J. Pharm. Pharmacol., 1965, 17, 794–804 Received June 21, 1965

The action of arsenic on *Bacillus cereus**

H. GEORGE MANDEL, JEROME S. MAYERSAK† AND MARGIT RIIS

Sodium arsenite (0·4 mM) inhibited the growth of exponentially growing *Bacillus cereus*, whereas sodium arsenate was growth-inhibitory only at 10 mM concentration. When the phosphate concentration was reduced sharply, 3·3 mM sodium arsenate inhibited growth, whereas the inhibitory effect of arsenite was independent of phosphate concentration. Neither arsenite nor arsenate produced any specific effects on the incorporation of precursors into ribonucleic and deoxyribonucleic acids, protein or cell wall, in support of the concept that their actions were at stages of energy utilisation rather than biosynthesis of macromolecules. Although some ^{74}arsenate may have been bound by cells during inhibition of growth, ^{74}arsenite was selectively and potently concentrated in micro-organisms. There was no evidence for the formation of arsenic-containing nucleic acids nor were labelled organic intermediates found. No instance of interconversion between pentavalent and trivalent states of arsenic could be established, and it was concluded that both compounds inhibited the growth of *B. cereus* by separate mechanisms.

THE chemical similarity between arsenic and phosphorus is believed to be responsible for many of the biochemical properties of the former element, particularly when in the pentavalent form. One example of this relationship is the process of arsenolysis (reviewed by Cohn, 1961), in which arsenic substitutes for phosphorus in intermediary metabolism. Although none of the low-molecular arsenic-containing analogues hypothesised as intermediates has yet been isolated, the possibility exists that if arsenic acts as a nucleic acid phosphorus analogue by replacement, stable cell constituents containing arsenic might be identified.

The use of radioisotopic arsenic with high specific activity offered a most sensitive technique for measuring such incorporation in arsenic-sensitive bacteria. Labelled arsenite was prepared from ^{74}arsenate, and information on the uptake of these drugs and their interconversions by exponentially growing cells was sought in relation to the effect of the drugs on growth, since pentavalent arsenicals are generally believed to become growth-inhibitory only after reduction to the trivalent form.

The *Bacillus cereus* system has served previously to demonstrate dissociations between various cell processes occurring during growth-inhibition produced by analogues of nucleic acid bases. Thus, 8-azaguanine specifically blocked the incorporation of amino-acids into proteins (Roodyn & Mandel, 1960a); 6-mercaptopurine and 6-thioguanine reduced the uptake of precursors into nucleic acids (Carey & Mandel, 1961; Mandel, Latimer & Riis, 1965); and 5-fluorouracil blocked the synthesis of deoxyribonucleic acid (DNA) (Reich & Mandel, 1964). Both 8-azaguanine and 5-fluorouracil have been shown to be incorporated extensively into polyribonucleotide derivatives in this system (Mandel, 1957; Reich & Mandel, 1964) and a trace of thioguanine was present in

From the Department of Pharmacology, The George Washington University, School of Medicine, Washington, D.C., U.S.A.

* This research was supported by Public Health Service Research Grant AI-04264 from the National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Maryland, U.S.A.

† Medical Student, Summer Research Scholar, supported by Lederle Laboratories.

nucleic acids (Mandel & others, 1965). Evidence for similar effects was therefore sought for arsenic.

A preliminary report has been presented (Mandel & Mayersak, 1962).

Experimental

MATERIALS AND METHODS

Growth and fractionation of bacteria. Bacillus cereus 569H cultures were grown as described previously (Mandel, 1961). When the growth medium was required to be low in phosphate, 95% of the potassium phosphate was replaced by 9 mM potassium sulphate and 16 mM tris-hydroxymethylaminomethane (TRIS) buffer. Growth was measured turbidimetrically in a Beckman spectrophotometer model DU at 540 mμ. Cultures were sampled by removing 2 ml aliquots, measuring turbidity, and mixing with 2 ml saline, 10% trichloroacetic acid (TCA) or N potassium hydroxide before filtration through Schleicher and Schuell coarse membranes. For cells grown in the presence of radioisotopes, radioassay after cold TCA-washing measured radioactivity in all cell fractions except the acid-soluble pool. Correspondingly, filtration of cells washed in hot TCA led to recovery on the filter of protein and cell wall. For cells grown with labelled uracil, the potassium hydroxide treatment at room temperature, resulting in loss of the ribonucleic acid (RNA) from cells, allowed the measurement of radioactivity in DNA on the filter (Roodyn & Mandel, 1960b).

To isolate and identify radioactive compounds in cell fractions, 25 ml samples of cells were harvested when suspensions had an optical density of 0·4 at 540 mμ (corresponding to about 0·4 mg dry weight of cells per ml), washed in 80% ethanol at 85° to remove the soluble pool and lipid fraction, and incubated with N potassium hydroxide overnight to hydrolyse RNA into mononucleotides. The ethanol-soluble fraction was chromatographed on paper using aqueous isopropanol (Markham & Smith, 1952) or ammonium acetate : ethanol (Paladini & Leloir, 1952). Mononucleotides were separated at pH 3·5 by paper electrophoresis (Markham & Smith, 1952).

Total activities in cells were estimated by centrifuging cell suspensions and counting aliquots of medium and packed cells. Radioactivity was readily lost upon washing the cells, and therefore it needed to be measured for unwashed bacteria.

For the fractionation of cell-free preparations, all at 0°, washed cells suspended in 4·5 mM magnesium acetate and 1 mM TRIS buffer at pH 7·4 were disintegrated in a Mickle apparatus, and remaining cells and cell walls removed by centrifugation. The supernatant solution was extracted twice with saturated aqueous phenol to precipitate proteins (Kirby, 1956) and with ether to remove phenol; the nucleic acids were then precipitated with 2 volumes of ethanol. The precipitate was dissolved in water and the solution dialysed overnight against the TRIS buffer.

Respiration studies. Oxygen uptake was assessed by the usual manometric technique (Umbreit, Burris & Stauffer, 1957).

Radioisotopes. Guanine-8-^{14}C, uracil-2-^{14}C, and DL-leucine-1-^{14}C were obtained from Isotopes Specialties Co., Burbank, California, U.S.A. Diaminopimelic acid-^{3}H was a gift from Dr. J. Strominger, Washington Univ., St. Louis, Mo., U.S.A.

A procedure was developed for the preparation of trivalent arsenic by reduction of ^{74}arsenate purchased from Radiochemical Centre, Amersham, Buckinghamshire, England (1 mc in approximately 10 μg).

To convert pentavalent arsenic to the trivalent state, 4 mg sodium arsenate, containing 160 μc ^{74}As, was mixed with 90 mg sodium metabisulphite in 0·65 ml water and 0·02 ml concentrated hydrochloric acid, and was placed in a water-bath at 60° for 1 hr. The product, together with 200 μg arsenic trioxide carrier, was streaked on Whatman No. 3 MM paper, neutralised with dilute ammonia, and subjected to descending chromatography in 90% aqueous isopropanol. The area of the paper corresponding to arsenite was identified by radioautography, eluted with water and concentrated by evaporation *in vacuo*.

Arsenic analytical procedures. Arsenic trioxide was dissolved in 1% sodium carbonate. Trivalent arsenic was detected on paper chromatograms as a yellow spot after dipping the paper in a solution of diphenylthiocarbazone (dithizone) in carbon tetrachloride and air-drying. Arsenic trioxide had virtually no mobility during paper electrophoresis at pH 3·5. At pH 7 or above (by treatment of the origin area of the paper with dilute ammonia) the compound exhibited an Rf of about 0·5 during descending chromatography on Whatman 3 MM paper in isopropanol: water (9:1). In isopropanol: water (7:3) its Rf was 0·6. A trace of arsenic always remained at the origin.

Arsenic pentoxide was dissolved by heating in diluted hydrochloric acid or potassium hydroxide. It was detected on chromatograms as a brown spot by spraying with a saturated solution of potassium iodide containing hydrochloric acid. Sodium arsenate, dissolved in water at equivalent concentrations, provided identical results and could be used interchangeably. At pH 3·5 the pentavalent ion migrated towards the anode during electrophoresis, and at pH 7 or above, remained at the origin during chromatography in the 90% isopropanol system. In aqueous 70% isopropanol arsenate had an Rf of 0·3.

In vitro complex formations. Dimercaprol and thioctic acid (6,8-dithioloctanoic acid) were from Calbiochem, Los Angeles, Calif., U.S.A. Sulphydryl compounds were detected by the nitroprusside method (Smith, 1960). The sulphur-containing compounds were dissolved in ethanol, diluted in water, and arsenite-containing ^{74}As marker was added. Chromatography of the mixture in water rather than organic solvents provided the best separation of the components.

Results

EFFECT OF ARSENITE AND ARSENATE ON GROWTH OF *Bacillus cereus*

Concentrations of 40 μg/ml arsenic trioxide (equivalent to 0·4 mM arsenite) produced inhibition of growth for about 30 min, after which

partial recovery took place (Fig. 1). Cells present during the inhibitory or recovery phases were indistinguishable in size and appearance from control cells after Gram staining. Whereas 0·1 mM arsenite produced practically no effect on growth, 1 mM arsenite essentially abolished any increase in turbidity of the bacterial suspension for at least 2 hr. These results were independent of the concentration of phosphate in the two growth media used.

Sodium arsenate, on the other hand, only became inhibitory to growth of *B. cereus* at 3 mg/ml (10 mM). When the phosphate buffer of the medium was replaced by TRIS, growth inhibition was observed at considerably lower arsenate concentrations. With only 5% of the usual concentration of phosphate, 3·3 mM arsenate produced inhibition of growth corresponding to that observed with 0·4 mM arsenite. At 1 mM arsenate in the TRIS medium inhibition was either brief or non-detectable.

UPTAKE OF RADIOACTIVITY BY CELLS GROWING IN THE PRESENCE OF LABELLED ARSENATE

Cultures of *B. cereus* were grown in phosphate medium with arsenate at subinhibitory concentrations of $1{\cdot}2 \times 10^{-4}$ mM (no carrier added), 0·16 mM or 2·6 mM, or in TRIS medium during inhibition by 3·3 mM arsenate. Up to $1{\cdot}3 \times 10^{6}$ cpm of ^{74}arsenate were present per ml of medium. Filters containing cells after washing with cold TCA usually contained 0·05% of the radioactivity of the medium filtered, apparently due to adsorption by the filters. During growth of the culture in the presence of arsenate the radioactivity on the filters did not increase, implying that no appreciable progressive accumulation of ^{74}As had taken place.

To determine the quantity of ^{74}arsenate in the whole cell, growth of a bacterial suspension was inhibited with 3·3 mM arsenate, the cells centrifuged off and their radioactivity counted. The radioactivity calculated per mg wet weight of cells suggested that there was some selective uptake of arsenate by cells. The results varied partially because of unavoidable contamination with traces of radioactive medium. Any selective absorption of arsenate was minor compared to that of the corresponding arsenite binding (Table 1).

Upon extraction of inhibited cells with hot 80% ethanol, only unchanged radioarsenate was recovered in the extract as determined by paper electrophoresis and chromatography. When carrier arsenite was added to the cells before the ethanol extraction (to exclude the possibility of loss by oxidation of any trace of radioarsenite during the extraction procedure) and the arsenite re-isolated and radioassayed, no evidence of reduction of radioarsenate to arsenite by the micro-organisms could be demonstrated in this fraction. Incubation of the ethanol-extracted cell residue with potassium hydroxide and separation of the liberated RNA mononucleotides by paper electrophoresis revealed no radioactive metabolite containing arsenic.

Lack of incorporation of radioarsenic into nucleic acids was confirmed using cell-free preparations, thus avoiding the ethanol and alkali

treatments. Cells from 300 ml of suspension inhibited by arsenate for 1 hr were disintegrated to destroy cell walls, and the supernatant cell-free fraction extracted with phenol to precipitate proteins. Almost all of the radioactivity was recovered in the aqueous phase containing nucleic acids. Dialysis against TRIS buffer removed most of the residual activity, but a trace (0·1%) of the original isotope resisted dialysis and was concentrated inside the dialysis bag. When normal cells were disintegrated as above, and then dialysed against ^{74}arsenate-containing buffer and then non-radioactive buffer, a small amount of isotope again was associated with the undialysed fraction. This trace of radioisotope was thought not to be incorporated during the biosynthesis of nucleic acids but to be adsorbed to some non-dialysable cellular component, though not necessarily a nucleic acid.

TABLE 1. RELATIVE ACCUMULATION OF ^{74}ARSENITE OR ^{74}ARSENATE IN CELLS

^{74}Arsenic Compound	Expt.	Radioactivity			Growth	
		Cells (cpm/mg wet weight)	Medium (cpm/μl)	Ratio, cells: medium	Effect	Generation time ratio
Arsenate	1	102	63·5	1·6	inhibition	
	2	1887*	98	19	inhibition	20
		1340*	102	13·1	recovery	1·5
		283	108	2·6	recovery	1·3
Arsenite	1	416	3·64	114	inhibition	
	2	227	1·50	151	inhibition	11·5
		66	1·56	42	recovery	2·2
		51	1·74	29	recovery	1·4
	3	1170	0·82	1430	inhibition	17
		322	1·33	242	recovery	2·8
		209	1·54	136	recovery	1·8

Growth of *B. cereus* in radioactive medium inhibited by 3·3 mM arsenate and 0·3 mM arsenite, followed by the usual spontaneous recovery of growth. Bacterial suspension was centrifuged, and one ml samples of supernatant solution plated on planchets, desiccated and radioassayed. Packed cells were plated directly on tared planchets, desiccated, weighed and radioassayed. Wet weight assumes 80% water content in cells. Growth was measured before harvest of cells and is expressed as ratio of generation times of drug-treated and control cultures.

* These values probably represent some entrainment of radioactivity from medium.

UPTAKE OF RADIOACTIVITY BY CELLS GROWING IN THE PRESENCE OF LABELLED ARSENITE

Cells growing in the presence of radioarsenite supplemented with carrier to 0·4 mM and 1 mM were filtered and washed with cold TCA before measuring incorporation of arsenic by the membrane technique. The radioactivity on the filters did not increase progressively during 2 hr of incubation. Usually about 0·1% of radioactivity, present in the medium filtered, was present on all membranes, including the zero-time sample, apparently due to adsorption.

Measurement of radioactivity in cells after centrifugation revealed a selective uptake of arsenite by cells. On a weight basis, cells contained hundreds of times the concentration of radioarsenite of the surrounding medium (Table 1). As growth recovered in the presence of arsenite (Fig. 1) the relative accumulation decreased. Extraction of this radioactivity with hot 80% ethanol or water, followed by paper chromatography, revealed only unchanged arsenite. No metabolites of arsenite could be

found in the nucleic acid fraction, as measured after electrophoresis of the mononucleotides.

FORMATION OF CHEMICAL COMPLEXES WITH TRIVALENT ARSENIC

Since the possibility existed that a complex of trivalent arsenic and thioctic acid might be present in cells, attempts were made to synthesise this compound. The interaction *in vitro* between these two compounds by varying pH, concentration, temperature, solvents and periods of interaction followed by chromatography led only to the recovery of the two reagents. By contrast, the interaction between dimercaprol and trivalent arsenic resulted in the immediate formation of an insoluble product with an Rf in water between that of dimercaprol and arsenite.

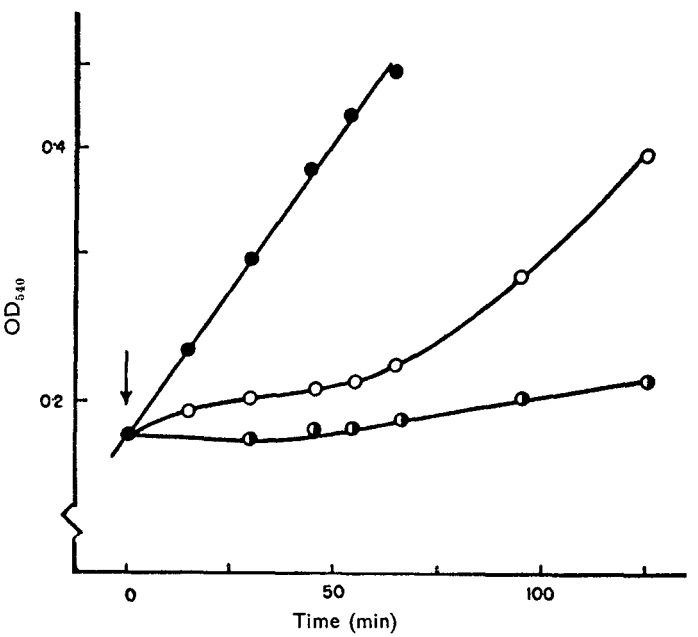

FIG. 1. Effect of arsenite on growth of exponentially growing *Bacillus cereus*. Concentration of arsenite: ●, 0; ○, 0·4 mM; and ◑, 1 mM.

RECOVERY FROM ARSENITE GROWTH INHIBITION

Cells inhibited by arsenite resumed growth after a lag period, the duration of which was related to the concentration of arsenite. The new rate of turbidimetric increase was less than that of control cells (Fig. 1), suggesting some residual cell effect which gradually was diluted out during subsequent growth. Cells which had adapted to grow in 0·4 mM arsenite could tolerate the addition of another 1 mmole of arsenite per litre without a significant decline in the growth rate. Greater additions again produced slowing of the rate of turbidimetric increase (Fig. 2).

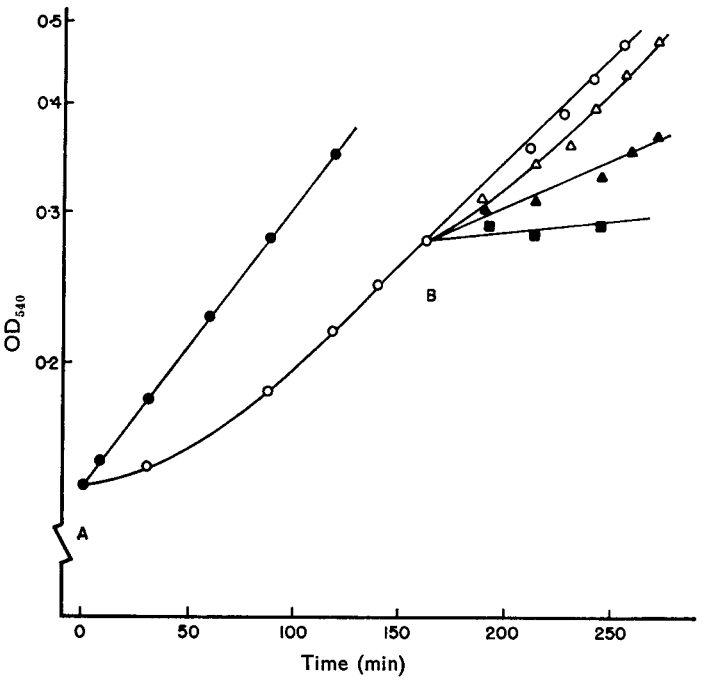

FIG. 2. Adaptation of cells of *B. cereus* to arsenite. At A, one culture received arsenite (0·4 mM) and was incubated until growth had recovered. At B, the culture was subdivided and more arsenite added. Final concentration of arsenite: ●, 0; ○ 0·4 mM; △, 1·4 mM; ▲, 2·4 mM; ■, 3·4 mM.

One possible explanation for the adaptation to arsenite would be its oxidation to arsenate, which does not inhibit growth at equimolar concentrations. Examination by chromatography and paper electrophoresis of growth medium of bacterial suspensions recovering from inhibition by ^{74}arsenite indicated the absence of labelled arsenate or any compound other than arsenite. There was still an inhibitory concentration of arsenite present in the medium in spite of the uptake of the drug by the cells, as also demonstrated by the inhibition of growth of fresh cells added to this medium. Thus, oxidation, which with less refined techniques at first appeared likely (Mandel & Mayersak, 1962) was not responsible for the adaptation. More recent experiments (Reich, M. & Mandel, H. G., unpublished) have suggested that cells in the presence of arsenite produce in the medium a substance which forms a dissociable complex with arsenite and thus reduces the effective concentration of the inhibitory arsenite.

EFFECT OF ARSENITE AND ARSENATE ON BIOSYNTHESES OF *B. cereus*

To determine whether arsenic treatment resulted in a specific effect on one of the major chemical components of cells, bacteria were grown in the presence of labelled precursors of various cell fractions in the presence

and absence of arsenite and arsenate at partially inhibitory concentrations. In Fig. 3, the uptake of radioactivity per ml of bacterial culture has been plotted against the progressive increase in bacterial turbidity. Such a parameter minimises the slower growth in the presence of the inhibitor and allows comparisons of relative utilisation of each isotope. It has been established from previous work that exogenous guanine-^{14}C labels RNA and DNA exclusively, leucine-^{14}C is taken up only by protein, uracil-^{14}C serves to measure exclusively DNA pyrimidines when cells are subsequently treated with potassium hydroxide, and diaminopimelic acid-^{3}H represents incorporation selectively into cell wall (Roodyn & Mandel, 1960b). Inhibition of growth by arsenite produced no specific effect on any of the reactions and the uptake of all of the isotopes was directly related to the formation of new cellular material. Correspondingly, when growth was completely inhibited by higher concentrations of arsenite, incorporation of any of the isotopic precursors was not evident.

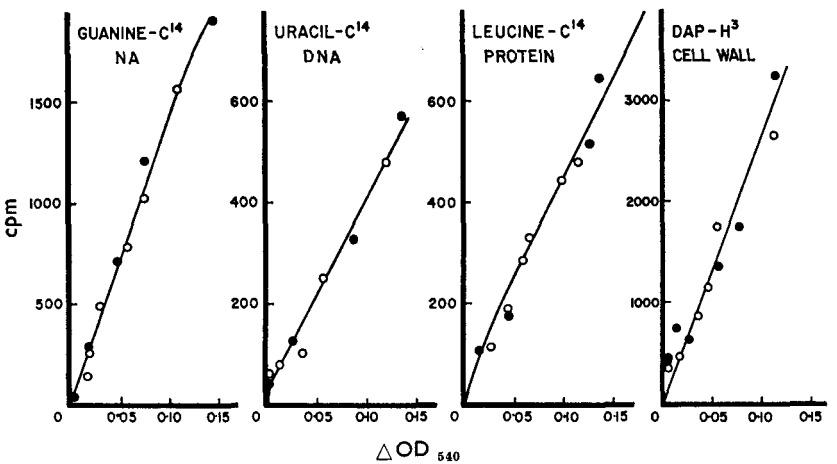

FIG. 3. Effect of arsenite on biosynthetic processes as a function of growth. Incorporation of ^{14}C-guanine measured labelling of nucleic acids, that of ^{14}C-uracil (followed by KOH digestion of RNA) for DNA formation, that of ^{14}C-leucine for protein synthesis, and that of ^{3}H-diaminopimelic acid for cell wall biosynthesis exclusively. Arsenite concentrations: ●, 0; ○, 0·4 mM.

Inhibition of growth by arsenate, similarly, did not involve a selective action on a particular cell fraction, and no dissociation of RNA, DNA or protein could be observed.

EFFECTS OF ARSENITE AND ARSENATE ON RESPIRATION

The rate of uptake of oxygen by cultures of cells growing in a Warburg apparatus in the presence of varying concentrations of arsenite (Fig. 4) and arsenate was found to be depressed when growth was inhibited. When comparisons were made after equivalent growth of the cultures, the consumption of oxygen exceeded that of a control culture, indicating that biosynthesis of macromolecules was curtailed to a greater extent by

the inhibitors than was oxygen uptake, and supporting the generally held concepts that the compounds act at early stages of energy utilisation rather than macromolecular biosynthesis.

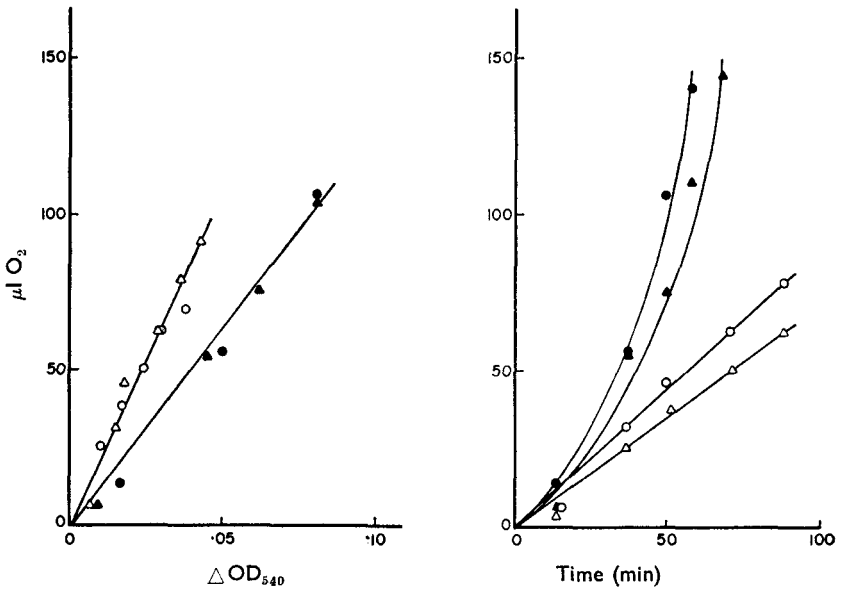

FIG. 4. Effect of arsenite on oxygen uptake of growing *B. cereus* cultures. Arsenite concentrations: ●, 0; ▲, 0·2 mM; ○, 0·4 mM; and △, 0·6 mM. Left, comparisons made with relation to increase in turbidity; right, rate of oxygen uptake.

Discussion

Effect of arsenite and arsenate on growth. The greater inhibitory action of arsenite than that of arsenate on growing cells has been reported for microbial and other systems (Loy, Schiaffino & Savchuck, 1961). The inverse relationship between arsenate action and phosphate concentration (Sussmann & Spiegelman, 1950; Rothstein, 1963) is in contrast to the independence of arsenite inhibition and phosphate concentration, as reported here.

Arsenite metabolism and binding. The interaction of trivalent arsenicals with sulphhydryl-containing tissue constituents, particularly the reduced form of thioctic (α-lipoic) acid in an enzyme-bound form, is believed to be extremely sensitive (Gunsalus, 1953; Sanadi, Langley & White, 1959). Although Reiss (1958) has reported evidence suggesting that γ-(*p*-arsenosophenyl)-n-butyrate *in vitro* formed a complex with dihydrolipoic acid, a complex between arsenite and thioctic acid was not detectable *in vivo* or *in vitro*, perhaps because of insufficient concentration of reduced thioctic acid, or the ready dissociation of such a complex. A complex between arsenite and dimercaprol could be prepared readily, however.

The trypanocidal action of particular organic arsenicals was observed to be related to the binding of the drugs to the parasite (Hawking, 1937),

and arsenosobenzene was found to be concentrated in susceptible trypanosomes 200 times more than its concentration in the supernatant solution (Eagle & Doak, 1951). In the present study with micro-organisms, the selective uptake of inorganic arsenite, but not that of arsenate, paralleled that of the organic derivatives, and appeared to be related to growth inhibition.

Arsenate metabolism and action. The elucidation of the metabolic alteration of arsenate has been severely limited by the suspected instability of organic arsenate esters in water, unlike that of the corresponding phosphates (suggested by Warburg & Christian, 1939). The inability to demonstrate any of the postulated radioactive organic arsenates in arsenate-treated *B. cereus* must be due to their instability, since the isotope technique is extremely sensitive. The observed arsenolysis of adenosine diphosphate to adenosine monophosphate in the presence of arsenate and polynucleotide phosphorylase (instead of polyadenylic acid synthesis) (Singer, 1963), probably is related to the compound's inhibition of nucleic acid synthesis, and the instability of such intermediates as adenosine-monophosphate-monoarsenate may explain the lack of incorporation of arsenic into polynucleotides of growing *B. cereus* cultures.

The relative lack of trypanocidal effectiveness of pentavalent arsenicals and the long delay in onset of action in comparison with the trivalent compounds led Ehrlich (1909) to suggest that the pentavalent compounds are reduced by the host to the biologically active trivalent derivatives. The lack of any such conversion in arsenate-treated *B. cereus*, as indicated by the absence of arsenite in the soluble fraction of cells, implies that the growth inhibition of arsenate is not due to reduction to arsenite. The separate actions of arsenate and arsenite is also demonstrated in the different susceptibilities to variations in phosphate concentration in the medium. It must be concluded, therefore, that in these experiments arsenate is growth-inhibitory *per se*, probably because of its antagonistic action to phosphate.

Recently, a report by Kay (1965) has appeared documenting the incorporation of ^{74}arsenate into nucleic acids of Ehrlich Lettré ascites carcinoma *in vitro*. Each of the mononucleotides isolated from nuclear and cytoplasmic RNA was labelled, and DNA also contained radioactivity. The possible incorporation of arsenate into messenger RNA was suggested.

References

Carey, N. H. & Mandel, H. G. (1961). *J. biol. Chem.*, **236**, 520–524.
Cohn, M., in Boyer, P. D., Lardy, H. & Myrback, K. (1961). *The Enzymes*, **5**, 179–206.
Eagle, H. & Doak, G. O. (1951). *Pharmacol. Rev.*, **3**, 107–143.
Ehrlich, P. (1909). *Ber. Dtsch. Chem. Ges.*, **42**, 17–47.
Gunsalus, I. C. (1953). *J. cell. comp. Physiol.*, **41**, Suppl. 1, 113–136.
Hawking, F. (1937). *J. Pharmacol.*, **59**, 123–156.
Kay, E. R. M. (1965). *Nature, Lond.*, **206**, 371–373.
Kirby, K. S. (1956). *Biochem. J.*, **64**, 405–408.
Loy, H. W., Schiaffino, S. S. & Savchuck, W. B. (1961). *Analyt. Chem.*, **33**, 283–285.
Mandel, H. G. (1961). *J. Pharmacol.*, **133**, 141–150.
Mandel, H. G. (1957). *J. biol. Chem.*, **225**, 137–150.

Mandel, H. G., Latimer, R. G. & Riis, M. (1965). *Biochem. Pharmacol.*, **14**, 661–682.
Mandel, H. G. & Mayersak, J. S. (1962). *Fed. Proc.*, **21**, 179.
Markham, R. & Smith, J. D. (1952). *Biochem. J.*, **52**, 552–557.
Paladini, A. C. & Leloir, L. F. (1952). *Ibid.*, **51**, 426–430.
Reich, M. & Mandel, H. G. (1964). *Science*, **145**, 276–277.
Reiss, O. K. (1958). *J. biol. Chem.*, **233**, 789–793.
Roodyn, D. B. & Mandel, H. G. (1960a). *Ibid.*, **235**, 2036–2044.
Roodyn, D. B. & Mandel, H. G. (1960b). *Biochim. biophys. Acta*, **41**, 80–88.
Rothstein, A. (1963). *J. gen. Physiol.*, **46**, 1075–1085.
Sanadi, D. R., Langley, M. & White, F. (1959). *J. biol. Chem.*, **234**, 183–187.
Singer, M. F. (1963). *Ibid.*, **238**, 336–343.
Smith, I. (1960). *Chromatographic and Electrophoretic Techniques, Vol.* 1, Chromatography, p. 98, New York: Interscience.
Sussman, M. & Spiegelman, S. (1950). *Arch. Biochem.*, **29**, 85–100.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*, Minneapolis, Minn.: Burgess Publishing Co.
Warburg, O. & Christian, W. (1939). *Biochem. Z.*, **303**, 40–68.