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Effects of sulfate on lactate and C_2-, C_3- volatile fatty acid anaerobic degradation by a mixed microbial culture

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Abstract

The effects of sulfate on the anaerobic degradation of lactate, propionate, and acetate by a mixed bacterial culture from an anaerobic fermenter fed with wine distillery waste water were investigated. Without sulfate and with both sulfate and molybdate, lactate was rapidly consumed, and propionate and acetate were produced; whereas with sulfate alone, only acetate accumulated. Propionate oxidation was strongly accelerated by the presence of sulfate, but sulfate had no effect on acetate consumption even when methanogenesis was inhibited by chloroform. The methane production was not affected by the presence of sulfate. Counts of lactate- and propionate-oxidizing sulfate-reducing bacteria in the mixed culture gave 4.5×10^8 and 1.5×10^6 viable cells per ml, respectively. The number of lactate-oxidizing fermentative bacteria was 2.2×10^7 viable cells per ml, showing that sulfate-reducing bacteria outcompete fermentative bacteria for lactate in the ecosystem studied. The number of acetoclastic methanogens was 3.5×10^8 viable cells per ml, but only 2.5×10^4 sulfate reducers were counted on acetate, showing that acetotrophic methanogens completely predominated over acetate-oxidizing sulfate-reducing bacteria. The contribution of acetate as electron donor for sulfate reduction in the ecosystem studied was found to be minor.

Introduction

The degradation of various intermediates in the anaerobic digestion of organic matter such as volatile fatty acids (VFA), alcohols and hydrogen by sulfate-reducing bacteria (SRB) in anaerobic habitats such as marine or lake sediments has been investigated by several authors (Banat et al. 1981; Smith & Klug 1981; Laanbrooek & Pfennig 1981; Balba & Nedwell 1982). The competition for hydrogen and acetate between sulfate reducers and methanogens in sediments or mixed cultures has also been extensively studied (Jørgensen 1977; Winfrey & Zeikus 1977; Abram & Nedwell 1978a, b; Mountfort et al. 1980). These data indicate that sulfate reducers do in fact compete with methanogens for both substrates. Propionate is now thought to be the main VFA accumulating during the anaerobic digestion of industrial waste water from bioethanol production plants (Bories 1981; Bories et al. 1982; Segretain & Moletta 1987; Qatibi & Bories 1988). In fact, the main organic waste components are glycerol and lactate, and propionate is their main metabolic intermediate. As in some other methanogenic ecosystems (Cohen et al. 1982), acetogenic fermentation of propionate is the

limiting step in overall waste methanization, but it is less frequent than limitation by acetate methanization (Kennedy et al. 1985). Acetate generally accumulates in higher concentrations in these waste waters than in marine or lake sediments. However sulfate reduction has not been investigated during the waste treatment process and more information is required if we want to understand anaerobic digestion more fully so as to be able to control this waste treatment process and clarify how these abundant substrates are consumed by sulfate reduction and methanogenesis. From the economic point of view, the methanization of this industrial waste is of particular interest: it can be used to control pollution while producing an immediately usable biogas as a potential energy sources (Bories 1981; Bories et al. 1982).

The present article deals with the role of SRB as scavengers for short-chain acids in a methanogenic microbial ecosystem developed for use with wine distillery waste water, and investigates the relationship between SRB and methanogenic bacteria.

Materials and methods

Media

The basal sulfate-free salt solution mineral-medium used for batch culture experiments had the following composition (g/l): K_2HPO_4, 0.4; KH_2PO_4, 0.4; NH_4Cl, 0.9; NaCl, 0.9; $MgCl_2 \cdot 6H_2O$, 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; cysteine-HCl $\cdot$ H_2O (as a reducing agent), 0.4; resazurin, 0.001. The gas atmosphere for batch cultures experiments was O_2-free N_2. Bicarbonate-buffered and sulfide-reduced medium was composed and prepared as described by Widdel & Pfennig (1977), modified by Widdel (1980) and supplemented with 0.1 g/l yeast extract (Difco) for bacterial counts. Methanogenic bacteria were counted in medium supplemented with 0.1 g/l yeast extract, 0.5 g/l L-cysteine hydrochloride and 5% (v/v) rumen juice; the gas atmosphere was a mixture of N_2 (80%) and CO_2 (20%)

Growth conditions and Sources of microorganisms

Batch anaerobic degradation of organic substrates was carried out in 60 ml serum bottles closed with butyl-rubber stoppers, incubated on an orbital stirrer at 37° C. The microbial culture was obtained from a 20 l-continuous fermenter fed with wine distillery waste water. Glycerol (by-product of alcoholic fermentation) and lactic acid (from malic acid fermentation) formed up to 60% of the soluble carbon in this waste; the sulfate concentration in the effluent varied between 6.0 and 12 mM. Organic substrates, sulfate, molybdate (20 mM) (an inhibitor of sulfate reduction) and chloroform (0.003% v/v) (an inhibitor of methanogenesis), were added from separately sterilized stock solutions.

Bacterial counts

Sulfate-reducing, fermentative and methanogenic bacteria were counted using the most probable number method (MPN). SRB were counted in the presence of 20 mM of sulfate, with the following substrates: lactate (20 mM); propionate (20 mM); acetate (20 mM). Fermentative bacteria were estimated with lactate (20 mM) and propionate (20 mM) without sulfate. Methanogenic bacteria were tested on acetate (20 mM), and H_2/CO_2 (80–20%) (3 bars) with acetate (5 mM) as carbon source. Total flora was estimated using the acridine orange epifluorescence method (Hobbie et al. 1977). Growth of sulfate-reducing, fermentative and methanogenic bacteria was determined from the sulfide production, the change in colour of the methylene blue previously added to the medium and the methane accumulation, respectively. Products were also qualitatively determinated.

Analytical methods

Lactate was assayed using the enzymatic method developed by Batlle et al. (1979); volatile fatty acids were analyzed by means of FID chromatography (glass column 2 m long, 1/8″ inner diameter,

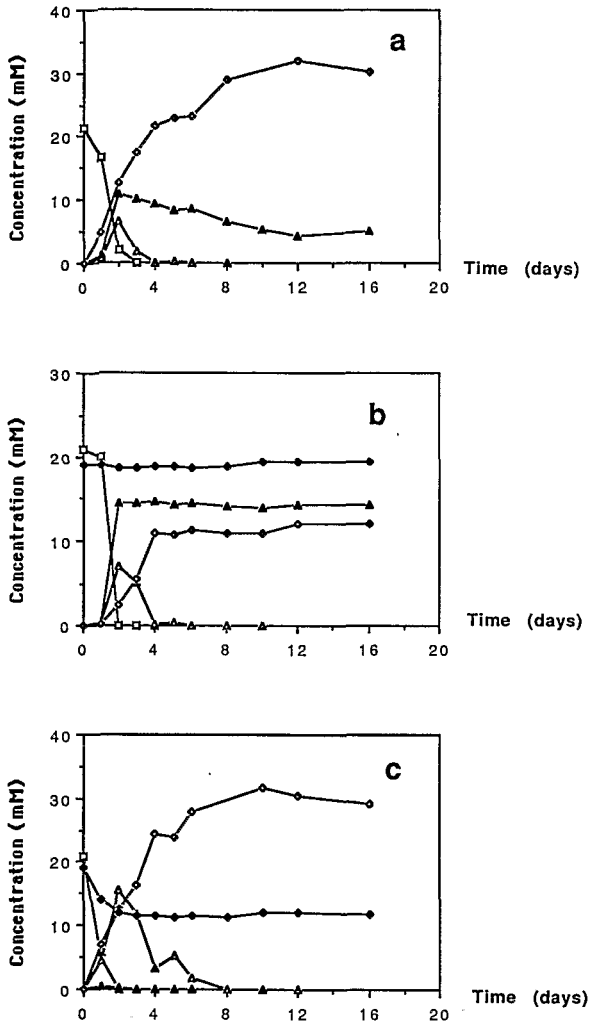

Fig. 1. Anaerobic lactate degradation by a mixed microbial culture in the absence of sulfate (*a*); in the presence of sulfate and molybdate (*b*) and in the presence of sulfate (*c*). Molybdate was added at a concentration of 20mM. Symbols: □, lactate; ▲, propionate; △, acetate; ◆, sulfate; ◇, methane.

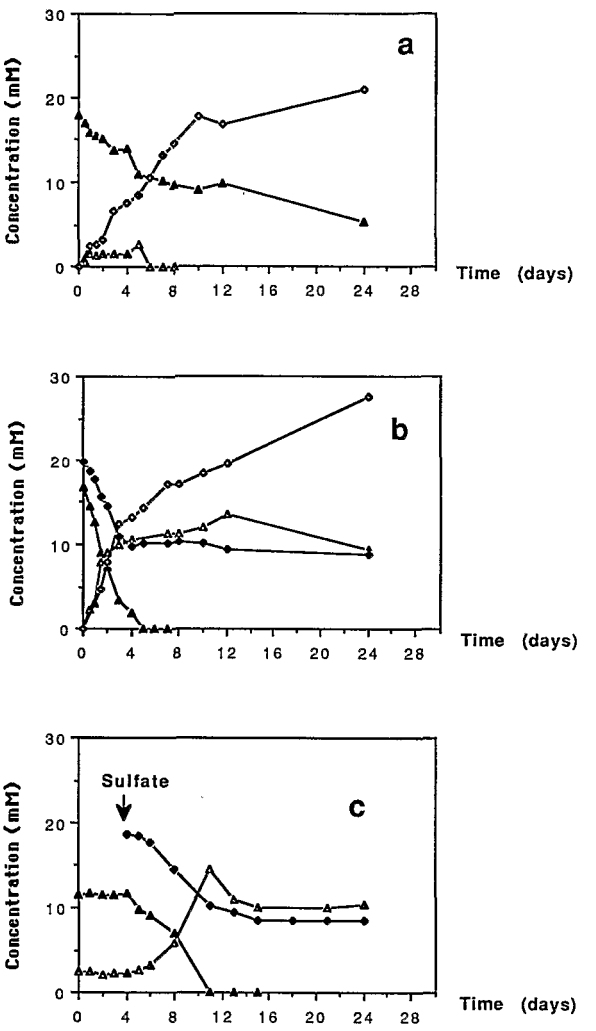

Fig. 2. Anaerobic propionate degradation by a mixed microbial culture in the absence of sulfate (*a*); in the presence of sulfate (*b*) and effects of sulfate addition on the anaerobic propionate degradation (*c*). Symbols: ▲, propionate; △, acetate; ◆, sulfate; ◇, methane.

filled with Chromosorb (SUPELCO Inc.), coated with 10% SP-1200 and 1% H_3PO_4, carrier gas: nitrogen; column temperature: 105°C; internal standard: ethyl 2-butyric acid; sulfate was quantified using the HPLC technique under the following conditions: pump, Shimadzu LC 6A: eluent, phtalic acid (3 mM) neutralised with Sodium-tetraborate at pH 4.9; flow rate, 2 ml mn^{-1}; injection loop, 100 μl; column, Vydac Anion Exchange Phase 302 IC (25 cm long); room temperature; conductivity detection was performed with a Shimadzu model CDD 6A detector; recorder integrator, Shimadzu chromatopak CR 3A; external standard; sulfide was determined spectrophotometrically as colloidal CuS (Cord-Ruwisch 1985); methane and hydrogen were measured by means of TCD gas chromatography INTERSMAT IGC 10 M. Gas separation was achieved with two successive columns: one stainles steel column 50 cm in length, 1/8″ in diameter, coated with silicagel 80/100 mesh, sep-

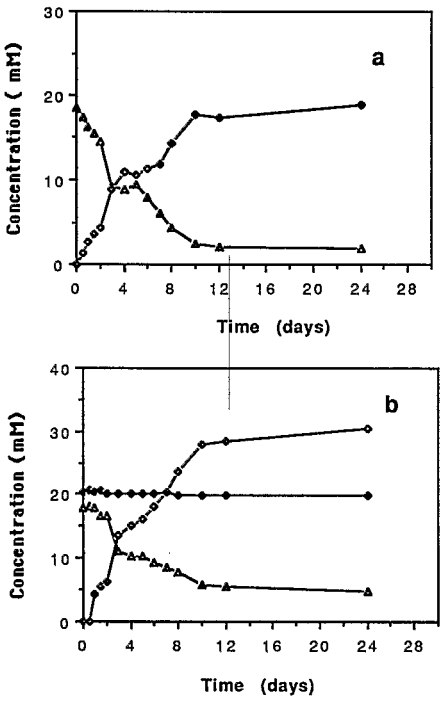

Fig. 3. Anaerobic acetate degradation by a mixed microbial culture in the absence of sulfate (*a*) and in the presence of sulfate (*b*). Symbols: △, acetate; ◆, sulfate; ◇, methane.

arated CO_2 from the remaining gas; one empty stainles steel column: 10 m in length, 1/8″ in diameter, was used to slow the gas elution in the detector; and one stainles steel column: 2 m in length, 1/8″ in diameter, coated with molecular sieve with a mesh size of 80/100 mesh, was used to separate out H_2 from CH_4; injection, 0.1 ml; carrier gas: argon 34 ml mn^{-1}; catharometric current intensity: 115 mA; external standard with known gas mixture.

Results

Lactate degradation

Lactate was rapidly degraded under all conditions (Fig. 1). Without sulfate (Fig. 1a) and with both sulfate and molybdate (Fig. 1b), acetate and propionate were produced by lactate decomposition in the ratio of approximately 0.6; on the other hand, no sulfate was utilized in the presence of molybdate, indicating that lactate degradation was largely due to fermentation. In the presence of sulfate, lactate degradation was accompanied by a concomitant consumption of the electron acceptor (Fig. 1c). This led to a shift in the ratio towards acetate; very little propionate was produced; these results suggested a high level of lactate utilization by sulfate-reducing bacteria. As shown in Fig. 1a and 1c, methane was not affected by the presence of sulfate, although less methane was produced in the presence of molybdate (Fig. 1b).

Propionate degradation

Experiments were performed in the absence or presence of sulfate without molybdate. The data on propionate degradation are given in Fig. 2; although the rate of propionate utilization by the mixed microbial population fluctuated somewhat from one experiment to another in the absence of sulfate, propionate oxidation proceeded at a slow rate and the amount of acetate accumulated was low (Fig. 2a). In the presence of sulfate, however, propionate oxidation was strongly affected (Fig. 2b, 2c); under these conditions, propionate was rapidly oxidized, sulfate was concomitantly consumed, and acetate formed. This clearly shows the effect of sulfate on propionate oxidation. Methane was produced even in the presence of sulfate (Fig. 2b). In some cases in the absence of sulfate, no propionate was oxidized during the 30-day incubation period. The differences between the propionate oxidation rates may be due to differences in the bacterial composition of the wastes.

Acetate degradation

As described in Fig. 3, acetate oxidation was independent of the presence of sulfate. The pattern of acetate oxidation was the same in the absence (Fig. 3a) and presence of sulfate without molybdate (Fig. 3b) and an equal amount of methane was produced in both cases. When sulfate was present without molybdate, it was not utilized even after 60 days of incubation. Furthermore, acetate oxidation

was never found to be dependent on sulfate reduction.

Effect of chloroform

To determine the relationship between sulfate reduction and methanogenesis, experiments were carried out in which chloroform was added to inhibit methanogenesis in the absence and presence of sulfate. As shown in Tables 1 and 2, methane production was inhibited when chloroform was added to the waste. When chloroform was added in the presence or absence of sulfate, methane production was suppressed and the acetate concentration in the waste was approximately the same at the end of incubation as at the beginning. In the presence of chloroform and in the absence of sulfate, hydrogen accumulated due to the decomposition of propionate, and disappeared when sulfate was added (Tables 1 and 2). The decomposition of lactate was not affected by chloroform addition. As in the case of propionate, with chloroform and without sulfate, hydrogen accumulated as the result of the decomposition of lactate, and disappeared when sulfate was added (Tables 1, 2).

Bacterial counts

As reported in Table 3, a total of approximately 10^{10} bacterial cells was counted per ml of the mixed culture using the acridine orange method. In low-sulfate tubes, 3.5×10^8 acetoclastic methanogens were counted per ml. In the last positive tubes, the only products were methane and presumably carbon dioxide. *Methanosarcina* and *Methanothrix* cells were observed by direct microscopic examination. In high-sulfate media, only 2.5×10^4 acetate-oxidizing sulfate reducers were counted per ml; the main microorganisms present were short rods, with rounded ends; they occurred singly or in pairs and looked like *Desulfobacter postgatei*. The degradation products were sulfide, methane and presumably carbon dioxide. In high-sulfate media, 4.5×10^8 lactate-utilizing sulfate reducers were counted per ml versus 2.2×10^7 lactate-fermenting per ml. Species of *Desulfovibrio* were identified as the main lactate degraders in the presence of sulfate, from their morphology and products (sulfide and acetate); whereas, in the absence of sulfate, *Propionibacterium* were observed in the last positive MPN tubes. 1.5×10^6 propionate-degrading sulfate-reducers per ml were counted; species of

Table 1. Effects of chloroform and sulfate addition on anaerobic acetate (lines 1–4), propionate (lines 5–8), and lactate (lines 9–12) degradation by a mixed microbial culture. Incubation was carried out at 37° C for 4 weeks.

Substrates	Concentration (mM)									
	Initial				Final					
	Lactate	Propionate	Acetate	Sulfate	Lactate	Propionate	Acetate	Sulfate	Methane	Hydrogen
C2	0	0	19.6	0	0	0	0	0	23.2	0
C2 + sul	0	0	19.2	16.4	0	0	0	14.4	22.5	0
C2 + chl	0	0	20.2	0	0	0	20.5	0	0	0
C2 + sul + chl	0	0	19.7	16.3	0	0	20	14.7	0	0
C3	0	18.4	0	0	0	7.6	0.6	0	22.7	0
C3 + sul	0	19.3	0	16.5	0	2.4	1.8	7.1	19.5	0
C3 + chl	0	19.2	0	0	0	18.9	0.8	0	0	0.6
C3 + sul + chl	0	19.2	0	16.1	0	8.2	10.9	7.9	0	0
Lac	21.4	0	0	0	0	2.4	0	0	29.1	0
Lac + sul	20.7	0	0	16.1	0	0	0	7.2	21.8	0
Lac + chl	21.1	0	0	0	0	15.8	10	0	0	0.5
Lac + sul + chl	20.5	0	0	16.5	0	0.8	24.9	4.5	0	0

Symbols: C2, acetate; C3, propionate; Lac, lactate; sul, sulfate; chl, chloroform.

Table 2. Effects of chloroform and sulfate addition on anaerobic lactate/acetate (lines 1–4), lactate/propionate (lines 5–8) and propionate/acetate (lines 9–12) degradation by a mixed microbial culture. Incubation was carried out at 37° C for 4 weeks.

Substrates	Concentration (mM)									
	Initial				Final					
	Lactate	Propionate	Acetate	Sulfate	Lactate	Propionate	Acetate	Sulfate	Methane	Hydrogen
Lac + C2	20.4	0	18	0	0	0	0	0	49.6	0
Lac + C2 + sul	20.2	0	18.9	15.9	0	0	0	6.3	50.7	0
Lac + C2 + chl	20.4	0	17.5	0	0	15	28.5	0	0	2
Lac + C2 + sul + chl	19.9	0	19.4	16.5	0	0.8	43.7	4	0	0
Lac + C3	20.8	21	0	0	0	0	0	0	71.9	0
Lac + C3 + sul	20.2	19.8	0	16.5	0	0.5	0.6	0	54.5	0
Lac + C3 + chl	20.6	18.8	0	0	0	34.9	9.6	0	0	2.4
Lac + C3 + sul + chl	20.6	19.3	0	15.6	0	16.4	27.6	1.5	0	0
C2 + C3	0	19.8	18.5	0	0	4.2	0.2	0	47	0
C2 + C3 + sul	0	19.4	20.5	15.7	0	0	6.2	4.7	41.1	0
C2 + C3 + chl	0	19.8	19.8	0	0	18.9	19.5	0	0	0.5
C2 + C3 + sul + chl	0	19.4	20.2	15.7	0	13.7	24.7	10.9	0	0

Symbols: C2, acetate; C3, propionate; Lac, lactate; sul, sulfate; chl, chloroform.

Desulfobulbus were observed in the last positive MPN tubes in which sulfide, acetate and presumably carbon dioxide were produced. 4.5×10^8 H_2-utilizing methanogenic bacteria were counted per ml; and the prevalent hydrogen-oxidizing methanogen was a straight, short, non-motile rod similar to those of the *Methanobacterium* species.

Table 3. Bacterial counts of the trophic groups involved in anaerobic l actate degradation by the mixed culture.

Substrates	Bacterial counts (viable cells/ml)	Products of degradation
− sulfate		
Lactate	2.2×10^7	Propionate + acetate
Propionate	nd	nd
Acetate	3.5×10^8	Methane
H/CO	4.5×10^8	Methane
+ sulfate		
Lactate	4.5×10^8	Acetate + sulfide
Propionate	1.5×10^6	Acetate + sulfide
Acetate	2.5×10^4	Sulfide + methane
Total flora	10^{10}	nd

nd, not determined.
Bacterial groups were counted using the most probable number (MPN). Total flora was using the acridine orange method (Hobbie et al. 1977). Tubes were incubated at 37° C for 4 weeks.

Discussion

During anaerobic digestion of wine distillery waste waters, VFA (mainly acetate and propionate) accumulate at concentrations higher than those pertaining in other anaerobic habitats such as marine or lake sediments. Their degradation plays a prominent part in the stability of the overall process (Mc Carty 1977; Zehnder 1978; Mackie & Bryant 1981). *Syntrophobacter wolinii* grows very slowly on propionate, when it is cocultured with *Methanospirillum hungatei* (Boone & Bryant 1980). SRB of the genus *Desulfovibrio* may replace the methanogens as hydrogen-consuming organisms in syntrophic cultures with hydrogen-producing, acetogenic bacteria in the presence of sulfate (Abram & Nedwell 1978a, b; McInerney et al. 1979). On the other hand, in sulfate-rich anaerobic ecosystems, sulfate reduction constitutes the final step instead of methanogenesis, with acetate and hydrogen as electron donors (Jørgensen 1977, 1982; Mountfort et al. 1980). Sulfate reducers prevent methane production from hydrogen and acetate by maintaining them at concentration levels that are too low for them to be metabolised by methanogenic bacteria

(Winfrey & Zeikus 1977; Lovley et al. 1982; Lovley & Klug 1983; Lovley 1985).

The rate of lactate metabolism was expected to vary depending on whether sulfate was absent or present. In fact, in the absence of sulfate, lactate was fermented to acetate and propionate, according to the usual fermentation pattern of *Propionibacterium*, whereas in the presence of sulfate, the products were acetate and sulfide. This suggests that lactate-utilizing species of *Desulfovibrio* may outcompete the *Propionibacterium*, which was confirmed by the bacterial counts. Furthermore, among lactate-utilizing bacteria, species of *Desulfovibrio* might be involved in lactate degradation by interspecies hydrogen transfer (Bryant et al. 1977).

Oxidation of propionate was found to be strongly dependent on sulfate reduction. It proceeded only at a very slow rate without added sulfate, but accelerated considerably in the presence of sulfate. This relationship was also observed with animal waste (Ueki et al. 1986). Propionate was oxidized even in the presence of chloroform when sulfate was added to the waste. The accumulation of propionate after addition of chloroform in the absence of sulfate indicates that the oxidation of propionate in the absence of sulfate probably depends on *S. wolinii*, which is known to oxidize propionate only in the presence of hydrogen-utilizing bacteria (Mackie & Bryant 1981; Koch et al. 1983; Mucha et al. 1988; Robbins 1988), and involves a low number of syntrophs. This was expected to be the case since propionate is generally assumed to be an important intermediate in our anaerobic fermenter.

Acetate degradation is independent of sulfate reduction. When chloroform and sulfate were added simultaneously to the waste, the sulfate was reduced slowly and acetate was not degraded. On the other hand, when chloroform was not added, methane was produced and acetate completely disappeared. This was confirmed by the very low number of acetate-oxidizing sulfate reducers. This indicates that acetate plays only a minor role if any as electron donor for sulfate-reducing bacteria at 37° C. These results are in agreement with those obtained by several authors (Ueki et al. 1986; Isa et al. 1986a, 1986b; Motoyuki et al. 1987). The low number of acetate-oxidizing sulfate-reducers was probably not due to a lysis induced by the temperature used, but rather to the low affinity of these bacteria for acetate at this temperature. The isolation and characterization of the main sulfate-reducing bacteria using lactate and propionate from the positive tubes will be described elsewhere (Quatibi 1990).

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