Journal of Organometallic Chemistry 139 (1977) 61–69

THE AEROBIC METHYLATION OF ARSENIC BY MICROORGANISMS IN THE PRESENCE OF L-METHIONINE-METHYL-d_3

WILLIAM R CULLEN, COLLEEN L FROESE, ANN LUI, BARRY C McBRIDE *, DAVID J PATMORE and MONICA REIMER

Chemistry Department, University of British Columbia, Vancouver, B C V6T 1W5 (Canada)

(Received April 4th, 1977)

Summary

The microorganisms *Scopulariopsis brevicaulis*, *Candida humicola*, and *Gliocladium roseum* growing aerobically, methylate a range of arsenic compounds to produce $R'R''As(CH_3)$ ($R' = R'' = CH_3$; $R' = n\text{-}C_4H_9$, $R'' = CH_3$). When L-methionine-methyl-d_3 is added to the cultures the CD_3 label is incorporated, intact, in the evolved arsine to a considerable extent, indicating that *S*-adenosylmethionine or some related sulphonium compound is involved in the biological process.

Introduction

It had been known for some time that various microorganisms acted on arsenic compounds to produce volatile arsines, "Gosio gas", but it was only after Challenger started work on the problem in 1931 that the metabolic product was correctly identified as trimethylarsine [1,2]. Challenger established that *Scopulariopsis brevicaulis*, growing on bread crumbs, methylates arsenate, arsenite, methylarsonate and dimethylarsinate to trimethylarsine *S brevicaulis* also methylates other arsenic containing acids $RAsO(OH)_2$ and $R'R''AsO(OH)$ yielding $RAs(CH_3)_2$ and $R'R''As(CH_3)$ ($R = CH_3CH_2$, $CH_3CH_2CH_2$, $CH_2{=}CHCH_2$; $R' = CH_3CH_2$, $R'' = CH_3CH_2CH_2$). When $R = ClCH_2CH_2$, trimethylarsine is produced Challenger also established that trimethylarsine is produced by other microorganisms such as *Penicillium notatum* and *P chrysogenum* but that unlike *S. brevicaulis* particular substrates are necessary. For example *P. notatum* does not methylate arsenate. Challenger usually identified the voltatile arsines by characterizing them as their mercuric chloride complexes which precipitated out when the gas stream was passed through a solution of mercuric chloride in

* Department of Microbiology, University of British Columbia (Canada)

hydrochloric acid (Biginelli's solution). He believed that "Biological methylation by *S. brevicaulis* is confined to the mould cell and does not take place in the medium."

In an attempt to establish the mechanism of alkylation Challenger et al. [3] added ^{14}C labeled *d,l*-methionine $^{14}CH_3SCH_2CH_2C(NH_2)HCOOH$ (1 mmol) to the *S. brevicaulis* culture medium and isolated ^{14}C labeled $(CH_3)_3As$ from As_2O_3 (0.25 mmol) The activity indicated 28 3% methylation defined by the ratio.

$$\text{methylation percentage} = \frac{100}{3} \times \frac{\text{activity of product per mol}}{\text{activity of methionine per mol}}$$

These authors suggested that the $^{14}CH_3$ group is probably transferred intact to arsenic by "active methionine." The latter is now known as *S*-adenosylmethionine [4].

In more recent studies Cox and Alexander [5,6] showed that trimethylarsine is produced from the same substrates, arsenate, arsenite, $CH_3AsO(OH)_2$ and $(CH_3)_2AsO(OH)$ by *Candida humicola* (Dazewska) Diddens and Lodder, growing in liquid medium. The *C humicola* was isolated from domestic sewage as was *Gliocladium roseum* Bain, and a *Penicillium* species The latter two organisms produced trimethylarsine, only from $CH_3AsO(OH)_2$ and $(CH_3)_2AsO(OH)$

Because of the widespread use of arsenicals, especially salts of dimethylarsinic acid and methylarsonic acid, as herbicides and pesticides, and preservatives [7], it is important to establish just how inert and immobile these compounds really are in the environment. Several reports described garlic-like odors above soils treated with these compounds [8,9]. One such study used arsanilic acid, 4-aminobenzenearsonic acid [10], a food additive for chickens, turkeys, and swine. Loss of arsenic from soils by volatilization has been established [9—11] Although benzenearsonic acid is not methylated by *S brevicaulis* [1,2], breakdown to arsenate and subsequent biological methylation is possible in soils [11]. Apparently this also happens with arsanilic acid [10].

This type of study, of course, does not establish the identity of the microorganisms responsible for the methylation and does not allow much to be deduced about the mechanism of the process. One of the principal objectives of the current investigation and our ongoing research is to elucidate the mechanisms of biological alkylations. In this paper we report on the effect of adding L-methionine and various arsenic compounds to *S brevicaulis*, *C. humicola*, and *G. roseum* growing aerobically in liquid medium

Experimental

S brevicaulis was obtained from the Microbiology Department at U B.C *C. humicola* and *G. roseum* were isolated by Cox and Alexander [5,6] All three were grown in the liquid medium of pH 5 (250 ml) described by Cox and Alexander [5,6]. The appropriate arsenic compound and L-methionine-methyl-d_3 [12], sterilized separately, were added to the autoclaved medium as indicated in Table 1. Arsenic substrate solutions were prepared by dissolving As_2O_3, $CH_3AsO(ONa)_2$, or $(CH_3)_2AsO(OH)$ in base and adjusting the pH to 5

During the growth period the cultures were maintained at ~25°C and were flushed by a slow stream of sterile air. During the fourth and fifth days after

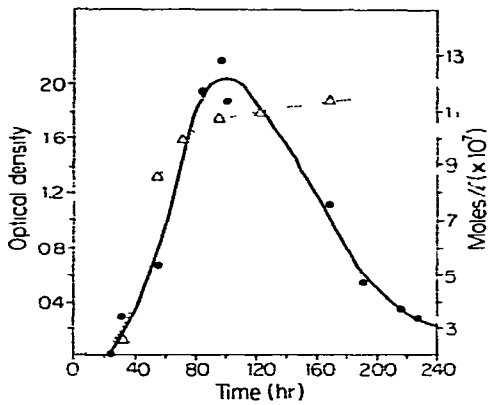


Fig 1 Production of trimethylarsine (●) as a function of growth of *C. humicola* (△)

inoculation the air stream was passed through a trap cooled in liquid oxygen (to condense out volatile components but prevent condensation of oxygen from the air). At the end of the experiment the contents of the trap were manipulated in a vacuum system to remove CO_2, and the less volatile fraction which stopped in a trap cooled to −112°C was condensed into a cold finger (~10 ml) fitted with a Teflon valve and serum stopper [13]. Vapor samples were usually examined by direct injection (1 ml) into the mass spectrometer of a Micromass 12 GCMS (Vacuum Generators-Micromass Ltd) although in some early experiments 100 μl vapor samples were injected into the mass spectrometer via the gas chromatograph. The raw data obtained by direct comparison of peak parent heights from the mass spectra are listed in Table 1

In other experiments the growth of the microorganism in a 1 l flask containing 250 ml of medium and arsenite (5 m*M*) was monitored by periodic removal of 5 ml samples for optical density measurements at 455 nm on a Turner Associates spectrometer. At the same time the trimethylarsine content of the head space in the flask was monitored by VPC using a Varian 1520 instrument fitted with a 6 ft 5% SE 30 stainless steel column. The flame ionization detector was responsive down to ~1 nmol in a 1 ml injection. Throughout these experiments the flask was flushed with a slow stream of sterile air. A typical curve from an experiment of this sort is shown in Fig. 1.

Results and discussion

Challenger [1,2] found that trimethylarsine is produced by *S. brevicaulis*, growing on bread crumbs, when treated with As_2O_3, $CH_3AsO(OH)_2$, and $(CH_3)_2AsO(OH)$. Using more modern techniques of collection of volatile arsines and investigation by mass spectroscopy we now find that the same microorganism grown in liquid culture, produces trimethylarsine from the same methylated substrates. Furthermore, there was no evidence for any other volatile arsenic containing metabolic product. Specifically, dimethylarsine could be expected to be present since this has been identified as being produced by cell extracts

of *Methanobacterium* strain M o.H. when treated with arsenate, H_2, ATP *, and methylcobalamin [14]. Dimethylarsine is also evolved by the same cell extracts from $(CH_3)_2AsO(OH)$ in the presence of H_2 and ATP. Whole cells, growing anaerobically in the presence of CO_2 and H_2, also metabolize arsenate to give an as yet unidentified arsine [14]. We have recently found that cell extracts of *Methanobacterium thermoautotrophicum* evolve both $(CH_3)_3As$ and $(CH_3)_2AsH$ from arsenate again with methylcobalamin as the carbon source [15] However, dimethylarsine would be expected to have a very short life time in an air stream, consequently even if it were formed as a metabolic product in the aerobic growths studied in the present investigation it would be quickly chemically transformed to dimethylarsenic acid and metabolized to trimethylarsine

Trimethylarsine (b p 52°C), although a spontaneously flammable liquid in air, is surprisingly stable in air at low partial pressures. A rough estimate of $10^{-6}\ M^{-1}\ s^{-1}$ for the rate constant of the reaction of $(CH_3)_3As$ with oxygen in the gas phase has been recently described [16].

The results of experiment 16 (Table 1) indicate that some loss of CD_3 from CD_3AsO_3Na is occurring since $(CH_3)_3As$ is produced. The exact significance of the numbers is discussed later; however the result seems to be genuine since chemical and spectroscopic analysis failed to indicate the presence of $CH_3AsO(Na)_2$ or unmethylated arsenic(III) or arsenic(V). A number of other indications of similar cleavage reactions are seen in other experiments with the same organism, e g. experiment 17, and *C humicola*, e g experiments 4 and 2

It is possible that the $As—CH_3$ bond cleavage is due to slow chemical reaction of the $(CH_3)_3As$.

Analogous reactions are known for other trialkylarsines [1,2,17,18] and Parris and Brinckman [16] report indications of alkyl group loss from trimethylarsines when exposed to air in methanol solution, although, in general, the fate of the lost alkyl group has not been established. Challenger has argued that loss of the $ClCH_2CH_2$ group from $ClCH_2CH_2AsO_3H_2$ during metabolism by *S brevicaulis* (trimethylarsine is the product) is of biological origin since the chemical reaction requires hot alkali [1].

As an extension of Challenger's work we find that disodium butylarsonate is reduced and methylated to butyldimethylarsine by *S brevicaulis* and that *C. humicola* does the same. Cox and Alexander [5,6] investigated the alkylation of methyl arsenicals only, by *C. humicola*

The most significant results in Table 1 show that when L-methionine-methyl-d_3 is added to the substrate, the CD_3 label is incorporated into the evolved arsine to a considerable extent. The percentages of the deuterated species listed in Table 1 were determined by comparing the relative peak heights of the parent ions. Spectra were usually run at fast scan speeds so the errors inherent in the method of measurement need to be considered. The two main sources of error seem to be. (1) the assumption that the responses of the deuterated arsines are the same as the undeuterated and (2) the assumption that the peak heights measured, reflect the composition of the mixture.

In order to check the validity of these assumptions a pure sample $(CD_3)_3As$ was

---

* ATP = adenosine triphosphate

TABLE 1

VOLATILE ARSINES PRODUCED BY MICROORGANISMS

Expt. number	Substrates (*mM*)				Deuterated species [a] (%)		
	Arsenic compound		L-$CD_3SCH_2CH_2CH(NH_2)COOH$	$(CH_3)_3As$	$CD_3As(CH_3)_2$	$(CD_3)_2AsCH_3$	$(CD_3)_3As$
Candida humicola							
1 [b]	As_2O_3	5	2	0.6	3 2	13 3	83
2 [b]	$CH_3AsO_3Na_2$	5	2	0 7	7 8	84.7	6.8
3 [b]	$CD_3AsO_3Na_2$	5	2	<1	2 7	10 1	87
4	$CD_3AsO_3Na_2$	5	—	8	92	—	—
5	$(CH_3)_2AsO_2H$	5	5	12 6	80 5	3 6	3 2
6	$(CH_3)_2AsO_2H$	5	0 5	57	39	2 6	1 4
7	$(CH_3)_2AsO_2H$	1	1	28 6	71 4	—	—
8	$(CH_3)_2AsO_2H$	1	0 1	59 3	40 7	—	—
9 [b]	$(CH_3)_2AsO_2H$	5	5	12 1	82 5	2 4	2 7
10 [c]	$(CH_3)_2AsO_2H$	5	2 5	11 6	85 3	2 1	1 0
11 [c]	$(CH_3)_2AsO_2H$	5	1 0	20	78	0 6	1 4
12 [c]	$(CH_3)_2AsO_2H$	5	0 5	24 5	74	0 7	0 7
13 [c]	$(CH_3)_2AsO_2H$	0 5	1 0	— [e]	—	no arsines detected	
14	n $C_4H_9AsO_3Na_2$	5	2 5	6 06	16 1 [f]	77 9 [g]	—
15	$(CH_3)_2AsO_2H$	5	2 5 [d]	—	—	no arsined detected	—
Scopulariopsis brevicaulis							
16 [c]	$CD_3AsO_3Na_2$	2 5	—	4 3	95 7	—	—
17	$CH_3AsO_3Na_2$	2.5	1 25	56 5	25 7	10 3	7 5
18	$(CH_3)_2AsO_2H$	2 5	2 5	33 9	41 3	24	0 8
19	n $C_4H_9AsO_3Na_2$	2 5	1 25	—	54 [f]	45 9 [g]	—
20	$(CH_3)_2AsO_2H$	2 5	2 0 [d]	—		no arsines detected	
Gliocladium roseum							
21 [h]	$CH_3AsO_3Na_2$	5	2 5	73	23	5 2	—
22	n $C_4H_9AsO_3Na_2$	5	2.5	—		no arsines detected	

[a] See text for a discussion of errors [b] VPC/mass spec determination, [c] Substrates added two days after inoculation, [d] Ethionine [e] $C_4H_9As(CH_3)_2$ [f] $C_4H_9As(CH_3)(CD_3)$ [g] $C_4H_9As(CD_3)_2$ [h] Collection was made over a seven day period

prepared in the belief that any difference in mass spectral response from $(CH_3)_3$-As would be greatest for this compound. Known 1/1 gas mixtures of various volumes of the two arsines were injected into an AEI MS-9 mass spectrometer and the ratio of the peak heights at *m/e* 120 and 129 determined. The average for these determinations was 0.86 and the range was from 0 77 to 0 92 (Table 2). It should be emphasized that the controls of the spectrometer had been optimized to record constant peak heights for selected background peaks The same procedure was used to determine the average ratio for other mixtures of the two gases (Table 2).

Errors due to differences in galvanometer response should be least for peaks of approximately the same height so the ratio 0.86 is an approximate experimental value for the difference in sensitivity of the mass spectrometer to the two arsines. This seems to be good to within ~10% for the other ratios investigated. A surprising feature of these experiments was the wide variation in the experimental peak height ratio determined for the same mixture in the mass spectrometer. These controlled experiments point to the possibility of substantial errors in determining percentage composition of the gases using peak heights alone under "controlled" conditions Since the results shown in Table 1 were obtained at non-optimized speeds, errors due to galvanometer response will be even greater. If we estimate this as ~20% then it is large enough to make an allowance for the different sensitivities of the components of the mixture, unnecessary, Thus, the interpretation of the figures in Table 1 should be made in the sense of high, medium, and low incorporation without too much reliance on the actual values. Nonetheless we do find what seems to be a greater incorporation of the CD_3 label from methionine than the 28.3% ^{14}C label incorporation from the same source described in the Introduction.

Returning to the experiments summarized in Table 1 the results of experiments 5, 6 and 18 seem to suggest that *C humicola* is better at incorporating the added methionine than *S brevicaulis*. The extent of utilization seems to be a function of the methionine concentration in experiments 5, 6, 7 and 8. However, when the substrates are added to the growing culture, methionine utilization seems independent of methionine concentration. Experiment 13 indicates that there is a minimum concentration of arsenic substrate necessary since this result was obtained under optimum growth conditions.

The *Gliocadium roseum* experiment, 21, was carried out in 2 parts, the figures

TABLE 2

MASS SPECTRAL RESPONSE FOR MIXTURES OF $(CH_3)_3As$ AND $(CD_3)_3As$ [a]

Injected ratio $(CH_3)_3As/(CD_3)_3As$	Peak height average ratio	Peak height ratio range
1/1	0 86	0 77 —0 92
1/4	0 21 (0 25 X 0 86 = 0 22)	0 19—0.23
4/1	2.97 (4 X 0 86 = 3 44)	3 26—2 68
1/10	0 10 (0 10 X 0 86 = 0 09)	0 098—0 106
10/1	7 57 (10 X 0 86 = 8 6)	6 8—8 08

[a] Using an AEI MS-9 mass spectrometer

given are for the first collection. A second collection of arsines was made six days later (18 days from inoculation) and the resulting distribution of arsines was almost identical with that recorded from the first collection * Thus the degree of incorporation does not seem to change with time. The absence of $(CD_3)_3As$ in this experiment is of interest with respect to $As—CH_3$ cleavage since some could be expected if the cleavage was of chemical origin. Its absence can be rationalized in two ways. Either the organism does not cleave $As—CH_3$ bonds via a biological pathway or the $As—CH_3$ cleavage is restricted to the first methyl group since arsenite and arsenate are not methylated by *G roseum* [5,6]

These results strongly suggest that methionine, or *S*-adenosylmethionine, or some related onium compound, is the source of the methyl groups in the biological alkylation of arsenic and thus support the oxidation—reduction pathway involving carbonium ions originally suggested by Challenger [1,2], Scheme 1 This pathway is chemically reasonable and is related to the well known Meyer

SCHEME 1

$$CH_3^+ + As^{III}(OH)_3 \xrightarrow{-H^+} CH_3As^{V}(OH)_2(O) \xrightarrow[-O^{2-}]{2e} As^{III}(CH_3)(OH)_2 \xrightarrow[-H^+]{CH_3^+}$$

$$(CH_3)_2As^{V}(OH)(O) \xrightarrow[-O^{2-}]{2e} As(CH_3)_2(OH) \xrightarrow[-H^+]{CH_3^+}$$

$$(CH_3)_3As^{V}(O) \xrightarrow[-O^{2-}]{2e} (CH_3)_3As$$

reaction [19,20] in which methyl iodide or dimethyl sulfate react with arsenite to yield methylarsonic acid New evidence for this pathway comes from experiments 15 and 20 where ethionine was added instead of methionine. The absence of any ethylarsines argues against a purely chemical transfer of an alkyl group from sulphur to arsenic, and the absence of any arsines at all argues strongly for a methionine based synthetic path since ethionine is a well known antagonist to methionine [21].

Other work in our laboratories has demonstrated that all steps in Scheme 1 can be duplicated if $(CH_3)_3S^+PF_6^-$ is used as the source of the methyl carbonium ion and SO_2 is used as the reducing agent [22].

McBride and Wolfe [14] proposed a mechanism for dimethylarsine production from arsenate by *Methanobacterium* in which methylcobalamin, usually a donor of the carbanion CH_3^-, is the methyl source. This scheme is based on the result that cell extracts utilize methylcobalamin as a methyl source to produce dimethyl arsine from arsenate, that methylarsonate seems to be an intermediate, and the as yet unsubstiantated belief that dimethylarsine is the volatile arsenical produced by the organism growing in an atmosphere of CO_2 and H_2 The essential feature of the scheme as written is the oxidative addition of CH_3^- to arsenic(III), clearly an unlikely occurrence. Other features in this scheme which has been widely restated by Wood [23] are unacceptable in terms of electron flow and oxidation

* This could be fortuitous however we are inclined to believe that it is an indication that our analytical technique is better than discussed above

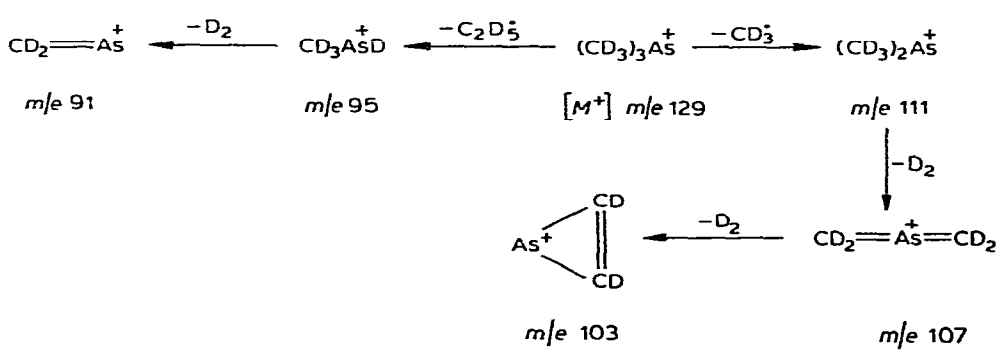
number as has been also pointed out by Zingaro and Irgolic [24]. At the present time there does not seem to be any strong evidence to support the claim that methylations by *Methanobacterium* follow a B_{12} dependent pathway. Indeed the B_{12} protein described by Wood and Wolfe [25] has been subsequently shown to belong to the non-methanogenic organism present in the original *Methanobacterium* culture [26]. Thus the possibility that the bacteria methylate arsenic by the onium mechanism in Scheme 1 cannot be ruled out

The growth curve in Fig. 1 shows, not unexpectedly, that in the case of *C humicola* maximum trimethylarsine production from arsenite follows maximum log phase growth and that it drops to a minimum in the resting phase. Peak production is reached about four days after inoculation which is the reason why this period was chosen for the experiments outlined in Table 1. The area under the curve in Fig. 1 can be taken as an approximate measure of the arsine production, since growth is taking place in a flow system, and corresponds to the conversion of ~1% of the available arsenic to trimethylarsine. (Challenger et al. [3] report an experiment with *S. brevicaulis* growing on bread crumbs which yielded ~85% of the available arsenic as trimethylarsine after more than a year of aspiration.) When these growth experiments with *C humicola* are conducted in stoppered flasks fitted with a septum for sampling by syringe, maximum arsine production from arsenite again occurs after about 80 h and corresponds with maximum growth. However the maximum concentration of the arsine on the head space, 6×10^{-7} mol/l, is less than that obtained in the "open" flasks After 80 h the arsine concentration slowly decreases but the microorganisms quickly repond by producing more if air is injected into the flask Experiments of this sort led to the decision to carry out growths in "open" flasks.

Although none of the experiments we describe are directly comparable to those of Cox and Alexander [6] it seems that we do find approximately the same ability for *C humicola* to methylate arsenic containing substrates.

Finally, it is of interest that the mass spectrum of $(CD_3)_3As$ has an identical cracking pattern to that of $(CH_3)_3As$ [27] except that H is replaced by D. Thus the main fragmentation path is as in Scheme 2. We also prepared $CD_3As(CH_3)_2$ for comparison pruposes and basically the same fragmentation pattern is obtained. Metastable peaks due to loss of both CD_3 and CH_3 from the parent ion

SCHEME 2



are observed affording $(CH_3)(CD_3)As^+$ and $(CH_3)_2As^+$ in high relative abundance.

Acknowledgements

This paper is a contribution from the Bioinorganic Chemistry Group, supported in part by operating and negotiated development grants from the National Research Council of Canada We thank Dr D.H Dolphin for a gift of L-methionine methyl-d_3, Dr D P. Cox for cultures of *C humicola* and *G roseum*, and Dr G Eigendorf for assitance with the mass spectroscopy.

References

1 F Challenger Adv Enzymology, 12 (1951) 429
2 F Challenger Chem Rev 36 (1945) 315
3 F. Challenger D B Lisle and P B Dransfield, J Chem Soc , (1954) 1960
4 S K Shapiro and F Schlenk (Eds) Transmethylation and Methionine Biosynthesis University of Chicago Press 1965
5 D P Cox and M Alexander Bull Environ Contam Toxicol 9 (1973) 84
6 D P Cox and M Alexander Appl Microbiol 25 (1973) 408
7 F A Woolson (Ed) Arsenical Pesticides American Chemical Society Symposium Series Number 7 Washington D C 1975
8 E A Woolson and P C Kearney Environ Sci Technol 7 (1973) 47
9 A R Isensee P C Kearney E A Woolson G E Jones and V P Williams Environ Sci Technol 7 (1973) 841
10 F A Woolson J Agric Food Chem 23 (1975) 677
11 R S Braman in E A Woolson (Ed) Arsenical Pesticides American Chemical Society Symposium Series Number 7 Washington, D C 1975 p 108
12 D H Dolphin and K Endo Analytical Biochem 36 (1970) 338
13 M C Waldman, J Chem Ed 46 (1969) 364
14 B C McBride and R S. Wolfe Biochemistry, 10 (1971) 4312
15 W R Cullen D Kent and B C McBride unpublished work
16 G E Parris and F E. Brinckman Environ Sci Technol. 10 (1976) 1128
17 A Menjanina and R A Zingaro Inorg Chem. 5 (1966) 187
18 M E P. Friedrich and C S Marvel, J. Amer. Chem. Soc 52 (1930) 376
19 A J Quick and R Adams, J Amer Chem Soc. 44 (1922) 805
20 W R Cullen, Advan. Organometal. Chem 4 (1966) 145
21 S Simmonds E B Keller J P. Chandler and V du Vigneaud J Biol Chem 183 (1950) 191
22 A.K Chopra, W R. Cullen and D H. Dolphin unpublished work.
23 J M Wood Science 183 (1974) 1049
24 R A Zingaro and K J Irgolic Science 187 (1975) 765l
25 J M Wood and R S Wolfe, Biochem , 5 (1966) 3598
26 M P. Bryant, E A. Wolin, M J Wolin and R S Wolfe Arch Mikrobiol 59 (1967) 20
27 R G Kostyanovsky and V G Plekhanov Mass spectrometry 6 (1972) 1183