BACTERIOLOGICAL STUDY OF CARBOXYLMETHOXYLAMINE HEMIHYDROCHLORIDE[1]

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Received for publication May 26, 1947

A voluminous literature including a number of comprehensive reviews has appeared as a result of current interest in antibacterial agents (Waksman, 1941; Dubos, 1942; van Niel, 1943; Martin, 1944; Woolley, 1945–1946). Those substances which are active in the presence of serum and have a high therapeutic index have attracted the greatest attention because of their application to clinical medicine. Recently a search for preservatives for concentrated protein solutions has led to the study of carboxylmethoxylamine hemihydrochloride. This hydroxylamine, hereafter called compound I, in concentrated protein solutions has been found to be bacteriostatic against a wide variety of gram-negative as well as gram-positive microorganisms. Although its clinical toxicity, described elsewhere (Favour, 1947), largely limits it to *in vitro* work, it nevertheless has many practical uses. Accordingly, its antibacterial properties are presented as the subject of this report.

EXPERIMENTAL RESULTS

Physical properties. Compound I is a slightly hydroscopic, white, crystalline powder which is readily soluble in the usual bacteriological culture media and in concentrated protein solutions. In aqueous solution it is an acid. It retains its antibiotic powers when buffered in neutral or alkaline solutions, after filtration or autoclaving, and during many months of storage in solution.

Procedures. Using the pour plate method and cultures in the logarithmic growth phase, a number of bacterial species were studied (table 1). Much of the detailed work was done with a strain of *Escherichia coli* the susceptibility of which to the compound was intermediate and constant, permitting its use for bioassay of stored and otherwise treated solutions.

In the course of the study we were supplied with two so-called "room temperature" coliform organisms which had been recovered from human albumin during processing. For some time they escaped detection by the usual cultural routines because they were inhibited or killed at 37 C but grew on ordinary media at 23 C. One of these was spontaneously resistant to the compound. The "indicator organism" (table 1) was recovered from a bottle of bromthymol blue. In the species studied there was no relation between relative resistance and ability to ferment sugars (see comments on mode of action). *Brucella*, for example, a non-carbohydrate fermenter, was among the most susceptible organisms studied.

[1] This work has been carried out under a contract, recommended by the Committee of Medical Research, between the Office of Scientific Research and Development and Harvard University.

Drug resistance. A single colony of the stock *E. coli* strain was subcultured in broth with an inoculum of 10,000 organisms per ml and transferred to tubes of broth containing ascending concentrations of the compound. The first tube

TABLE 1

Approximate amount of compound I required to inhibit 1,000 organisms per ml in broth and in albumin

	TRYPTIC DIGEST BROTH	25% HUMAN ALBUMIN
	mg %	*mg %*
Escherichia coli	20	20
Eberthella typhosa	30	20
Salmonella paratyphi	20	20
Salmonella schottmuelleri	20	20
Salmonella choleraesuis	20	20
Shigella paradysenteriae	20	20
Shigella sonnei	20	20
Proteus vulgaris	50	30
Proteus Oxk	20	20
Proteus Ox19	20	20
Proteus Ox2	20	20
Pseudomonas aeruginosa	50	20
Vibrio metchnikovii	10	10
Brucella abortus	30	30
Corynebacterium diphtheriae	10	10
Corynebacterium hofmanii	10	10
Bacillus subtilis	10	10
Bacillus anthracis	10	10
Staphylococcus aureus	10	10
Staphylococcus albus	10	10
Streptococcus pyogenes	10	10
Streptococcus viridans	10	10
Diplococcus pneumoniae type III	10	10
Mycobacterium tuberculosis	Albumin-oleic-acid medium with 20 mg % compound	
Note: Spontaneously resistant organisms follow:		
"Room temperature organism" 1	1,000	?
"Room temperature organism" 2	100	30
"Indicator organism"	150	?
Yeasts	1,000	
Molds	Grew on plates containing 50 mg %	
Viruses	See discussion	

showing visible growth was similarly subcultured in a second series of ascending concentrations of the compound and the process carried through 18 such subcultures. In the first subculture the *E. coli* resistance jumped from 20 to 125 mg per cent, at which it remained thereafter. Coincidentally, the organisms

which did grow were small, grew very slowly, and on the first subculture on drug-free medium usually failed to take up the red dye from McConkey's medium or to form the usual surface colonies. The first or second subculture on normal medium restored cultural morphology and the original drug sensitivity. Fermentation studies on organisms taken from the small phase were the same as those from normal *E. coli*, perhaps because of the rapid return to their previous growth characteristics.

Sporeforming organisms (*Bacillus subtilis* and *Bacillus anthracis*) in the vegetative phase behaved like *E. coli*. Inhibited cultures, however, could be subcultured even when exposed to 100 mg per cent compound for 13 days. Ten mg per cent compound was inactive against spores even when the temperature was held at 58 C for 2 hours.

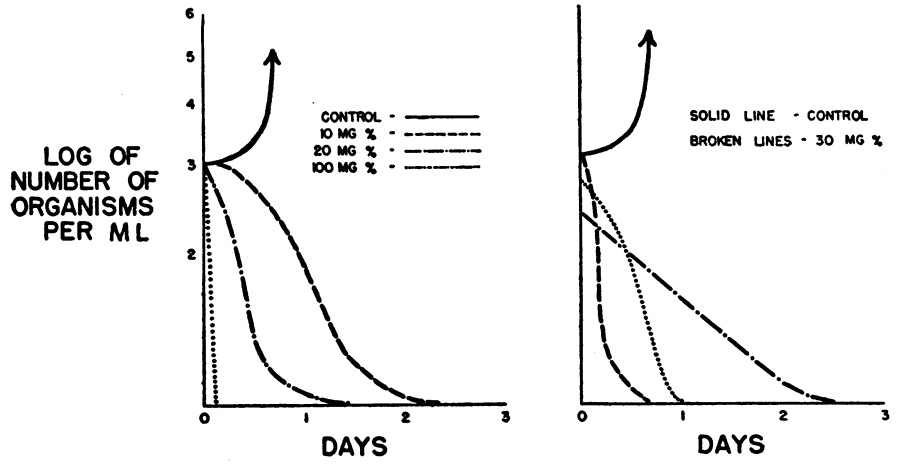


FIG. 1. IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE IN 25 PER CENT ALBUMIN ON E. COLI

Sporeforming organisms (*Bacillus subtilis* and *Bacillus anthracis*) in the vegetative phase behaved like *E. coli*. Inhibited cultures, however, could be subcultured even when exposed to 100 mg per cent compound for 13 days. Ten mg per cent compound was inactive against spores even when the temperature was held at 58 C for 2 hours.

Drug concentration. With the use of small inocula of *E. coli* in a relatively poor medium (25 per cent human albumin) a difference between bacteriostatic and bactericidal drug concentrations can be seen (figure 1). The difference between these concentrations is greater for a relatively resistant organism such as *Proteus vulgaris* (figure 2) and less for a susceptible organism such as the hemolytic streptococcus (figure 3).

Size of inocula. From 2,000 to 10,000 organisms per ml, depending on other factors, are prevented from growing, but the larger inocula are not inhibited by proportional increases in drug concentration unless the medium is very poor in growth factors (figure 4). On the other hand, very small inocula in a poor me-

dium may survive longer than a larger inoculum in the same drug concentration (figure 1). Likewise a given inoculum in a poor medium may survive longer than a larger inoculum in the same drug concentration (figure 6). In addition, a

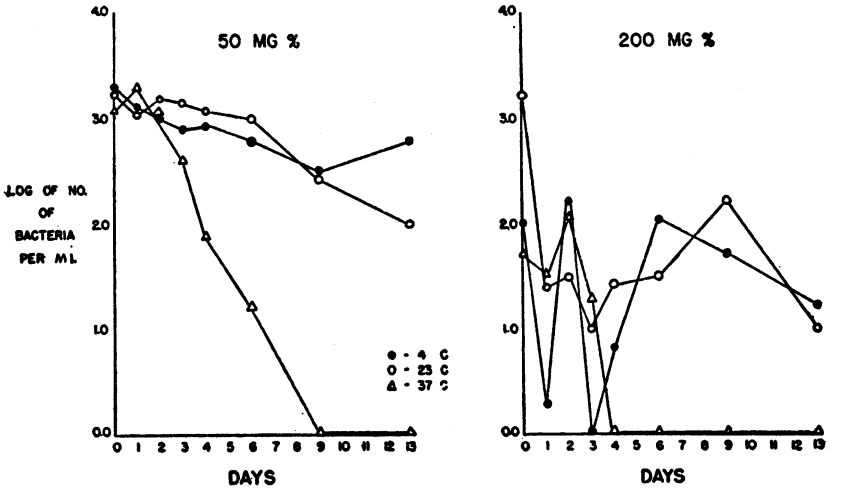


FIG. 2. EFFECT OF CARBOXYLMETHOXYLAMINE ON PROTEUS VULGARIS TRYPTIC DIGEST BROTH

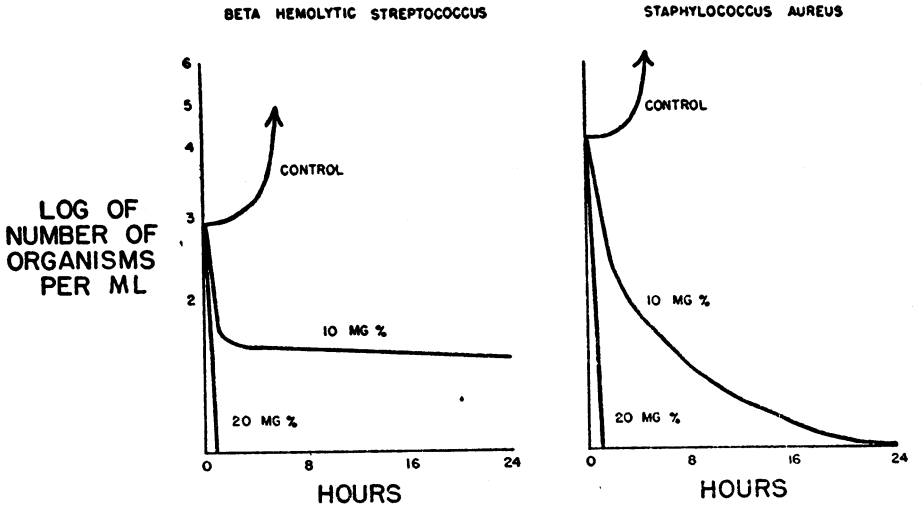


FIG. 3. IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE IN 25 PER CENT ALBUMIN

given inoculum in saline will survive longer at an intermediate drug concentration than at a high or low concentration (figure 5) and a moderate inoculum in water will survive longer with less than the optimal amounts of growth factors (figure 6).

Blocking antibiotic effect. When sodium pyruvate and compound I, mg for mg, are added to a medium, the antibiotic effect of the drug is completely

blocked. Acetone in somewhat higher concentrations also will block the action of the compound (see discussion). Thioglycolate (Brewer's medium) with or without methylene blue completely blocks the antibiotic action of the compound.

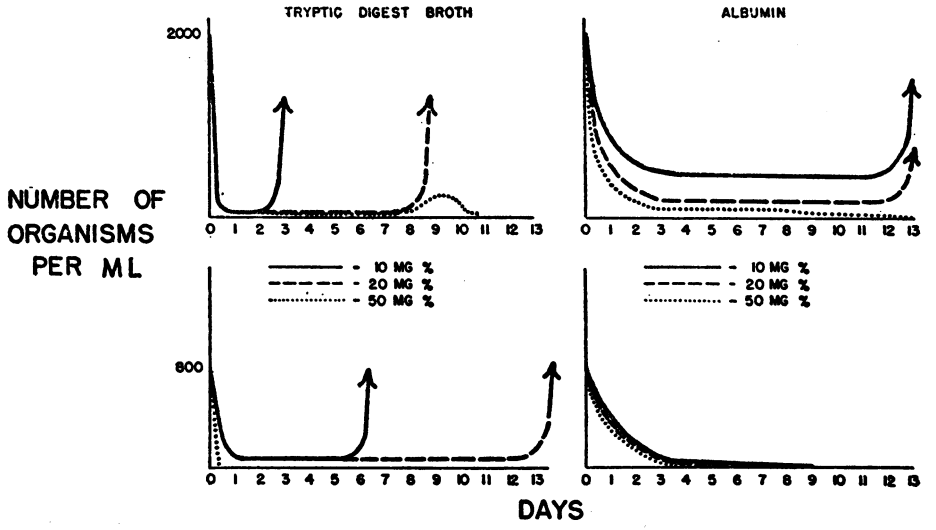


FIG. 4. IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE ON E. COLI

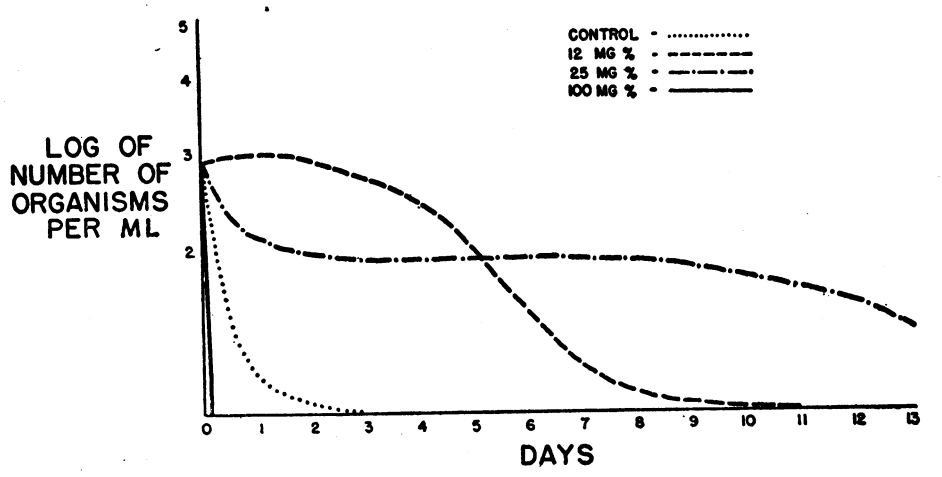


FIG. 5. IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE ON WASHED E. COLI IN .085 PER CENT SODIUM CHLORIDE

Efforts to potentiate antibiotic effect. Because of the carbohydrate cycle suggested for tissues by Toenniessen and Brinkmann (1930), an effort was made to inhibit succinic dehydrogenase at the same time compound I was present, using iodoacetic acid (Oxford, 1942) and malonate. With *E. coli* in tryptic digest broth, 400 organisms per ml, and varying concentrations of compound I from 2 to 20 mg per cent, it was found that similar concentrations of iodine-free iodo-

acetic acid, equal concentrations of sodium malonate, or the combination of the two did not enhance the effect of the compound.

Gamma globulin solutions (fractions II and III), 18 per cent, have a natural antibacterial power which varies with the lot and the bacterial species. Growth

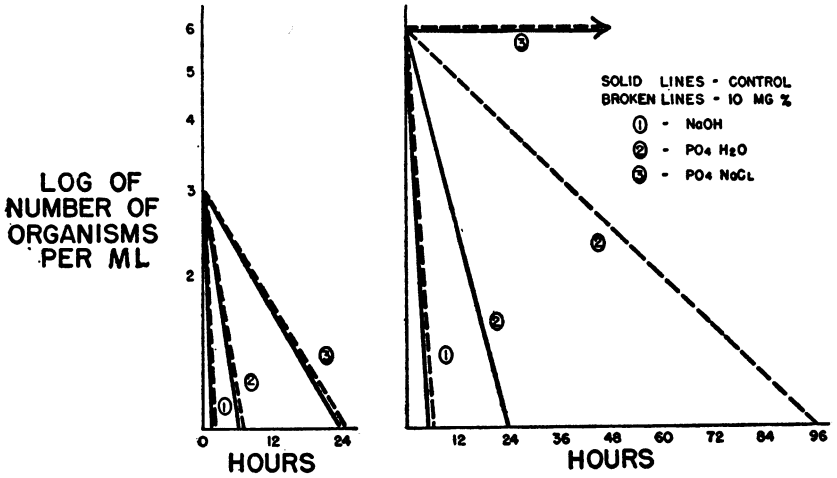


FIG. 6. IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE ON WASHED E. TYPHI

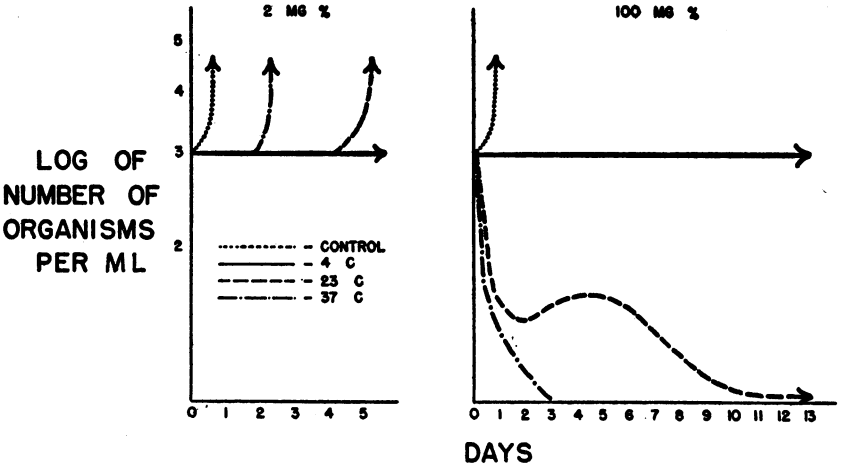


FIG. 7. IN VITRO ACTIVITY OF CARBOXYLMETHOXYLAMINE IN 25 PER CENT ALBUMIN ON E. COLI

inhibition or bacterial death takes place during 3 to 5 days at 37 C. Thereafter, bacteria grow luxuriantly in globulin unless inhibited by a preservative.

Time of drug exposure. In time bacteriostatic concentrations of compound I are bactericidal but not necessarily so (figure 6). Slides of cultures taken in the bacteriostatic phase show pleomorphism, gigantism, and failure of complete fission. Pour plate studies in this interval show no increase in the number of viable organisms. (See, also, comments on efforts to induce drug resistance.)

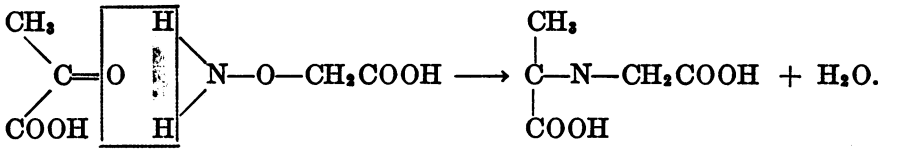
Temperature. Unlike some metabolic inhibitors, such as mercury derivatives, compound I is relatively inactive at low temperatures (figure 7).

Media. A small inoculum is more readily suppressed and killed in a poor medium than in a medium with many growth factors (figure 4). Nonnutrient medium does not necessarily potentiate compound I on proportionately larger inocula (figure 6). Even the presence of the small amount of phosphate ions used to adjust the pH to neutral may alter the survival time of bacteria (figure 6). On the other hand, twice molar glucose or equimolar lactate in broth, each indirect sources of pyruvate in the body, do not alter the antibacterial properties of the compound.

DISCUSSION

The present studies on carboxylmethoxylamine hemihydrochloride indicate that it belongs to the group of metabolic inhibitors. Like the arsenicals it has a low therapeutic index, and like the sulfonamides it is bacteriostatic and must be used in relatively high concentrations. Like penicillin (Oxford, 1942) it prevents cell division and promotes bacterial pleomorphism. And finally, like the salts of heavy metals, its wide range of biological toxicity is matched by its antibacterial effects on a broad range of bacterial species. Unlike most such agents, its effects are limited to small inocula. Although it is too toxic for use as a preservative in large-volume, parenteral solutions, it has certain special uses in which other substances are ineffective. Among these is the preservation of blood-typing serum, bacterial vaccines, and other preparations with a high protein content. No doubt, in time still other uses will be found. An unexplored field is virus work. The influenza virus, PR8 strain, is not inhibited by bacteriostatic concentrations of the compound (Stanley, 1947).

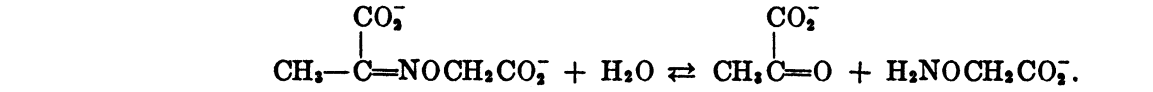
Another subject for further study is the mechanism of action of the compound on both tissues and on bacteria. Previous reports on the antibiotic effect of hydroxylamines have indicated their wide range of species toxicity (Mayer and Oechslin, 1937; Burton *et al.*, 1940). Clarke and associates (1947) demonstrated the ability of pyruvate to block the *in vivo* antibiotic effect of compound I on cellular metabolism. From these observations it would seem that carboxylmethoxylamine is a metabolic inhibitor by virtue of its ability to combine with alpha ketones in living cells, the most important of these being pyruvate:



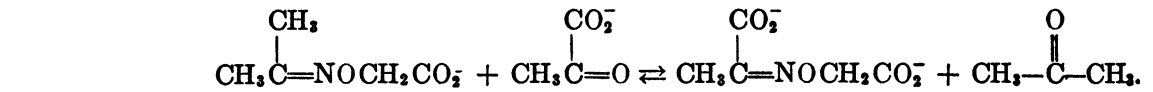
Acetone has a similar blocking action. This, Clark feels, is due to the fact that oximes undergo appreciable hydrolysis:

$$(CH_3)_2C{=}NOCH_2{\cdot}CO_2^- + H_2O \leftrightarrows (CH_3)CO + H_2NOCH_2CO_2^-$$

The pyruvic compound undoubtedly acts similarly, though the composition at equilibrium may not be the same:



If a second aldehyde or ketone is added, the equilibrium is disturbed with the regeneration of some of the original carbonyl compound and the formation of a new carboxymethoxime. For example, if acetone carboxymethoxime be added in excess to pyruvate, an appreciable proportion of the pyruvate will be removed from the system as its carboxymethoxime and a corresponding amount of acetone will be liberated:



This would explain why acetone carboxymethoxime possesses vestigial antibacterial properties and why the carboxymethoxime of pyruvate, which of course is incapable of tying up any pyruvate it may encounter, is quite inactive. Aldehydes and alpha-keto acids other than pyruvic acid should act in a similar fashion.

SUMMARY

Carboxylmethoxylamine hemihydrochloride belongs to the group of metabolic inhibitors. It is bacteriostatic in small concentrations (30 mg per cent) and bactericidal in large concentrations (100 to 1,000 mg per cent).

Its antibiotic activity is presumably due to its ability to combine with alpha ketones, the most important in living organisms being pyruvate. Its toxicity *in vitro* is counteracted by pyruvate, acetone, and thioglycolate, but not by lactate, glucose, anaerobic conditions, methylene blue or its leuco form, cysteine aerobically or anaerobically, iodoacetic acid, or malonate.

Inocula (1,000 organisms or less) of a wide variety of common gram-negative and gram-positive organisms are inhibited. Occasional spontaneously resistant coliform organisms have been encountered. Viruses, yeasts, and fungi are not affected by the compound. Only the vegetative forms of sporeforming bacteria are affected.

The compound is too toxic for large-volume parenteral use. However, it can be used as a preservative for concentrated protein solutions such as typing sera or in vaccines as a supplement to routine aseptic techniques in order to combat small chance contaminations.

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