

## Microbial growth on 2-bromobutane

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SPERL, G. T. and MCKAE, J. 1980. Microbial growth on 2-bromobutane. *Antonie van Leeuwenhoek* 46:331–341.

A member of the genus *Arthrobacter* was isolated which grew at the expense of 2-bromobutane as sole source of carbon and energy. Evidence is presented which suggests that the initial conversion of 2-bromobutane to 2-butanol is a spontaneous chemical hydrolysis and not mediated by the organism. Further evidence from oxygen consumption experiments indicates that 2-bromobutane is oxidized through 2-butanol, methyl ethyl ketone, ethyl acetate to acetate and ethanol. Results of experiments with cells grown on pathway intermediates reveal that the enzymes necessary for the oxidation of 2-butanol, methyl ethyl ketone, ethyl acetate, ethanol and acetaldehyde are not coordinately, but individually induced by their respective substrates.

### INTRODUCTION

The escape of toxic chemicals into the biosphere occurs daily and sometimes leads to serious problems. This has led several workers to study the possible fates of these chemicals in our environment. Alexander (1965) suggested the term recalcitrant for chemicals which are resistant to degradation by microorganisms. Still other chemicals posing problems of toxicity are readily degraded and completely degraded. The metabolic fates of petrochemical industry compounds with varying toxicities are known, especially in the group of aromatic hydrocarbons (Chapman, 1971, Gibson, 1971, McKenna, 1971).

The initial stages of this study were involved in answering two questions. The first was whether reputed carcinogenic compounds could be degraded and thus detoxified by microorganisms, and the second was whether microbial dehalogenases could account for some of the detoxification of halogenated organic compounds released into the biosphere. Several workers have noted dehalogenating enzymes from soil microbes which use Dalapon (2,2-dichloropropionic acid), fluoroacetic acid, and  $\gamma$ -hexachlorocyclohexane, each of which is quite toxic (Kearney, Kaufman and Bell, 1963; Goldman, 1969; Haider and Jagnow, 1975; Chapman, 1976; Senior, Bull and Slater, 1976). However, carcinogenic

organohalides have not been well studied with respect to microorganisms.

One of the results of our earlier work was the isolation of an *Arthrobacter* sp. which uses 2-bromobutane as sole source of carbon and energy. The observations that this organism grew on a reputed chemical carcinogen, and also liberated bromide during growth lead us to pursue this for three reasons: 1) The bromine is removed from 2-bromobutane before growth is complete, suggesting a possibly active role by the organism in removal of the halogen, 2) 2-bromobutane is a reputed carcinogen (Stecher, 1968) and, 3) 2-bromobutane has been used in agriculture especially for the increased production of latex from rubber trees (Pakishathan, 1976). In this paper we report our initial studies with this organism and, because of our experiences with 2-bromobutane as well as other halogenated organic compounds, a caution for workers who use halogenated compounds in their research.

## MATERIALS AND METHODS

### *Cultures*

The organism *Arthrobacter* 2BB was isolated by direct enrichment culture using soil as an inoculum and the basal medium of Sperl and Hoare (1971). The organism was routinely maintained and grown at 30°C on the above medium. Vapors of 2-bromobutane were supplied from a small sealed tube with a 1 mm diameter hole in it suspended from the flask's stopper.

### *Analytical procedures*

Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as a standard. Since our medium contains chloride (about 500  $\mu\text{M}$ ), qualitative tests of halide removal (bromide) were obtained using the method of DeGeiso, Rieman and Lindenbaum (1954) employing an ion exchange resin (Dowex 1X-8) for the separation of chloride from bromide. The technique showed high levels of bromide when precipitated with silver nitrate. Bromide was quantitatively determined by the method of Binkley (1947), using strychnine which is specific for bromide even in the presence of excess chloride. Acid cleaned glassware rinsed well with deionized distilled water is a necessity. All chemicals were of reagent grade and used without further purification.

### *Growth studies*

It was necessary to constantly monitor the pH of the cultures since the hydrolysis of 2-bromobutane leads to the production of HBr with a concomitant lowering of the pH and cessation of growth. Batch cultures of the organism were grown in erlenmeyer flasks of the same medium described above, but containing phenol red (20 mg/l) for a visible control of pH change. Acid (1N HCl) or base (1N NaOH) was added aseptically to maintain the pH at neutrality. Substrates were

supplied at a concentration of 0.1% (w/v) except for 2-bromobutane which was supplied in the vapor form as described above, and methyl ethyl ketone at a concentration of 0.05% (w/v). Higher methyl ethyl ketone concentrations inhibited growth of the organism. Other studies were performed in flasks fitted with a sidearm tube for measurements directly with the Klett-Summerson colorimeter using a green filter.

#### *Respirometry studies*

Oxygen consumption experiments were performed as follows. Cells were harvested by centrifugation at  $7500 \times g$  for 10 min and washed three times in 50 mM potassium phosphate buffer, pH 7.0. After washing the cells were resuspended in the same buffer to a final concentration of 400–500 Klett units (green filter). Samples of these suspensions were saved for protein determinations at a later time. A Yellow Springs model 4004 oxygen electrode with a mitochondrial chamber (Gilson Medical Electronics) was used for measurements of oxygen consumption. All oxygen consumption experiments were carried out at 30°C. The output from the electrode was connected to a linear recorder in order to be able to determine stoichiometry and rates of oxygen consumption. Substrates are introduced into the chamber by injection through a capillary tube using a Hamilton syringe of 10 or 50  $\mu$ l total volume. The mitochondrial chamber volume was measured at 1.62 ml.

## RESULTS

The *Arthrobacter* sp. grew well with 2-bromobutane when the pH of the medium was kept at neutrality. The results of the amounts of bromide liberated in relation to the growth of the culture are shown in Fig. 1. The culture volume was 100 ml and the total 2-bromobutane supplied was 1.0 ml. If all the 2-bromobutane vaporized, dissolved in the medium and was hydrolyzed, then the highest concentration that bromide could be in the medium would be 91.6 mM. The final bromide concentration was 7 mM and therefore only about 8% of the possible available substrate was dehalogenated. However, since 2-bromobutane is not very soluble (about 9 mM at saturation), and very volatile (1 ml of 2-bromobutane will completely evaporate from a sealed tube with a 1 mm diameter hole in it in less than 24 h at 30°C), the possibility of a 92% loss due to evaporation is not unreasonable. The other important observation from Fig. 1 is that almost all the bromide is liberated before the organism shows macroscopic signs of growth; just about the time exponential growth begins. This seems to indicate that the dehalogenating mechanism might not be due to the organism. However, it is possible that the organism has to detoxify the compound by removing the bromine before growth can occur.

Since there was a possibility that the organism was removing the halogen, the

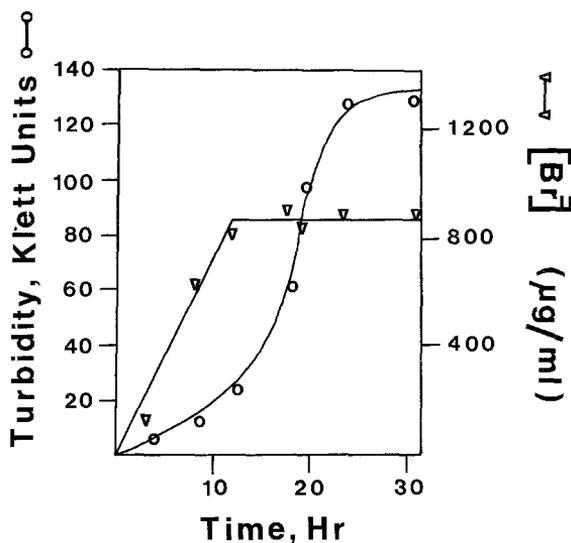


Fig. 1. Growth of *Arthrobacter* 2BB on 2-bromobutane and the release of bromide from 2-bromobutane. The pH was adjusted to 7.0 at each observation by the addition of 1N NaOH.

intermediate from which the halogen was removed had to be determined. There are two possibilities for the oxidation of alkanes by microorganisms (temporarily neglecting the bromine). The first follows a terminal oxidation using molecular oxygen of butane to 1-butanol with succeeding steps through butyraldehyde and butyric acid. The butyric acid is then oxidized by  $\beta$ -oxidation through crotonic acid,  $\beta$ -hydroxybutyric acid and  $\beta$ -ketobutyric acid to 2 moles of acetyl-S-Coenzyme A per mole butane oxidized (McKenna, 1971). Although 1-butanol (as well as most primary alcohols) and butyraldehyde are oxidized, several of the crucial intermediates in this scheme are not oxidized by cells grown on 2-bromobutane. These are butyric acid, crotonic acid and  $\beta$ -hydroxybutyric acid.

The second alternative is the subterminal oxidation of butane (again neglecting the bromine for the purposes of discussion) to 2-butanol with succeeding steps through methyl ethyl ketone, ethyl acetate, ethanol, acetaldehyde and acetate (Allen and Markovetz, 1970; Markovetz and Kallio, 1971; Klug and Markovetz, 1971). All these intermediates are oxidized by 2-bromobutane grown cells. We therefore accepted this scheme as a working hypothesis for the pathway of utilization of 2-bromobutane.

When during this subterminal oxidation scheme, is the bromine released? Were compounds such as 3-bromo-2-butanol made; or 2-bromoethyl methyl ketone? If the bromine were on the same carbon which was being subterminally oxidized, then it would either come off in the first or second step in order to yield

a ketone group on that carbon. If it were on the adjacent C<sub>3</sub> position, then the bromine could conceivably remain until the compound was split into 1-bromoethanol and acetate. After a search of the chemical literature we found that the possible brominated intermediates (or those substituted with any other halogen) were either unstable in an aqueous environment or unknown. This in conjunction with the possibility that a bromine atom in place of a hydrogen must impose some serious steric problems on enzymes using these compounds as substrates, led us to propose that the bromine comes off at either the first or second step.

In the literature are several papers dealing with the hydrolysis of secondary halides (Coburn, Grunwald and Marshall, 1953; Winstein, Grunwald and Jones, 1951). The rates reported for these hydrolyses were rapid enough to be of significance and further strengthened our proposed pathway. However, the first step, the hydrolysis of 2-bromobutane to 2-butanol, now appeared likely to be not enzymatic, but a purely chemical hydrolysis.

We then performed an experiment to determine if the initial hydrolysis was enzymatically mediated or strictly chemical (Table 1). The mitochondrial chamber was set up using a constant amount of 2-bromobutane injected directly from the bottle to avoid hydrolysis before coming into contact with the cells. Varying cell concentrations were tested with this constant substrate level (excess of 2-bromobutane even for the most concentrated cell concentration). If the hydrolysis were cell mediated then the rate of oxygen consumption should increase in proportion to the cell concentration or the amount of oxygen consumed/mg protein should be constant. As is seen in Table 1, this is not the case. The oxygen consumption increased to a constant level of about 20 nmoles oxygen consumed per minute. Thus it appears that the initial hydrolysis is strictly a chemical hydrolysis and not enzymatically mediated.

Quantitative studies on the stoichiometry of oxygen consumed per mole of substrate agree with the hypothesis that the bromine was probably released at the

Table 1. Rates of oxygen consumption by 2-bromobutane-grown cells in the presence of 2-bromobutane

2-Bromobutane ( $\mu\text{mol}^1$ )	Cells (amount in $\mu\text{g}$ protein)	Rate (nmol O <sub>2</sub> consumed/min)	$\mu\text{mol}$ O <sub>2</sub> consumed per $\mu\text{g}$ protein
5.65	5.25	6.44	1.23
5.65	10.5	12.2	1.16
5.65	21.0	19.3	0.91
5.65	42.0	18.6	0.44
5.65	84.0	20.4	0.24
5.65	168	19.9	0.12

<sup>1</sup> 2-Bromobutane (1  $\mu\text{l}$ ) was injected directly from the bottle into the mitochondrial chamber to avoid previous hydrolysis.

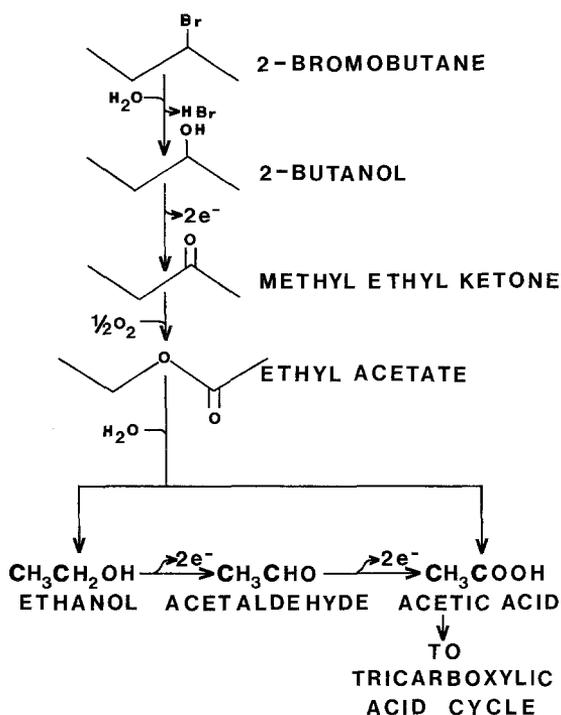
first step (see Table 2). We thus propose the metabolic pathway for the utilization of 2-bromobutane by *Arthrobacter 2BB* shown in Fig. 2. The final "product" of this pathway is 2 moles of acetate from each mole of 2-bromobutane oxidized. These acetate molecules are probably then oxidized to  $\text{CO}_2$  via a combination of the tricarboxylic acid cycle and the glyoxylate bypass. Theoretically each acetate molecule could drive the uptake of 2 moles of oxygen. As can be seen from Table 2 this is not the case. However, prior to the level of acetate, it would be expected that acetaldehyde would drive the uptake of 0.5 moles of oxygen/mole acetaldehyde oxidized, ethanol 1.0 mole of oxygen, ethyl acetate 1.0 mole of oxygen, methyl ethyl ketone 1.5 moles oxygen, 2-butanol and 2-bromobutane 2.0 moles of oxygen each. The actual results (Table 2) of 0.6, 1.0, 1.1, 1.6, 2.0 and 2.1, respectively, are in excellent agreement with the theoretical values for the proposed pathway (compare Fig. 2 and Table 2).

Another, more indirect, experiment supporting the proposed pathway is presented in Table 3. *Arthrobacter 2BB* was capable of growth on all of the intermediates as sole source of carbon and energy, of the proposed pathway, except for acetaldehyde. In addition 2-chloro- or 2-iodo-butane also served as carbon and energy sources for this organism. We assume that acetaldehyde

Table 2. Stoichiometry of oxygen uptake by 2-bromobutane-grown cells of *Arthrobacter 2BB*

Substrate	Level of substrate supplied (nmol)	nmol $\text{O}_2$ consumed	mol $\text{O}_2$ consumed <sup>1</sup> per mol substrate
2-Bromobutane	{ 10	{ 45.5	4.3
	{ 20	{ 80.8	
2-Butanol	{ 10	{ 42.7	4.2
	{ 20	{ 83.6	
Methyl ethyl ketone	{ 10	{ 37.5	3.8
	{ 20	{ 76.4	
Ethyl acetate	{ 10	{ 33.3	3.3
	{ 20	{ 66.0	
Ethanol	{ 20	{ 43.2	2.1
	{ 50	{ 102.1	
Acetaldehyde	{ 20	{ 35.7	1.7
	{ 50	{ 80.5	
Acetic acid	{ 20	{ 22.8	1.1
	{ 50	{ 53.5	

<sup>1</sup> Average of the determinations at both substrate levels.

Fig. 2. Proposed pathway for 2-bromobutane utilization by *Arthrobacter* 2BB.Table 3. Growth of *Arthrobacter* 2BB on proposed-pathway intermediates

Source of carbon and energy <sup>1</sup>	Presence of growth	$\mu^2$ (h <sup>-1</sup> )	g <sup>3</sup> (h)
2-Bromobutane	+	0.17	4.05
2-Butanol	+	0.42	1.65
Methyl ethyl ketone	+	0.04	18.0
Ethyl acetate	+	0.43	1.62
Acetaldehyde	-	0	0
Acetate	+	0.50	1.38
Ethanol	+	0.55	1.25
2-Chlorobutane	+	0.16	4.21
2-Iodobutane	+	0.18	3.90

<sup>1</sup> Supplied at a concentration of 0.1% (w/v) except for methyl ethyl ketone which was supplied at a concentration of 0.05% (w/v). Concentrations of acetaldehyde from 0.01% to 0.5% (w/v) failed to support growth.

<sup>2</sup> Growth rate constant.

<sup>3</sup> Mean generation time:  $g = \ln 2/\mu$ .

Table 4. Rates of oxygen consumption by cells grown on pathway intermediates ( $\mu\text{mol O}_2$  consumed/min  $\cdot$  mg protein)

Substrate <sup>1</sup>	Growth substrate						
	2-bromo-butane	2-butanol	methyl ethyl ketone	ethyl acetate	ethanol	acetate	peptone
2-Bromobutane	1.83	1.83	0.12	0.14	0	0	0
2-Butanol	2.86	3.58	0.16	0.16	0.06	0	0
Methyl ethyl ketone	1.27	1.75	1.95	0	0	0	0
Ethyl acetate	2.31	1.91	3.50	5.93	0.72	0	0
Ethanol	2.54	1.27	1.67	1.43	5.01	0	0
Acetaldehyde	1.66	1.81	1.59	1.37	1.83	0	0
Acetic acid	0.88	0.80	1.11	1.27	2.31	1.99	5.56
Peptone	4.41	4.93	5.92	6.16	5.49	3.26	6.20

<sup>1</sup> 500 nmoles of substrate were supplied in the mitochondrial chamber for each substrate except for peptone where 10  $\mu\text{l}$  of a 1% (w/v) solution was used.

presents some toxicity that the organism is not capable of overcoming, since acetaldehyde is oxidized by cells grown on 2-bromobutane and other pathway intermediates (Table 2). The growth rate on methyl ethyl ketone is also very slow (18 h mean generation time), but growth does occur at reduced substrate concentrations. Methyl ethyl ketone may also be too toxic at the concentration with which the other substrates were tested.

Results of oxygen consumption experiments using these cells grown on pathway intermediates (Table 4) show a general trend that all the enzymes of the pathway are induced by their respective substrates. There is some leakage with respect to the secondary alcohol dehydrogenase oxidizing 2-butanol in ethyl acetate and methyl ethyl ketone grown cells, but there is still a 93–95% decrease from the fully induced levels. The other leak is in the esterase activity in ethanol grown cells. There is about an 85% decrease in activity, but a significant amount is formed. However, the general trend is toward individual enzymes being induced by their respective substrates. Cells grown on peptone (10% w/v in deionized distilled water) were used as a control for the non-induced state. Of all the pathway intermediates, only acetate was oxidized by peptone-grown cells.

## DISCUSSION

The alarming evidence of Ames and coworkers (McCann et al., 1975, 1976) which suggests that cancer may be, to a large extent, caused by toxic chemicals encountered in the environment has brought up the question of the fates of these

chemicals in the biosphere. Also, many of the chemicals showing mutagenic activity in the *Salmonella* microsome test (McCann et al., 1975) contain halogens. Halogenation is historically a well known technique for the production of pesticides from otherwise less toxic substances (e.g. 2,2-dichloropropionate or Dalapon is an herbicide while propionate at the same level is relatively non-toxic). We have begun our studies of the fates of toxic chemicals by isolation of cultures capable of using these compounds as sole source of carbon and energy. From these studies we isolated *Arthrobacter* 2BB which uses 2-bromobutane. Our purpose in the work reported here was to determine the metabolic fate of this chemical. We succeeded by showing that it is oxidized by subterminal oxidation to 2 moles of acetate per mole 2-bromobutane metabolized. However, we also discovered that many non-aromatic halogenated chemicals hydrolyze spontaneously in an aqueous environment at rates which are significant (Buczowski and Sperl, unpublished results). This could pose problems for researchers who use halogenated compounds in their work where the results depend on the chemical integrity of the halogen, and are unaware that these hydrolyses are quite common. From this and other unpublished work we now believe that most of the halide release from organohalide compounds contaminating the biosphere is of a chemical nature and that specific dehalogenating enzymes are the exception rather than the rule.

In the case of 2-bromobutane, it has been shown that the rate of hydrolysis under the conditions of growth and respiration experiments is about  $4.5 \times 10^{-8}$  moles/1·s at 30°C. The rate is equal to the product of the rate constant and the concentration of the compound (see Table 3). The rate constant is given as  $2.5 \times 10^{-5}$ /s by Winstein et al. (1951) for isopropyl bromide. The rate constant for 2-bromobutane is about one-half this value due to a more bulky ethylene group next to the halogenated carbon, or  $1.3 \times 10^{-5}$ /s (D. W. Emerson, personal communication). This calculates to a rate of 6.9 nmoles 2-bromobutane hydrolyzed to 2-butanol/min in the mitochondrial chamber. Each nmole of 2-butanol will drive the uptake of about 4.2 nmoles of oxygen. This means that the maximum rate of oxygen uptake by cells in the presence of 3.45 mM 2-bromobutane would be about 28.9 nmoles O<sub>2</sub> consumed/min in the chamber. This corresponds well with the figure of 21.4 nmoles O<sub>2</sub> consumed/min in the chamber (average, Table 3). Thus it appears that the ability of *Arthrobacter* 2BB to use 2-bromobutane is due solely to the hydrolysis of 2-bromobutane in an aqueous environment.

From an environmental and toxicological point of view, these results are more comforting. The work of Winstein et al. (1951) shows that secondary or tertiary halides are very susceptible to hydrolysis and thus should not pose serious environmental problems if the products of the hydrolysis are not in themselves toxic. Primary halides are more stable but still susceptible to hydrolysis at rates about 100 to 1000 times slower, and could, in the short term, prove to be a problem from an environmental perspective. The type of halogen present is also

a factor with the rates of hydrolysis of halides following the order  $I > Br > Cl$  (Winstein et al., 1951).

The actual results of the metabolic experiments are rather straightforward with 2-bromobutane being oxidized by a process of subterminal oxidation of butane, except in this case the initial hydroxylation is replaced by the hydrolysis of the bromine. The pattern of control over the pathway is well known and not unusual. These pathway does, however, contain an enzyme which converts methyl ethyl ketone to ethyl acetate by the addition of one oxygen atom. This process is analogous to the Baeyer-Villiger rearrangement (Friess and Pinson, 1952) due to oxidation by a peracid. We are investigating this enzyme at the present time.

We wish to thank Dr David W. Emerson for his discussions and help with the problems in organic chemistry.

*Received 8 January 1980*

#### REFERENCES

- ALEXANDER, M. 1965. Biodegradation problems of molecular recalcitrance and microbial fallibility. — *Adv. Appl. Microbiol.* **7**: 30–80.
- ALLEN, J. E. and MARKOVETZ, A. J. 1970. Oxidation of n-tetradecane and 1-tetradecene by fungi. — *J. Bacteriol.* **103**: 426–434.
- BINKLEY, F. 1947. A colorimetric reaction of chloride ion. — *J. Biol. Chem.* **148**: 403–406.
- CHAPMAN, P. J. 1971. Bacterial degradation of phenolic compounds. *In*: Degradation of synthetic organic molecules in the biosphere. — National Academy of Sciences, Washington, D.C.
- CHAPMAN, P. J. 1976. Microbial degradation of halogenated compounds. — *Biochem. Soc. Trans.* **4**: 463–466.
- COBURN, W. C., GRUNWALD, E. and MARSHALL, H. P. 1953. Some solvent and salt effects on the solvolysis of s-butyl bromide. — *J. Am. Chem. Soc.* **73**: 5735–5736.
- DEGEISO, R. C., RIEMAN, W. and LINDENBAUM, S. 1954. Analysis of halide mixtures by ion exchange chromatography. — *Anal. Chem.* **26**: 1840–1842.
- FRIESS, S. L. and PINSON, R. 1952. Reactions of peracids VI. The reaction of acetylcyclohexane with perbenzoic acid. — *J. Am. Chem. Soc.* **74**: 1302.
- GIBSON, D. T. 1971. Initial reactions in the degradation of aromatic compounds. — *In*: Degradation of synthetic organic molecules in the biosphere. — National Academy of Sciences, Washington, D.C.
- GOLDMAN, P. 1969. The carbon fluorine bond in compounds of biological interest. — *Science* **164**: 1123.
- HAIDER, K. and JAGNOW, G. 1975. Abbau von  $^{14}C$ -,  $^3H$ - und  $^{36}Cl$ -markiertem  $\gamma$ -Hexachlorocyclohexan durch anaerobe Bodenmikroorganismen. — *Arch. Microbiol.* **104**: 113–122.
- KEARNEY, P. C., KAUFMAN, D. D. and BELL, M. L., 1963. Enzymatic dehalogenation of 2,2-dichloropropionate. — *Biochem. Biophys. Res. Commun.* **14**: 437–441.
- KLUG, M. J. and MARKOVETZ, A. J. 1971. Utilization of aliphatic hydrocarbons by microorganisms. — *Adv. Microbial Physiol.* **5**: 1–43.
- LOWRY, O. H., ROSENBOUGH, N. J., FARR, A. L. and RANDALL, 1951. Protein determination with the Folin phenol reagent. — *J. Biol. Chem.* **193**: 265–275.

- MARKOVETZ, A. J. and KALLIO, R. E. 1971. Subterminal oxidation of aliphatic hydrocarbons by microorganisms. — *CRC Crit. Rev. Microbiol.* **1**: 225–237.
- MCCANN, J., CHOI, E., YAMASAKI, E. and AMES, B. N. 1975. Detection of carcinogens as mutagens in the *Salmonella*-microsome test: Assay of 300 chemicals. — *Proc. Nat. Acad. Sci. US* **72**: 5135–5139.
- MCCANN, J. and AMES, B. N. 1976. Detection of carcinogens as mutagens in the *Salmonella* microsome test: Assay of 300 chemicals: Discussion. — *Proc. Nat. Acad. Sci. US* **73**: 950–954.
- MCKENNA, E. J. 1971. Microbial metabolism of normal and branched chain alkanes. *In*: Degradation of synthetic organic molecules in the biosphere. — National Academy of Sciences, Washington, D.C.
- PAKISHATHAN, S. W. 1976. Haloparaffin stimulation of rubber yield in *Hevea brasiliensis*. — *Brit. Pat.* 1,255,991.
- SENIOR, E., BULL, A. T. and SLATER, J. H. 1976. Enzyme evolution in a microbial community growing on the herbicide Dalapon. — *Nature* **263**: 474–476.
- SPEL, G. T. and HOARE, D. S. 1971. Denitrification with methanol: A selective enrichment for *Hyphomicrobium* species. — *J. Bacteriol.* **108**: 733–736.
- STECHEP, P. G. (ed.). 1968. *The Merck Index*, 8th Ed. — Merck and Co., Inc., Rahway, N.J.
- WINSTEIN, S., GRUNWALD, E. and JONES, H. W. 1951. The correlation of rates and the classification of solvolysis reactions into mechanistic categories. — *J. Am. Chem. Soc.* **73**: 2700–2708.