THE REDUCTION OF FORMALDEHYDE BY BACTERIAL CELLS

By PAUL H. KOPPER

With the Technical Assistance of Leon Robin

(From the Department of Microbiology and Public Health, The Chicago Medical School, Chicago)

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In the course of an investigation of the metabolic activities of an atypical, creatinine-decomposing strain of *Pseudomonas aeruginosa*, it was shown by Kopper and Robin (1950) that this organism decomposed sarcosine by oxidative demethylation to formaldehyde and glycine. The presence of formaldehyde, however, could be detected only when autolyzed bacteria were allowed to act on the substrate. Resting bacterial cells produced glycine, but no formaldehyde from sarcosine. On the basis of further observations in connection with a study of the enzyme responsible for the breakdown of sarcosine it was suggested by Kopper (1950) that living cells might be capable of disposing of formaldehyde through reduction, a process made possible by the reducing potential of the cell. The purpose of this report is to present experimental evidence in support of this hypothesis.

EXPERIMENTAL

1. Preparation of the Bacterial Cell Suspension

The organisms were grown on meat extract agar at room temperature for 40 hours. They were then washed off with distilled water and centrifuged. Following one more washing and centrifugation, the bacteria were suspended in distilled water. An aliquot of this suspension was adjusted turbidimetrically to give a reading of 53 on the scale of the Leitz photoelectric colorimeter (C filter). By means of nutrient agar plate surface counts, this optical density was found to correspond to approximately 3×10^9 viable cells per ml. Unless indicated otherwise, 3×10^{10} cells in 0.1 ml. of distilled water were used throughout the course of the experiments.

2. Materials and Methods

Commercial formalin (40 per cent formaldehyde, U.S.P.) was diluted to the desired concentration with distilled water. The quantitative determination of HCHO was carried out with chromotropic acid reagent, as previously described (Kopper, 1950).

Two methods were employed for measuring small amounts of methyl alcohol. Reaction mixtures from which all HCHO had disappeared were treated with $KMnO_4$ in accordance with the procedure outlined by Boos (1948). The chromotropic acid

test was then performed on the oxidized material, and the resulting color intensities were compared with standards obtained with known amounts of CH_3OH identically treated. This test is specific for CH_3OH, as it is based on the color reaction of its oxidation product, HCHO, with chromotropic acid. The second method used for the determination of CH_3OH was described by Harger (1934). It involves distillation of the alcohol, its oxidation, and titration of the residual oxidant. This method proved suitable for measuring the amount of CH_3OH evolved from only partial reduction of HCHO, as the latter could be prevented from distilling over by the addition to the reaction mixture of chromotropic acid reagent. A blank was run with the reagent and a correction made for the reducing substances given off by it in the process of distillation.

Bacterial carbohydrate was determined as glucose by the method of Folin and Wu (1920) following hydrolysis of the organisms for 2 hours in 2 N HCl at 100°C.

Carbon dioxide was measured volumetrically in a Van Slyke carbon dioxide apparatus.

TABLE I

Reduction by Atypical P. aeruginosa of Formaldehyde to Methyl Alcohol as a Function of Time and Temperature

Temperature	Time of exposure	HCHO taken up	CH_3OH recovered	Recovery
°C.	*min.*	*mg.*	*mg.*	*per cent*
37	15	5.0	2.0 to 3.0	37.4 to 56.1
	30	5.0	1.3 to 3.5	24.3 to 65.4
	60	5.0	1.3 to 3.8	24.3 to 70.6
3	120	3.8	3.7	90.9
	180	5.0	5.2	97.2

Experiments were conducted in the following manner: Bacteria and formaldehyde were mixed in test tubes, and the volume was adjusted to 2 ml. with distilled water. The tubes were placed in a 37°C. water bath and shaken at 275 to 285 oscillations per minute for 30 minutes. They were then quickly removed and centrifuged; the supernatants were decanted for further analysis. Under these experimental conditions 400 to 480 μg. of HCHO were taken up by 3×10^{10} cells of the atypical strain of *P. aeruginosa*. This will be referred to as the reducing capacity of this organism.

RESULTS

For the determination of reaction products 6×10^{11} *Pseudomonas* cells were shaken with 5 mg. of HCHO at 37° and 3°C. The rate of uptake of HCHO and its conversion into CH_3OH are illustrated by the data shown in Table I.

It is difficult to explain why at 37°C. HCHO was recovered as CH_3OH in such variable amounts as indicated in Table I. No measurable quantity of CH_3OH is driven off in the experimental procedure used. Since the bacteria were able to reduce HCHO to CH_3OH but proved to be completely inactive against CH_3OH in the absence of HCHO, one might assume a synthesis of

CH_3OH— and residual HCHO— molecules into higher carbon compounds, which could subsequently be broken down to satisfy the energy requirements of the organism. Additional evidence for this assumption may be found in the results of experiments carried out under anaerobic conditions. The reaction process was then slowed down by about 50 per cent and was arrested at the CH_3OH— stage as it was at 3°C. Yet the avidity of the system for a hydrogen acceptor was shown to be considerable when methylene blue in a final concentration of 1:20,000 was added to a mixture of bacteria and HCHO in evacuated Thunberg tubes. Decolorization of the dye was complete in 5 minutes or less. However, the amount of oxidized compound, whatever its nature, was not large enough to cause a detectable deficit in the quantity of CH_3OH recovered from HCHO.

Under aerobic conditions, when the recovered CH_3OH failed to account for as much as 75 per cent of the HCHO that had been taken up by the bacteria, considerable amounts of additional reaction products would be expected to appear, and a search for these was undertaken. The results were largely negative. Unlike the strain of *Achromobacter*, which was investigated by Paretsky and Werkman (1950), atypical *P. aeruginosa* does not synthesize reserve food of a carbohydrate nature from HCHO. Hydrolysis of bacteria after an uptake of 5 mg. of HCHO and of bacterial controls yielded practically identical amounts of reducing sugar. Bial's orcinol test, which gave positive results with the fermentation liquor of *Achromobacter* organisms, was negative when performed on the supernatants of the reaction mixtures in which atypical *P. aeruginosa* had acted on HCHO.

No carbon dioxide could be detected in the supernatants; however, they were found to be slightly acid as compared with those of the bacterial controls. Following the uptake of 5 mg. of HCHO the titratable acidity was equivalent to 0.3 to 0.5 ml. of N/100 HCl. The significance of the appearance of such small amounts of acid is difficult to assess, especially since some acid was also produced at 3°C., where HCHO was recovered almost completely as CH_3OH.

The data presented in Tables II and III show the relationship of substrate and bacterial concentration respectively to the uptake of HCHO by the bacterial cells. The uptake is independent of the concentration of the substrate up to a certain point. Beyond this point the toxicity of HCHO would seem to interfere with the reducing activity of the cell. The same conclusion is to be drawn from an examination of the values for HCHO uptake obtained with varying bacterial concentrations. The quantities of HCHO disposed of by higher bacterial concentrations are several times larger than the difference in the number of bacteria would indicate.

Attempts were made to relate the reducing capacity of the cells to various internal and external factors and to change some of these factors in order to gain an understanding of the underlying process.

Table IV shows the effect of aging on the uptake of HCHO by the bacteria. There is a considerable drop in reducing capacity between the 2nd and 3rd day of incubation at room temperature. This may be related to a slowing down of

TABLE II

Relationship of Substrate Concentration to the Uptake of Formaldehyde by Atypical P. aeruginosa

Substrate concentration	HCHO taken up
μg.	μg.
400	400
600	398
1000	395
1400	248
1800	168

TABLE III

Relationship of Bacterial Concentration to the Uptake of Formaldehyde by Atypical P. aeruginosa*

No. of bacteria	HCHO taken up
	μg.
0.3×10^{10}	2
0.6×10^{10}	35
1.2×10^{10}	160
1.8×10^{10}	273
2.4×10^{10}	388

* Substrate concentration—400 μg.

TABLE IV

Effect of Aging on the Uptake of Formaldehyde by Atypical P. aeruginosa

Age of culture	Substrate concentration	HCHO taken up
days	μg.	μg.
3	400	322
7	400	307
14	200	140
21	100	88
28	100	0

synthesizing processes, as would be expected to occur in the old, but still viable cells of the stationary phase of the bacterial growth curve. Bacterial counts remained fairly constant during the 1st week. Not until the end of the 2nd week had they decreased by about 50 per cent; at the end of the 4th week only 1 per cent of the original number of bacteria proved viable.

The reducing capacity of the organism was found to remain unaffected over

a wide pH range, from pH 3 to 11. In order to gain information about the effect of acids and bases on cellular function, 3×10^{10} cells were incubated at 37°C. for 30 minutes with varying concentrations of HCl and NaOH. After adjustment of the pH to neutrality the cells were tested for HCHO uptake in the usual way. The results are presented in Table V.

N/100 HCl destroys all bacterial life and with it the reducing capacity of the organisms; N/100 NaOH induces a 40 per cent decrease in HCHO uptake, apparently without affecting the viability of the cells exposed, which might lead one to speculate that the damage caused to the cell can be repaired in a nutritive medium such as nutrient agar, in which plate counts were made, or is of such a nature as not to affect cellular reproduction.

Raising the temperature of incubation up to 48°C. exerted no ostensible effect on the reducing capacity of the bacteria, but merely accelerated the

TABLE V

Effect of Acids and Bases on the Uptake of Formaldehyde by Atypical P. aeruginosa*

Acid or base added	Concentration	HCHO taken up
		μg.
HCl	N/100	0
	N/1000	400
	N/10,000	400
NaOH	N/100	239
	N/1000	400

* Substrate concentration—400 μg.

reaction. The uptake of 400 μg. of HCHO by *Pseudomonas* cells required 50 to 60 minutes at 20°C., 15 to 20 minutes at 37°C., and less than 10 minutes at 45°C. The effect of heat on reducing capacity and viability was demonstrated by exposure of bacterial suspensions to various temperatures for 10 minutes, and subsequent determination of their ability to take up HCHO and to multiply in a nutrient agar medium. The results are reported in Table VI.

The data presented in Table VI would seem to indicate that certain synthetic enzyme systems involved in bacterial reproduction are more severely damaged by higher temperatures than that structural barrier, whatever its nature, which prevents the reducing substances of the cell from becoming freely accessible to the oxygen in its environment, thereby maintaining that difference in the level of redox potentials between the inside and outside of the cell upon which many cellular operations appear to be dependent.

In another series of experiments it was attempted to determine the effect of a number of nutrient substances on the uptake of HCHO by the organism. The compounds were added in amounts of 0.01 millimole; casitone, a pan-

creatic digest of casein, in a concentration of 0.05 per cent. The bacteria were incubated with these substances for 30 minutes at 37°C. prior to the addition of HCHO and performance of the tests in the usual way. The results are presented in Table VII.

In the evaluation of the results a knowledge of the utilization by the organism of the various compounds listed in Table VII seems to be relevant. Sucrose

TABLE VI

Effect of Preheating on the Uptake of Formaldehyde by Atypical P. aeruginosa*

Temperature of preheating	No. of viable bacteria	HCHO taken up
°C.		μg.
—	3.0×10^{10}	400
44	3.0×10^{10}	379
46	4.6×10^{9}	292
48	2.0×10^{8}	232
50	3.5×10^{7}	129
52	5.2×10^{5}	26

* Substrate concentration—400 μg.

TABLE VII

Effect of Preincubation with Nutrients on the Uptake of Formaldehyde by Atypical P. aeruginosa*

Nutrient substance added	HCHO taken up
	μg.
None	460
Sucrose	460
Sodium citrate	463
Glycine	463
dl-Valine	485
l-Histidine	526
l-Proline	576
Casitone	538

* Substrate concentration—600 μg.

is not utilized at all; sodium citrate is readily oxidized, 3 times as rapidly, for example, as proline, as determined by methylene blue reduction times in evacuated Thunberg tubes; glycine is broken down to methylamine and CO_2 (Kopper, 1949); valine is a poor source of both carbon and nitrogen; histidine, proline, and casitone each support adequately the growth of the organism.

In order to have a stimulating effect on the uptake of HCHO, the nutrients must be added to the bacterial suspensions some time prior to the setting up of the tests with HCHO. No increase in reducing capacity was observed upon simultaneous addition of nutrient and HCHO to the organisms. Reducing

capacity appears thus to have a definite relationship to processes preparatory to cell multiplication, no actual increase in the number of cells taking place during the short period of incubation with the nutrients.

Data on the uptake of HCHO by a number of other microorganisms are presented in Table VIII. Suspensions of the bacteria in distilled water were adjusted to the same optical density as that of atypical *P. aeruginosa* suspensions, and it was assumed that they would approximate the latter in the number of cells per milliliter. No viable counts were made. In all experiments the number of cells of the respective organism which were allowed to act on the

TABLE VIII

Uptake of Formaldehyde by Different Microorganisms

Name of organism	Substrate concentration	HCHO taken up	CH_3OH recovered
	μg.	μg.	μg.
Pseudomonas aeruginosa	200	200	70
	400	254	
Pseudomonas aeruginosa (ATC)	200	200	75
	400	270	
Pseudomonas fluorescens	200	200	65
	400	314	
Pseudomonas fluorescens (ATC)	200	200	70
	300	271	
Escherichia coli	400	117	37
Proteus vulgaris	100	20	
Salmonella typhosa	100	34	
Shigella paradysenteriae	100	10	
Chromobacterium violaceum	100	33	
Corynebacterium xerose	200	44	41
Streptococcus haemolyticus	100	16	
Staphylococcus aureus	100	30	12
Clostridium perfringens	100	0	

substrate corresponded to 3×10^{10} cells of atypical *P. aeruginosa*. Because of the experimental difficulties involved in measuring accurately very small amounts of CH_3OH by either of the methods described earlier, this determination was omitted in some instances.

The differences in HCHO uptake can be correlated with other differentiating features of the bacteria listed in Table VIII. *Pseudomonas* is a predominantly aerobic organism, is able to utilize a large variety of oxidizable carbon compounds for growth, and also appears to have the highest reducing capacity. At the other end of the scale is found *C. perfringens*, which is an obligatory anaerobe; it is very fastidious in its nutritional requirements and takes up no HCHO. The remainder of the organisms of Table VIII occupy an intermediate position in gaseous and nutritional requirements and in HCHO-reducing capacity.

Aldehyde oxidases or mutases were excluded from consideration because of the dependence of HCHO uptake on cell viability. Cells autolyzed with toluene were unable to act on HCHO, while the enzymes attacking aldehydes have been described as being unaffected by this reagent.

The mode of action of some antibiotics has been linked to interference with the activity of sulfhydryl groups. It seemed, therefore, pertinent to attempt to relate antibiotic activity to the reducing capacity of the bacteria. Only aureomycin and chloromycetin, acting on both Gram-positive and Gram-negative bacteria, were tested for their inhibitory activity against the organisms of Table VIII. It was found that the several strains of *Pseudomonas* were least sensitive to both antibiotics, 30 to 60 μg. of aureomycin and 125 to 500 μg. of chloromycetin per ml., respectively, being required to inhibit the growth in nutrient broth of 0.1 ml. of a 1:10 dilution of a 24 hour culture of the organisms. Other bacteria exhibited a more scattered type of sensitivity to these compounds. For example, the minimal inhibitory doses of aureomycin for *E. coli*, *P. vulgaris*, and *C. perfringens* were found to be 10, 30, and 0.01 μg. respectively, whereas 2.5 μg. of chloromycetin was the smallest amount required to arrest the growth of each of the three organisms in broth culture. The relative lack of toxicity of the two antibiotics for *P. aeruginosa* and *P. fluorescens* may thus be due not to sulfhydryl groups, but to protective mechanisms of an entirely different character which would be present in these bacterial species.

DISCUSSION

The significance of the concept of cellular reducing capacity presented here and illustrated by the uptake of HCHO by various microorganisms is difficult to assess at this time. That cells exhibit reducing activity has long been known. The reduction of nitrate to nitrite, for instance, is a differential characteristic of bacteria, widely used in their classification. *P. aeruginosa* and *P. fluorescens* are especially active in reducing nitrate beyond nitrite to nitrogen and ammonia. The quantitative determination of cellular reducing capacity appears not to have been attempted thus far. Formaldehyde was selected as a substrate in the present investigation because of the incidental circumstances which led to its initiation. It would not be suitable for the study of the reducing capacity of living systems in which aldehyde oxidases or mutases are encountered.

Porter (1946) points out that redox potentials of cell suspensions are probably largely due to cellular products, and do not give information about the potential of the cell interior. According to his data, for example, suspensions of *Clostridia* have a potential of −0.3 to −0.41 volt; *E. coli*, −0.4 volt; *Saccharomyces cerevisiae* +0.22 volt. On the other hand, the HCHO-reducing capacity of *C. perfringens* was found to be 0, of *E. coli* and *S. cerevisiae* about

the same, 100 μg. This would seem to preclude any relationship between the two types of determinations.

The concept of reducing capacity may also prove of importance in the study of plant and animal cells. Welch and Sakami (1950), using the radioactive tracer technique in their investigations of animal metabolism, obtained proof of the transformation of formate into the CH_3— group of methionine, and du Vigneaud, Ressler, and Rachele (1950) report the appearance of C^{14} of HCHO and formic acid in the CH_3— groups of choline. The emergence of these and other metabolic products may eventually be shown to be related to the reducing activity of living cells.

The activity of intracellular enzymes is known to be considerably depressed in cell-free preparations. Even greater difficulties present themselves in researches on synthesizing enzymes. The latter may be especially sensitive to changes in the redox potential of their environment, but if this were known, it might be possible to investigate their mode of action apart from the functional complexities of the living cell. For such various reasons it would seem desirable to initiate a more intensive study of the reducing capacity of bacterial, plant, and animal cells in the normal and diseased states.

SUMMARY

Formaldehyde is reduced to methyl alcohol by living cells of a creatinine-decomposing strain of *Pseudomonas aeruginosa*. The amount of HCHO taken up in 30 minutes by 3×10^{10} cells of this organism is termed its reducing capacity. It was found to be 400 to 480 μg.

The reaction takes place over wide pH and temperature ranges, is independent of the concentration of formaldehyde as long as the latter is not high enough to exert a toxic effect on the cell, but depends on the number of bacteria present.

In cultures, 3 to 7 days old, there is a decline in reducing capacity without a proportionate decrease in the number of viable cells. On the other hand in cultures, 2 to 4 weeks old, the number of viable cells determines the amount of HCHO taken up.

N/100 NaOH depresses the reducing capacity of the organism without affecting bacterial viability.

Exposure of bacterial suspensions to temperatures of 46–52°C. for 10 minutes causes a far more rapid decrease in the number of viable cells than in reducing capacity.

Incubation of cellular suspensions with nutrient substances, which are able to support adequately the growth of the organism, for 30 minutes prior to the addition of HCHO, induces increases in reducing capacity.

The uptake of HCHO by different microorganisms seems to be related to their gaseous and nutritional requirements. Strains of *P. aeruginosa* and *P.*

fluorescens are predominantly aerobic, least fastidious in their nutritional requirements, most active against HCHO, and least susceptible to the action of aureomycin and chloromycetin.

The potential significance of cellular reducing capacity is discussed.

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ADDENDUM

Since this paper was submitted for publication, information has been obtained about the action of the creatinine-decomposing strain of *P. aeruginosa* on acetaldehyde. This compound, instead of being reduced to ethyl alcohol, is oxidized to acetic acid. The enzyme responsible for the reaction behaves like other bacterial oxidases; it is, for example, unaffected by toluene and catalyzes the oxidation of the substrate at a rate proportional to its concentration. Of the other organisms previously investigated only *E. coli* was tested for its action on CH_3CHO. It, too, proved capable of oxidizing the compound, converting it, presumably by way of CH_3COOH, into non-acidic reaction products the identity of which remained undetermined.

These additional observations appear significant because they draw attention to the high degree of specificity which regulates the behavior of the cell toward substrates as closely related chemically as HCHO and CH_3CHO. In attempts at generalization this is often forgotten, and an acetaldehyde oxidase may be labelled aldehyde oxidase, though, as in the instances here reported, it fails to attack formaldehyde.