitrogen = 2.75 × 10^{9} cocci, strain C203M)

RESPIRATION SYSTEMS		WASHED STREP. INTRODUCED IN TERMS OF MGM. OF NITROGEN (× 10^{-3})	RESPIRATION				GROWTH				PER CENT INHIBITION BY SULFANILAMIDE	
			Aerobic									
			Cmm. O_2 consumed				Increase in terms of mgm. of nitrogen (× 10^{-3})				For 3 hour period	
			Zero hour	1st hour	2nd hour	3rd hour	Zero hour	1st hour	2nd hour	3rd hour	Respiration	Growth
ml.												
Aerobic												
1. 6 mgm. yeast extract..... 1.0	Control	118	(a) 0	88	132	148	0	0	0	0		
Phosphate buffer pH 7.4..... 4.0		118	(b) 0	91	135	154	0	0	0	0		
0.3 M glucose..... 0.4												
	Containing 0.04 M	118	(a) 0	57	75	91	0	0	0	0	39	
	sulfanilamide	118	(b) 0	56	75	90	0	0	0	0	42	
2. 3 mgm. yeast extract..... 0.5	Control	212	(a) 0	153	222	271	0	0	0	0		
Normal horse serum..... 0.5		212	(b) 0	152	208	284	0	0	0	0		
Phosphate buffer..... 4.0												
0.1 M glucose..... 0.4	Containing 0.04 M	212	(a) 0	72	117	160	0	0	0	0	41	
	sulfanilamide	212	(b) 0	80	117	163	0	0	0	2	43	
3. 15 mgm. yeast extract dissolved	Control	118	(a) 0	98	209	266	0			155		
in normal horse serum..... 5.0		109	(b) 0	131	285	453	0	51	94	124		
0.3 M glucose..... 0.4		136	(c) 0	138	325	478	0	87	136	169		
	Containing 0.04 M	118	(a) 0	64	101	127	0			118	52	24
	sulfanilamide	109	(b) 0	73	116	164	0	32	38	38	64	71
		136	(c) 0	79	125	171	0	46	46	61	64	63

			Anaerobic								Per cent inhibition by sulfanilamide	
			Cmm. CO_2 evolved				Increase in terms of mgm. of nitrogen ($\times 10^{-3}$)				For 3 hour period	
											Respiration	Growth
Anaerobic												
1. 6 mgm. yeast extract 1.0	Control	118	(a) 0	133	193	220	0	0	0	0		
Physiological saline solution 4.0		118	(b) 0	182	303	352	0	0	0	0		
0.3 M glucose 0.2												
2.0 M $NaHCO_3$ 0.2	Containing 0.04 M	118	(a) 0	104	132	165	0	0	0	0	25	
	sulfanilamide	118	(b) 0	134	228	276	0	0	0	0	30	
2. 3 mgm. yeast extract 0.5	Control	124	(a) 0	197	391	568	0	9	9	19		
Normal horse serum 0.5		124	(b) 0	178	388	559	0	9	19	19		
Physiological saline solution 4.0												
0.2 M glucose 0.2	Containing 0.04 M	124	(a) 0	152	278	388	0	6	6	9	31	
2.0 M $NaHCO_3$ 0.2	sulfanilamide	124	(b) 0	139	230	388	0	0	0	0	31	*
3. 15 mgm. yeast extract dissolved in	Control	118	(a) 0	360	946	1295	0			160		
normal horse serum 5.0		109	(b) 0	248	719	1292	0	38	83	106		
0.6 M glucose 0.2		100	(c) 0	233	663	1297	0	50	103	106		
2.0 M $NaHCO_3$ 0.4												
	Containing 0.04 M	118	(a) 0	209	598	816	0			90	37	44
	sulfanilamide	109	(b) 0	130	437	758	0	5	52	63	41	40
		100	(c) 0	136	428	813	0	14	53	71	37	43

* Since the increase in growth nitrogen (transcribed from turbidity readings of 3 to 5 units) is so small, this may be taken as being within the experimental error. If these figures actually represent a slight growth, this slight difference is too small to characterize accurately.

active respiration of 2.7×10^9 washed organisms in the presence of small quantities of yeast extract, glucose, and serum, there was no measurable increase in the number of organisms over that of the initial number (table 3). This was determined by making turbidity measurements with a Klett-Summerson photoelectric colorimeter as described below. A possible explanation might be that the food is attacked and energy liberated as shown by the O_2 and CO_2 exchange, but that this energy is not utilized because in an environment with limited amount of nutrient such a large number of organisms could not multiply. It is also possible that the increase in the number of organisms was too small to detect by this method.

Ely (1939) in a similar experiment with *E. coli* studied the oxygen consumption in a synthetic medium. He stated that there was considerable growth in this medium when small inocula were used. However, in suspensions of the concentrations used (3.5×10^9 to 4.5×10^9 organisms per ml.) a rather rapid and uniform respiration occurred with no increase in population as determined by the plate counting method.

In our subsequent experiments the serum, yeast extract, and glucose content of the systems were considerably increased (see table 4). Then aerobic and anaerobic respiration and growth were measured in the presence and absence of sulfanilamide. The growth was estimated by determining the increase of turbidity, after 1, 2, and 3 hours of respiration, by means of the photoelectric colorimeter.

Twelve Warburg vessels were used, one vessel for each determination. The content of each vessel was washed out completely into a standard tube and brought to a definite volume before turbidity readings were taken. The weight of organisms introduced into each system was determined at the start by determining the total nitrogen content and turbidity values of the measured samples. The readings on the apparatus were calibrated by nitrogen values of the suspensions of various concentrations. The range of turbidity readings, from 0 to 240, plotted against the total nitrogen values ranging from 0.053 to 0.953 mgm. nitrogen, was a straight line.

TABLE 4

Correlation of the inhibition of aerobic (cmm. O_2 consumed) and anaerobic (cmm. CO_2 evolved) respiration with the inhibition of the aerobic and anaerobic growth by sulfanilamide

(133 × 10^{-3} mgm. N = 2.75 × 10^9 cocci, strain C203M)

RESPIRATION AND GROWTH SYSTEMS	TIME	MGM. STREPTOCOCCAL N = $\times 10^{-3}$		NUMBER OF COCCI = $\times 10^9$		RESPIRATION DURING GROWTH		PER CENT INHIBITION OF	
		Normal growth	Growth in 0.04 M sulfanilamide	During normal growth	Growth in 0.04 M sulfanilamide	Normal	In 0.04 M sulfanilamide	Respiration	Growth
ml.	*hours*								
Aerobic									
Streptococci..... 0.4	0	(a) 109	109	2.25	2.25	0	0	0	0
Yeast extract 15		(b) 136	136	2.81	2.81	0	0	0	0
mgm. in serum. 5.0		(c) 240	240	4.96	4.96	0	0	0	0
Glucose 0.3 M.... 0.4									
	1	(a) 160	141	3.31	2.92	131	73	44	37
		(b) 223	182	4.61	3.76	138	79	43	47
	2–2½	(a) 203	147	4.20	3.03	285	116	59	59
		(b) 272	182	5.62	3.76	325	125	62	66
		(c) 444	311	9.18	6.43	720	234	68	65
	3	(a) 233	147	4.82	3.03	453	164	64	69
		(b) 305	197	6.31	4.07	478	171	64	64
Anaerobic*									
Streptococci..... 0.4	0	(a) 109	109	2.25	2.25	0	0	0	0
Yeast extract 15		(b) 100	100	2.07	2.07	0	0	0	0
mgm. in serum. 5.0		(c) 133	133	2.75	2.75	0	0	0	0
Glucose 0.6 M.... 0.2									
$NaHCO_3$—2 M... 0.2	1	(a) 147	114	3.03	2.36	248	130	48	87
		(b) 150	114	3.10	2.36	233	136	42	72
	2	(a) 192	161	3.97	3.33	719	437	39	37
		(b) 203	153	4.20	3.16	663	428	35	49
	3–3½	(a) 215	172	4.45	3.56	1292	758	41	41
		(b) 206	171	4.26	3.54	1297	813	37	33
		(c) 331	254	6.88	5.24	1651	957	43	39

* 5% CO_2 + 95% N.

The results of both respiration and growth measurements with the same streptococcal suspensions are given in table 4 and in figures 1 and 2. The data show that the inhibition of respiration

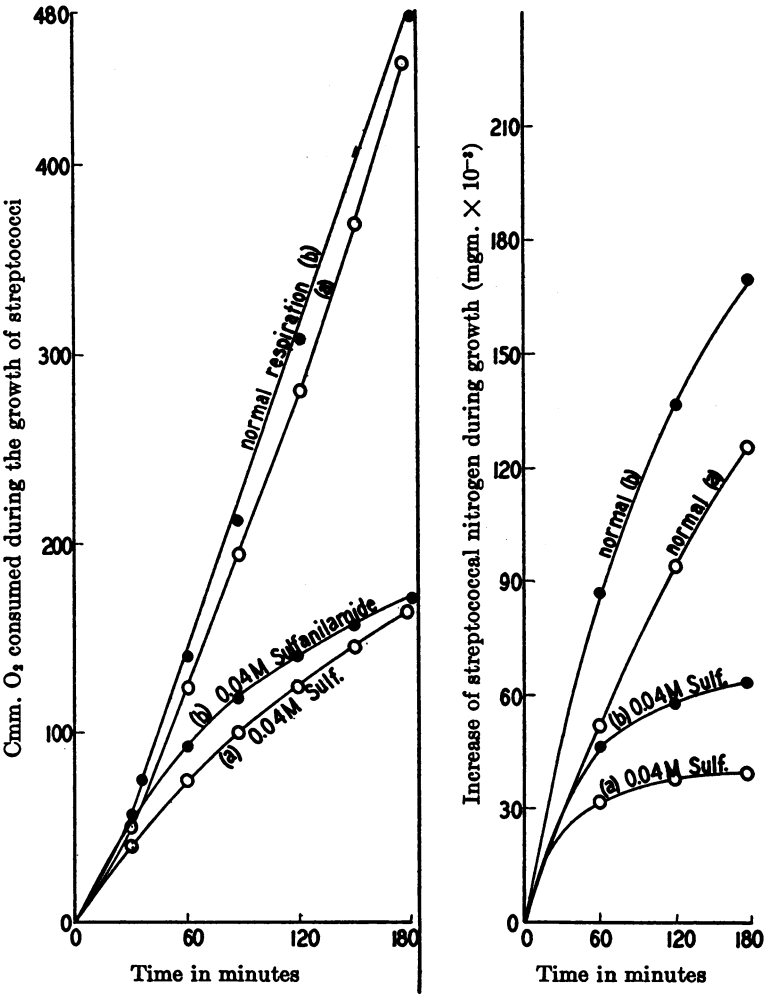

FIG. 1. CORRELATION OF THE INHIBITION OF AEROBIC RESPIRATION AND GROWTH

results in the inhibition of growth aerobically and anaerobically. The degree of the inhibition of respiration is of the same order of magnitude as the inhibition of growth by sulfanilamide.

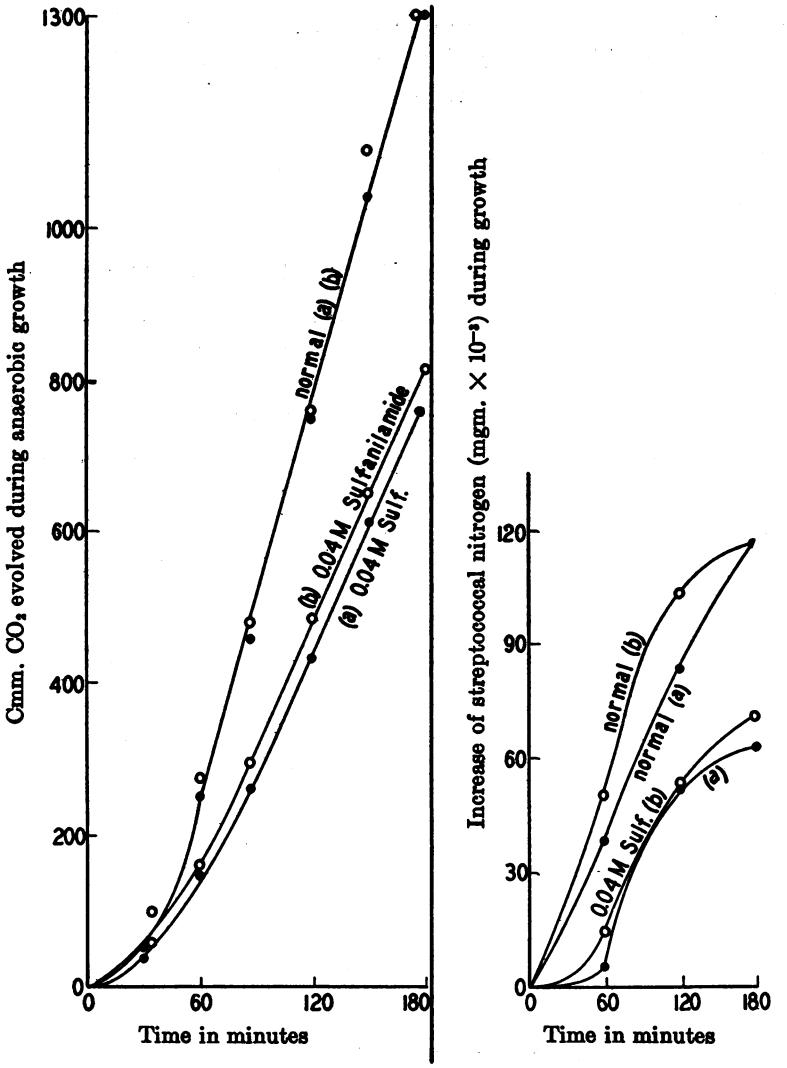

FIG. 2. CORRELATION OF THE INHIBITION OF ANAEROBIC RESPIRATION AND GROWTH

2. p-Hydroxylaminobenzenesulfonamide

Mayer reported that p-hydroxylaminosulfonamide is 100 times more effective *in vitro* than sulfanilamide. Bratton, White, and Marshall (1939) on the other hand reported that in *in vitro* experi-

ments it was 10 times more effective than sulfanilamide. These investigators did not take into consideration the toxic effect of hydrogen peroxide which results from the oxidation of hydrox-

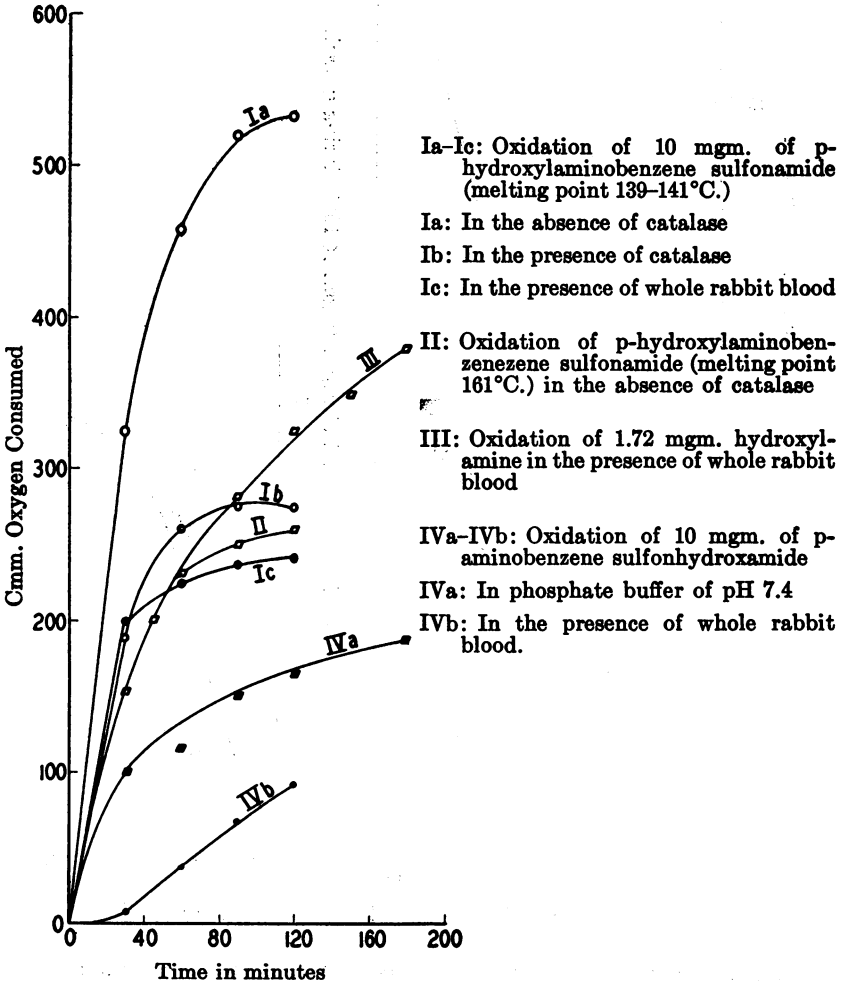

FIG. 3. OXYGEN CONSUMPTION OF THE DRUGS PER SE

ylaminosulfonamide in *in vitro* experiments in the absence of catalase. Furthermore the discrepency between Mayer's findings and those of the latter group appears to be due to the fact that Mayer used a hydroxylaminosulfonamide melting at 161°C. and the others experimented with the one melting at 139.5 to 140.5°C.

Our findings show that the higher melting point substance is more resistant to air oxidation and perhaps for that reason exercises greater inhibition on the respiration than the one with the lower melting point (fig. 3, Ia, II).

p-Hydroxylaminobenzenesulfonamide inhibits completely the aerobic respiration of streptococci in blood, and in serum and yeast extract mixture after elimination of hydrogen peroxide by catalase (table 1). Anaerobic respiration in blood is inhibited 80 to 100 per cent, and in serum and yeast extract 15 to 50 per cent (table 2). The reaction products of oxidation of hydroxylaminobenzenesulfonamide are hydrogen peroxide and probably azoxydibenzenesulfonamide (Bamberger, 1898, 1900). Experiments were carried out to determine the inhibiting effect of the oxidation product on respiration. One ml. of a 1 per cent solution was allowed to oxidize completely in the Warburg apparatus. When the system ceased to use oxygen the resulting hydrogen peroxide was eliminated by catalase and the effect of the reaction mixture was tested on respiration. The oxidation product free of hydrogen peroxide, was only 25 per cent effective in aerobic respiration in contrast to 100 per cent inhibition obtained with the dry drug introduced just before the measurements started. The oxidation product was without inhibiting effect on the anaerobic respiration of streptococci.

Properties of the drug. Part of the p-hydroxylaminobenzenesulfonamide used in our experiments was kindly supplied by Dr. R. O. Roblin, Jr. of the American Cyanamide Company. It was crystalline and melted at 139.5 to 140.5°C. Our preparation (preparation C) prepared according to the method of Bratton, White and Marshall (1939) was also crystalline and melted at 139 to 140°C. A second crystalline substance prepared by a modified method melted at 160 to 161°C. (preparation A), which corresponds to that reported by Mayer. Both of these were prepared from the same p-nitrobenzenesulfonamide. In what other respects these two substances differ will be discussed in another communication. It will suffice to state at present that they appear to be isomers.[3]

[3] While this paper was in press, Burton (1941) reported (June 14, 1941) the preparation of two p-hydroxylaminobenzenesulfonamides, one melting at 139–140°, the other at 160–161°. Our preparations seem to be similar. However,

Both preparations are quite stable in anaerobic systems. They oxidize readily in aerobic systems. In studies to determine their effect it is *essential* that the drug be kept dry and introduced from the side arm into the respiration system just before the measurements are started.

It has been shown that the solutions of hydroxylaminobenzenes oxidize in the following manner (Bamberger, 1898, 1900):

$$C_6H_5NHOH + O_2 \longrightarrow C_6H_5N{=}O + H_2O_2$$

$$C_6H_5N{=}O + C_6H_5NHOH \longrightarrow C_6H_5{-}N(O){-}N{-}C_6H_5 + H_2O$$

The quantity of hydrogen peroxide found in the reaction mixture satisfied the amount required theoretically. The oxidation product of the above substance is orange yellow. That these substances undergo the same type of oxidation as found by Bamberger for hydroxylaminobenzenes is evident from the following considerations. Ten mgm. of the substance (melting point 139.5 to 140.5°C.) should theoretically require 590 cmm. oxygen for complete oxidation. Manometric measurements showed that in two hours time 10 mgm. substance consumed 535 to 574 cmm. oxygen (fig. 3), which corresponds to 91.7 to 97.2 per cent of the theoretical figures. Of this, 60 to 82 per cent was recovered as hydrogen peroxide. Evidently part of the hydrogen peroxide is used up in some manner. The oxidation products are orange yellow (Bamberger, Bratten, White and Marshall).

In the presence of catalase or blood the volume of oxygen measured during the oxidation of the above p-hydroxylaminobenzenesulfonamide is about one half of the volume measured in their absence (fig. 3, Ia, Ib, Ic). This is due to the following reaction:

$$2H_2O_2 + \text{catalase} \rightarrow 2H_2O + O_2$$

The oxygen liberated returns to the system.

All of our experiments with p-hydroxylaminobenzenesulfonamide showed that about 60 per cent of it is oxidized during the first 30 minutes (fig. 3). For this reason the method proposed by Rosenthal and Bauer (1939) for determining the amount of this substance in the presence of aromatic amines after 30 minutes pretreatment with acetic anhydride cannot be employed. The ease with which this substance is oxidized

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the method we used differs from that of Burton. He stated that these are dimorphic forms of the same substance. Our results, as described above, show that these two preparations are chemically and biologically different.

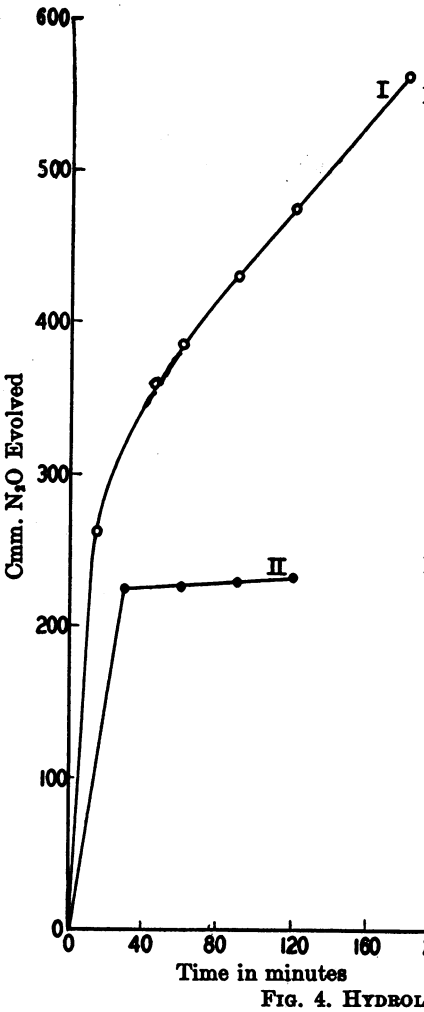
would make the demonstration of its formation in biological systems likewise difficult.

3. p-Aminobenzenesulfonhydroxamide, 4. Benzenesulfonhydroxamide, 5. Benzhydroxamic acid

These substances and their acyl-derivatives have been shown by Moore, Miller and Miller (1940), and Hampil, Webster and Moore (1941) to possess antistreptococcal activity. The studies on the effect of these substances on the aerobic and anaerobic respiration of streptococci (tables 1 and 2) show that they exercise greater inhibition than sulfanilamide.

Properties of the drugs. Of the above three substances the first is oxidizable in air (fig. 3), the other two have been found to be resistant under identical conditions. It was desirable, therefore, that the first substance be introduced in dry form from the side arm into the respiratory system just before the measurements were started. The oxidation product is lemon yellow. According to Piloty (1896) benzenesulfonhydroxamide is oxidized by ferric chloride, hypochlorite, or iodine giving a lemon yellow substance. He proposed the formula of $(C_6H_5SO_2)_2NOH$ resulting from two molecules of $C_6H_5SO_2NHOH$ and involving the use of one atom of oxygen. In our experiments 10 mgm. of p-aminobenzenesulfonhydroxamide used 190 cmm. oxygen during three and one half hours time. Theoretically it should require 300 cmm. oxygen. Assuming that the reaction under our conditions follows the course proposed by Piloty we can account for 60 per cent of the oxygen theoretically required. Hydrogen peroxide is not formed during the oxidation of this substance. In anaerobic systems in the presence of $NaHCO_3$ all the above three substances evolved a gas not absorbable by KOH (fig. 4). Piloty found that benzenesulfonhydroxamide decomposes in KOH solution yielding $2C_6H_5SO_2 + H_2N_2O_2$. Under the conditions of our experiment p-aminobenzenesulfonhydroxamide yields a gas. This gas, possibly resulting from the decomposition of $H_2N_2O_2$, may be assumed to be N_2O from the fact that 10 mgm. $H_2N—C_6H_4SO_2NHOH$ should theoretically yield 593 cmm. N_2O. We measured the evolution of 575 cmm. gas corresponding to 98 per cent of the amount required by theory. Since the solution of $H_2N—OH$ is stable and does not evolve any gas under identical conditions, it appears that one of the hydrolytic products of the above substance is not

H_2N—OH, and that N_2O is not derived from H_2N—OH as an intermediary product.



I. Hydrolysis of 10 mgm. of p-aminobenzene sulfonhydroxamide anaerobically in the presence of *M*/15 $NaHCO_3$

II. Hydrolysis of 10 mgm. of benzhydroxamic acid anaerobically in the presence of *M*/15 $NaHCO_3$

FIG. 4. HYDROLYSIS OF DRUGS

The inhibiting effect of the oxidation products of p-aminobenzenesulfonhydroxamide and the non-gaseous hydrolytic products of the above three substances was tested on respiration. The first was oxidized aerobically for two hours in a Warburg set-up and the

reaction mixture was then tested on aerobic respiration. It showed slight decrease in the inhibiting effect. Another sample was completely hydrolyzed anaerobically in the presence of $NaHCO_3$ and then tested on anaerobic respiration. The inhibiting effect was completely lost.

6. Hydroxylamine hydrochloride

It has been shown that neutral solutions of hydroxylamine exercise powerful inhibiting effect on catalase (Jacobson, 1892; Blaschko, 1935). Smythe (1939) stated that hydroxylamine strongly inhibited yeast fermentation. Frank and Gaffron stated that hydroxylamine inhibited the photosynthetic reactions by plants and bacteria (Frank and Goffron, 1941).

We have found that H_2NOH in dilutions up to 0.001 M inhibits the respiration of streptococci to a high degree (see tables 1 and 2, footnote §).

In our control experiments a neutral solution of the substance is resistent to air oxidation. It is also stable in the presence of $NaHCO_3$ under anaerobic conditions. In contrast to the resistance of H_2NOH to oxidation under conditions cited above, it oxidizes in whole blood as will be seen in figure 3, curve III.

DISCUSSION

The inhibitory action of sulfanilamide drugs on bacterial growth has been the subject of numerous studies. As the processes involved during growth are numerous and complex, no clear insight as to the possible mechanism of the action of these drugs has been gained, as yet. The inhibition of growth by any agent can be assumed to have resulted from the inhibition of one or more of the enzyme systems—respiratory, proteolytic, amylolytic, lipolytic, etc.,—of growing bacteria. A few studies dealing with this subject have shown that these drugs exercise a slight inhibiting effect on the oxygen uptake of the washed suspensions of certain bacteria in the presence of glucose. The experiments devoted to these studies were limited in number and scope. The experimental conditions were not comparable to the conditions existing during growth *in vitro* or *in vivo*. Furthermore, the slight inhibition of

oxygen uptake has not been correlated with the simultaneous inhibition of growth. For such reasons, perhaps, these findings have not generally received the consideration due them.

The metabolic activity of bacteria in the "resting state" results in oxygen consumption aerobically and the evolution of carbon dioxide anaerobically, in the presence of a substrate such as glucose. During such activity, though the washed cells do not multiply, the degree of their activity indicates potentiality for multiplication when favorable conditions for growth are offered. The respiration of a non-proliferating bacterial suspension can be inhibited by various agents, which likewise may prove to be growth-inhibiting. On the other hand it is a well established fact that the bacteria killed by various bacteriocidal agents do not respire, indicating that there is a direct relation between the effect on the respiration and multiplication, and *vice versa*.

During the respiration of the "resting state," (and also most probably when multiplication takes place), there is present endogenous activity, involving the oxygen uptake and the evolution of carbon dioxide. The type, age, and the condition under which respiration (and growth) takes place are factors in determining the degree of endogenous activity. In general it is due to the processes of disintegration or oxidative autolysis, for which reason the greater the endogenous activity the smaller the activity in metabolizing a given substrate (Wieland and Sevag, 1933; Sevag, 1933). Killed organisms may consume a small amount of oxygen, or anaerobically reduce methylene blue, or similar dyes, for the reason that reducing substances are liberated from the cells undergoing autolysis. Such substances are also to be found in cell-free culture fluid depending on the extent of the autolysis of the cells grown in it (Avery and Neill, 1924 a, b, c, and d). This, as well as the oxygen consumed during endogenous activity, however, is in no way related to the respiration of the "resting state" of the cells which alone can serve as a potential source of energy utilizable for multiplication.

The present study has concerned itself mainly with the mechanism of sulfanilamide action on the respiration of streptococci, and the various points discussed above have been taken into considera-

tion in evaluating the results obtained. The measurements were carried out under conditions most favorable for active respiration. The systems contained glucose, yeast extract, with or without serum, or defibrinated whole blood. Under these conditions sulfanilamide, p-hydroxylaminobenzenesulfonamide, p-aminobenzenesulfonhydroxamide, etc., and hydroxylamine hydrochloride exercised strong inhibiting effect on both the aerobic and anaerobic respiration. The inhibition in the presence of a constant amount of sulfanilamide was found to be inversely related to the number of organisms present in the system. The inhibiting effects of 0.04 M sulfanilamide on the aerobic respiration of 4.1, 2.05, and 1.02 mgm. of streptococci were, respectively, 22, 31, and 48 per cent, and anaerobically 11, 15, and 25 per cent. Similarly, varying amounts of sulfanilamide vary in their inhibiting effect on the respiration of a given number of organisms (table 1 and 2). These results are in agreement with the findings of numerous other investigators that the effect on growth is directly related to the concentration of the drug.

Even when minimal amounts of glucose and yeast extract, with or without serum, were present in the respiration system the washed cells respired actively; however, an increase in the number of organisms under these conditions could not be demonstrated. The nutritional conditions were either too limited to allow growth, or the increase in number was too small to be detected. Nevertheless under these conditions all the drugs mentioned above exercise strong inhibition (table 3). When the optimal amounts of glucose, yeast extract and serum were added to the respiratory systems the respiration was active and also the increase in the number of organisms could readily be measured (table 4 and figures 1 and 2). The increase in the number of streptococci after 1, 2, and 3 hour periods of aerobic growth was, respectively, 50, 100, 120 per cent; anaerobically the growth was slightly less, but the general trend of growth was of similar nature. The inhibition of the aerobic respiration was, respectively 43.5, 60 and 64 per cent, the inhibition of growth during the corresponding periods was also, respectively, 42, 62, and 67 per cent. Under anaerobic conditions the inhibition of respiration at the end of the

third hour was 40 per cent, and inhibition of growth 37 per cent. These results show that the inhibition of both the aerobic and anaerobic respiration results in proportional inhibition of growth.

During the three hour period of growth about 1.0×10^7 organisms consumed 1 cmm. oxygen which corresponds to 2.5×10^9 molecules of oxygen consumed per coccus. Similarly a single coccus is responsible for the evolution of 7.3×10^9 molecules of carbon dioxide under anaerobic conditions. Since these values are to be interpreted as a measure of energy relationship of the various reactions involved during growth, the effect of sulfanilamide has been to reduce the production of energy utilizable for growth aerobically by 63 per cent, and anaerobically by 40 per cent.

It has been generally accepted that there is a period of 2 to 6 hours before *in vitro* effect of a drug on growth becomes apparent (Long and Bliss, 1939; Lockwood, 1938; Osgood, 1938; Spring, et al., 1940). In all of the studies referred to a small initial inoculum has been used to demonstrate the bacteriostatic or bactericidal properties of chemotherapeutic agents *in vitro*.

In contrast to the above studies Libby (1940) reported that using large inocula (12 to 50 million organisms), in every instance where a drug has shown activity at the four-hour incubation period it has been possible to demonstrate activity at the two-hour incubation period. In all our experiments the initial inoculum has been over a billion organisms. In every instance the drug has had an inhibiting effect on respiration as early as the first quarter or one-half hour period. The measurement of growth taken after the first hour of respiration showed that the inhibition of growth by sulfanilamide was in proportion to the inhibition of respiration. From our findings it would appear that the effect of the drug on growth is exercised as soon as the effect on respiration becomes effective.

For the demonstration of an appreciable inhibition of respiration by sulfanilamide the *volume* of O_2 consumed and CO_2 evolved by streptococci, or pneumococci (see Article III), was an important factor to be considered. In systems respiring weakly the inhibition was in general relatively small, and therefore of uncer-

tain nature. For a system to respire actively, it required 2 to 5 billions of young cocci per test, and therefore a proportionate amount (0.02 to 0.04 M), of sulfanilamide. The use of this and of higher concentrations of enzyme inhibitors is in accordance with general practice in studies on enzymes (Elvehjem and Wilson, *et al.* (1939)).

That the inhibiting effect of 0.04 M sulfanilamide on the respiration and growth of streptococci was not dependent on the alteration of the colloidal properties of the cells, or on a possible osmotic concentration effect was evident from the fact that: (a) the degree of inhibition by a given concentration of sulfanilamide was in inverse ratio to the number of cocci, and *vice versa*; and, also, (b) both the inhibition of respiration and growth were reversible. This reversibility of the inhibition appeared to be responsible, as discussed in Article III, for the anti-sulfanilamide action of p-aminobenzoic acid and possibly of other substances.

SUMMARY

Our findings show that the aerobic and anaerobic respiration of *Streptococcus pyogenes* is inhibited by sulfanilamide, hydroxylamine, and its sulfonamide derivatives.

The inhibition of respiration takes place even in the absence of growth. In the presence of growth the inhibition of respiration results in the proportional inhibition of growth.

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