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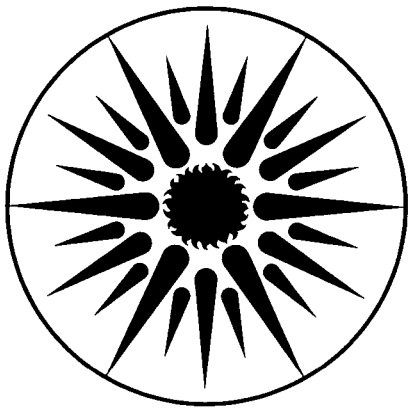

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BACTERIAL CHROMATE REDUCTION AND PRODUCT CHARACTERIZATION

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KWIC Index: *Bacillus subtilis*, chromate reduction, Cr(V) detection, Cr(III) accumulation, chromium speciation, chromate bioremediation

ABSTRACT

Bacillus subtilis reduced hexavalent chromate to trivalent chromium under either aerobic or anaerobic conditions. Reduction of Cr(VI) and appearance of extracellular Cr(III) were demonstrated by electron spin resonance (ESR) and spectrophotometry. Chromate reduction was stimulated more than five-fold by freeze-thawing, indicating that intracellular reductases or chemical reductants reduce chromate more rapidly than do intact cells. Moderately concentrated cells (10% pellet volume after centrifugation) reduced approximately 40 μM chromate/min (2 mg Cr/l·min) when exposed to 100 μM

chromate (5 mg Cr/l). Highly concentrated cells (70% pellet volume) reduced more than 99.8% of 2 mM chromate (100 mg Cr/l) within 15 min. This rate of chromate reduction was of the same order of magnitude as the rate of respiration in aerobic cells. A substantial fraction of the reduction product (ca. 75%) was extracellular Cr(III), which could readily be separated from the cells by centrifugation. At high chromate concentrations, some fraction of reduced Cr(VI) appeared to be taken up by cells, consistent with a detection of intracellular paramagnetic products. At low chromate concentrations, undefined growth medium alone reduced Cr(VI), but at a slow rate relative to cells. Under appropriate conditions, *B. subtilis* appears to be an organism of choice for detoxifying chromate-contaminated soil and water.

INTRODUCTION

Chromate, i.e. hexavalent chromium (Cr(VI)), is an established human carcinogen, whose modes of action may include a catalysis of free radical reactions and crosslinking of DNA (Shi et al. 1990). Cr(VI) is generally a potent microbial toxin, although examples of resistant microbes are known. The stable reduction product of chromate, the chromic Cr(III) species, generally shows considerably less toxicity. Several aerobic and anaerobic micro-organisms are known to reduce chromate (Ishibashi et al. 1990). It has been suggested that such organisms may have utility in environmental restoration. Here we show that *Bacillus subtilis*, a widely distributed aerobic soil bacterium posing no known hazards to humans and capable of growing under a variety of conditions, is a potent chromate reducing, and hence, detoxifying, organism.

MATERIALS AND METHODS

ESR assays:

Although chemical species-specific chromium assays with indicator dyes have been applied to the study of microbial chromate metabolism (Ishibashi et al. 1990), these methods do not lend themselves to studies of high chromate exposure levels, where uncharacterized complexes of metabolic products, e.g., complexed Cr(III) species may interfere with the absorbance measurements. To overcome this potential problem and to provide tools for analyzing the localization of chromate metabolism we developed and applied to *B. subtilis* a variety of novel electron spin resonance (ESR) assays . Included was an assay for chromate ions, capable of detecting as little as 5 μg Cr/l in buffer solutions, described in more detail below. We also developed a less sensitive assay for chromic ions (sensitivity about 10 mg Cr/l), assays for both intra- and extracellular univalent reductase activities, and an assay for intracellular paramagnetic species, suitable for detecting the accumulation of chromic ion complexes. These assays were supplemented by previously established ESR methods for measuring cell volumes, spectrophotometric analyses of chromate and EDTA-chelated Cr(III) as well as x-ray fluorescence (XRFS) measurements of total chromium and other trace elements.

Cell preparations

Cells grown to an optical density of 125 Klett units in an enriched medium (modified from Pipper et al 1977 by dilution with 1 vol deionized water) were packed by centrifugation, resuspended in about 10 volumes of fresh growth medium (referred to as 10% pellet volume cells), and used for all experiments except where an additional centrifugation step was employed to increase cell density. After harvesting the cells were

maintained on ice for up to 1 hour before transfer to the ESR laboratory. Thereafter the cells were stirred on ice. Typically 1 ml samples were withdrawn from the stirred suspension with a micropipettor and treated with chromate or other reagents in a 1.5 ml Eppendorf tube.

ESR measurement of Cr(VI)

The Cr(VI) assay consisted of analyzing a transient paramagnetic Cr(V)-complex that arises from the univalent reduction of Cr (VI) by thioglycerol and complexing of Cr(V) by glycerol and thioglycerol. Reaction cocktails for the reduction and complex formation, designated as RC1 and RC2, respectively, were developed empirically to avoid a suppression of the ESR species by reaction intermediates and products. RC1 consists of 0.5 M $NaHCO_3$, 0.1 M sodium phosphate, pH 7.4, while RC2 consists of 0.4 M glycerol and 20 mM thioglycerol in 10 mM sodium phosphate, pH 7.4. The assay consisted of treating 25 µl of an unknown sample with 0.5 µl of 10 mM sodium ferric-EDTA, 5 µl of RC1 and 20 µl of RC2 and placing the mixture into a 100 µl glass capillary for ESR analysis. The ESR signal arising from the reaction was scanned at 2 min intervals to determine its maximum magnitude. Quantification of chromate was relative to ESR signals arising from defined additions of potassium chromate to the unknown solution (recovery experiment). In some experiments, larger ESR signals were obtained by replacing thioglycerol in RC1 by the reduced nitroxide TOLH (1,4-dihydroxy-2,2,6,6-tetramethyl piperidine).

RESULTS

Effect of chromate on growth of *B. subtilis* and elimination of chromate from aerobic growth media

Cultures of *B. subtilis* grew normally in the presence of 50 μM K_2CrO_4 (2.5 mg Cr/l) and suffered only modest growth inhibition in the presence of 0.5 mM K_2CrO_4 (Fig 1). ESR analyses of supernatant fractions from centrifuged cells grown with 50 μM K_2CrO_4 indicated that more than half of the chromate had been removed from the batch culture media during the logarithmic phase of growth. The supernatant fractions of cells grown for 2 hrs with 50 μM K_2CrO_4, contained 18 μM residual Cr(VI) (1 mg Cr/l). There was no detectable loss of Cr(VI) in parallel samples of uninoculated growth media. Supernatant fractions of cells grown to stationary phase with 25 μM K_2CrO_4 (3 hours of growth) contained less than 0.15 μM chromate (8 μg Cr/l). Parallel samples of uninoculated growth media contained 15 μM residual chromate. Thus reduction of low chromate concentrations by growth medium can be detectable and may have to be analyzed separately to accurately quantify cellular chromate reduction rates at low chromate levels (ca 1 mg Cr/l). At 0.5 mM K_2CrO_4, the chromate concentration in cell supernatant fractions did not change appreciably during the 2 hour logarithmic growth period. Residual chromate was quantified by recovery experiments (linear extrapolation of ESR signals similar to those shown in Fig 2), since the magnitudes of the ESR signals were affected by changes in the medium during cell growth. Analysis of total chromium concentrations by XRFS after centrifugation indicated that the chromium concentration in cell pellets was about the same as the initial chromium concentration in the medium (Table I, after correcting for the effects of lyophilization and allowing for variability in pellet density). Washing removed much of this chromium, indicating that the bulk of the reduction product during logarithmic growth (low cell density) was present in the

extracellular domain. Since the cells were grown under aerobic conditions, these results indicate that chromate detoxification by *B. subtilis* is feasible in aerobic environments.

Chromate reduction by anaerobic suspensions of concentrated cells

To gain insight into the potential extent of chromate reduction, we studied concentrated cells by ESR. Cells that had been stirred on ice for about six hours after harvest (500 µl), were pelleted by 2 min centrifugation at 10,000g, diluted with 200 µl of growth medium (70% cell pellet) and treated with 2 mM K_2CrO_4 (100 mg Cr/l). ESR analysis of the residual chromate showed that these cells had removed more than 99.8% of the chromate from the extracellular aqueous phase within 15 min (Fig 2). The final chromate concentration in the supernatant fraction, designated by "0", was below detectability; therefore the residual chromate levels may have been substantially lower than the level shown in sample "1", which had been treated with 1 µM (50 µg Cr/l) fresh chromate. Visual observation supported the ESR assays, i.e., the yellow color seen in the ESR capillaries containing freshly chromate-treated cell suspensions had faded over a period of several minutes and assumed a grayish hue when chromate became undetectable by ESR. Polarographic measurements showed that respiratory activity in these cells consumed dissolved oxygen in less than one min; thus most of the chromate removal occurred under anaerobic conditions. This finding establishes the feasibility of chromate removal with anaerobic *B. subtilis*. Visual observation also indicated that the rate of chromate fading increased as a function of incubation time of the cell suspension on ice.

Extracellular nitroxide reductase activity -- inhibition by chromate

The extensive chromate reduction seen in *B. subtilis* was surprising in light of the well-documented toxicity of chromate for many cells. This finding suggested that the lack of chromate toxicity might be due to an exclusion of chromate ions by the cells and would imply that chromate was removed by to an extracellular reduction system. To test this hypothesis, we applied a nitroxide ESR assay to extracellular univalent reductases. The ESR assay utilized a membrane-impermeable nitroxide probe, PECU-Glucam, a persistent free radical derivative of glusosamine previously developed for membrane permeability studies with erythrocytes and other cells (Mehlhorn & Packer 1983) Univalent reduction of this probe eliminates its ESR signal. To confirm that this probe did not enter *B. subtilis*, we utilized published methodology for quantifying intracellular aqueous volumes (Mehlhorn & Packer 1983). No evidence of an ESR signal of this probe was evident when cells were treated with the extracellular line-broadening agent Mn-EDTA, consistent with the expectation that this probe is impermeable to membranes of *B. subtilis*.

Supernatant solutions from centrifuged cells (pelleted within three hours of harvesting) did not reduce the nitroxide appreciably, indicating negligible nitroxide reductase activity by growth medium or by substances released from cells. In cell suspensions not supplemented with chromate, the nitroxide was reduced at a rate of 7.6 μM/min, corresponding to about 6% of the rate of respiratory oxygen consumption. In the tenth-millimolar concentration range of chromate, nitroxide reduction was transiently inhibited; then nitroxide was reduced at the same rate as had been observed in the absence of chromate (Fig 3A). If the nitroxide was incubated with cells in the absence of chromate until the ESR signal had been eliminated, the signal could be fully restored by treatment with chromate, indicating that the nitroxide reduction product was reoxidized

by chromate. The lag phase in nitroxide reduction was a function of the chromate concentration (Fig 3B), consistent with a rapid reoxidation of reduced nitroxide by chromate until virtually all of the chromate had been depleted. By relating the rate of chromate reduction, estimated from the lag phases, to an ESR analysis of cell volumes, a chromate reduction rate of 2.5 nM/min per μl of cell volume was calculated for a chromate concentration of 100 μM (5 mg Cr/l). This rate of chromate reduction is comparable to the rate of nitroxide reduction (see also Fig. 3A) and one might assume that the nitroxide mediated chromate reduction under these conditions. However, at higher chromate concentrations, the chromate reduction rate was significantly greater than the reduction rate for the nitroxide (Fig 3A vs. 3B), suggesting that most of the chromate reduction occurred independently of the nitroxide. The increasing rate of chromate reduction with increasing chromate concentrations (Fig 3B) suggests that the putative reductase activity was not saturated at the lower chromate concentrations.

The lag phase in nitroxide reduction was decreased more than five-fold when cells were freeze-thawed (three cycles of freezing in liquid nitrogen and thawing at 25°C). Thus intracellular reducing agents have considerably more chromate reducing activity than does the putative extracellularly oriented reductase.

As noted earlier, virtually complete elimination of 2 mM chromate (100 mg Cr/l) by highly concentrated cells occurred within 15 min, a significantly faster rate of chromate disappearance than the 20 min time frame for chromate reduction in the submillimolar range shown in Fig 3B. The primary reason for this difference is probably the higher cell density of cells used for removing the 2 mM chromate (70% vs. 10% cell pellets). As noted above, aged concentrated cells exhibited higher rates of chromate reduction than cells tested shortly after harvesting; this may have played a role in the

extensive chromate reduction observed with the highly concentrated cells (less than 0.2% residual chromate).

Appearance of extracellular Cr(III)

Although it seemed likely that the mechanism effecting chromate disappearance was an extracellular reduction of Cr(VI) to the stable reduction product Cr(III), direct evidence for the formation of this species was desirable. Since previous ESR studies had identified Cr(III) trioxalate as an effective line-broadening agent for nitroxide free radicals, we pursued an analytical strategy based on this observation. When stock solutions of $CrCl_3$ were freshly diluted into 0.1 M sodium oxalate containing an ^{15}N perdeuterated nitroxide (characterized by narrow intrinsic line widths and therefore very sensitive to line broadening) a time dependent line broadening of the nitroxide was observed, with the linewidth attaining its maximum value in about 10 min. The increase in linewidth was proportional to the concentration of Cr(III) and was detectable at about 0.5 mM $CrCl_3$ (25 mg Cr/l). We assumed that a time- and chromate treatment-dependent increase in linewidths of the nitroxide after incubation of cell supernatant fractions with sodium oxalate would be indicative of Cr(III). Application of this assay to centrifuged chromate-treated cells yielded line broadening consistent with a reduction of chromate to Cr(III), of which a substantial fraction remained in the extracellular aqueous phase.

Detection of intracellular chromic ions

ESR volume measurements of chromate-treated cells showed a decrease of the intracellular signal intensities, which was accompanied by an increase in linewidths (Fig 4). The maximum magnitude of the linewidth increase was a function of the chromate concentration, consistent with a conversion of chromate to an intracellular paramagnetic

chromium species. It is noteworthy that ESR volumes were not eradicated by any of the tested chromium concentrations, i.e., the observed linebroadening (two-fold) was consistent with the lineheight decreases (four-fold) in the first-derivative ESR spectra. This observation indicates that a substantial fraction of the *B. subtilis* membranes remained intact throughout this chromate treatment.

Spectrophotometric assays of Cr (VI) loss and Cr(III) appearance

The observation of a fading of the yellow chromate color observed in the ESR experiments was pursued with a spectrophotometric study of chromate-treated *B. subtilis* (ca. 30% cell pellet volume) by use of a spectrophotometric assay of the EDTA complex of Cr(III) (I. Fry and R. Mehlhorn, unpublished). After a 20 min incubation period with 2 mM K_2CrO_4 (100 mg Cr/l) cells were centrifuged and supernatant solutions were analyzed spectrophotometrically. A residual chromate concentration of 250 μM was estimated (absorbance at 350 nm, corrected for absorbance by non-chromate components of growth medium). Thereafter the supernatant solutions were treated with 20 mM EDTA, pH 7.0, incubated for three days at room temperature and the absorbance at 550 nm was determined to estimate a Cr(III)-EDTA concentration of 1.5 mM. These results support the interpretation that the major species at the beginning and end of the incubation periods were Cr(VI) and Cr(III), respectively. It can also be concluded that the bulk of the chromate reduction product appears in the extracellular phase. This creates the opportunity for developing schemes for chromium immobilization, e.g., using ion exchange matrices (work is in progress).

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Fig 1. Growth curves of *B. subtilis* in the absence and presence of chromate. Control (■), 50 μM K_2CrO_4 (2.5 mg Cr/l;◊) and 0.5 mM K_2CrO_4 (25 mg Cr/l;♦).

Fig 2. ESR assay demonstrating disappearance of chromate from aqueous solution by concentrated suspensions of *B. subtilis.* Concentrated cells (yielding a 70% packed volume after 2 min centrifugation at 10,000 g) were treated with 2 mM K_2CrO_4 for 15 min, diluted two-fold with 50 mM $MgCl_2$, 10 mM TRIS-HCl, pH 7.0, centrifuged 2 min at 10,000 g and the supernatant solution was assayed for chromate. The supernatant solution was treated with the thioglycerol reaction mixture (see Methods and Procedure) and treated with K_2CrO_4 (recovery experiment) yielding 0, 1, 2, or 3 μM of freshly added chromate (0, 50, 100, 150 μg Cr/l), as indicated. Magnetic field interval shown in millitesla (mT).

Fig 3. Kinetics of chromate reduction by *B. subtilis*, deduced from the inhibition of the reduction of the membrane-impermeable nitroxide PECU-Glucam by chromate. (A) ESR signals of the probe as a function of time showing lag in nitroxide reduction in the

presence of 200 μM chromate (10 mg Cr/l); (B) Lag phase in nitroxide reduction vs. chromate concentration.

Fig 4. Appearance of intracellular paramagnetic species in *B. subtilis* treated with 5 mM K_2CrO_4 (250 mg Cr/l). *B. subtilis* cells (internal aqueous volume of 1.7%) were treated with 100 μM $^{15}N,^{2}H$ Pyrad, 4 mM $K_3Fe(CN)_6$, 90 mM $(Me_4N)_2$Mn-EDTA. (A) two scans without chromate, successive spectra shifted to the left, 2 min between scans, instrument gain $2.5x10^4$; (B) two scans after treatment with 5 mM K_2CrO_4, instrument gain $2.5x10^4$; 2 min between scans, (C) four more scans of chromate-treated samples; instrument gain $2.5x10^4$, 4 min between scans.

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Table I. Trace elements (μg/g dry weight) of pelleted *Bacillus subtilis* grown in chromate solutions*

Element	Control (5/28/92)	2.5 mg Cr/l chromate grown	2.5 mg Cr/l chromate & wash	Control (6/2/92)	2.5 mg Cr/l chromate grown	25 mg Cr/l chromate grown
Ti	8±4	<12	13±4	<12	<12	<12
Cr	<5	53±3	3.7±1.4	2.8±1.4	27±2	1010±50
Mn	33±2	32±2	33±2	33±2	35±2	40±3
Fe	48±2	42±2	44±2	112±6	33±2	33±2
Ni	1.3±0.3	0.7±0.3	<0.9	<0.9	<0.9	0.4±0.3
Cu	8.2±0.5	6.9±0.3	5.1±0.3	9.7±0.5	5.5±0.3	4.5±0.3
Zn	66±3	68±3	61±3	77±4	84±4	95±4
Se	0.3±0.1	0.4±0.1	0.7±0.1	0.3±0.1	0.4±0.1	0.5±0.1
Br	2.1±0.1	2.0±0.1	6.6±0.3	0.4±0.1	0.4±0.1	0.2±0.1
Rb	13.3±0.7	12.5±0.6	12.7±0.6	24±1	16±1	22±1
Sr	3.4±0.3	3.9±0.3	5.0±0.3	3.0±0.3	3.9±0.3	3.8±0.3
Pb	<2	<2	<2	0.7±0.5	<2	0.6±0.5

*Trace elements were determined by X-ray fluorescence. Columns 2-4 and columns 5-7 refer to two separate experiments. Wash refers to centrifuged cells after resuspension in chromate-free medium.

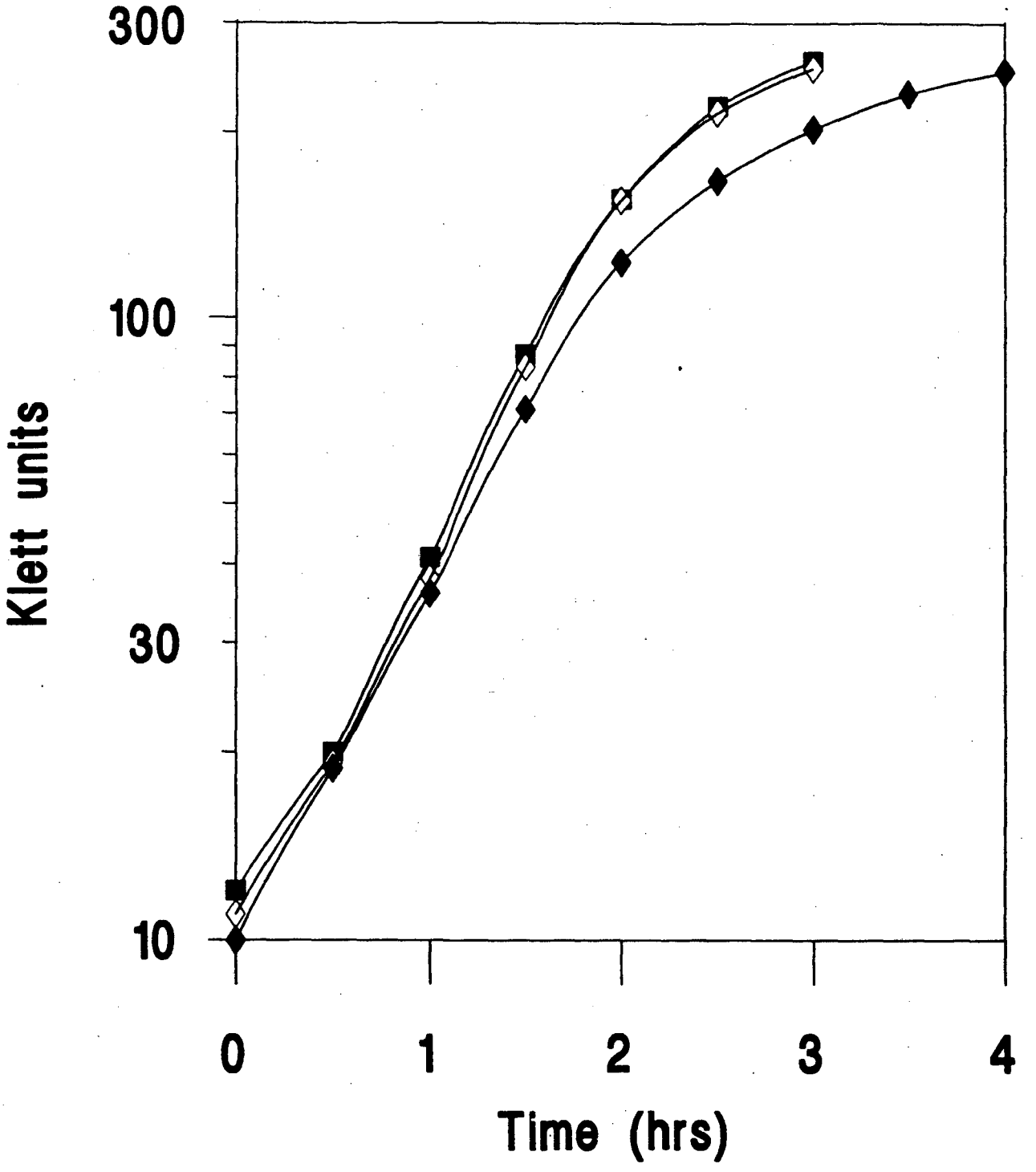

Figure 1

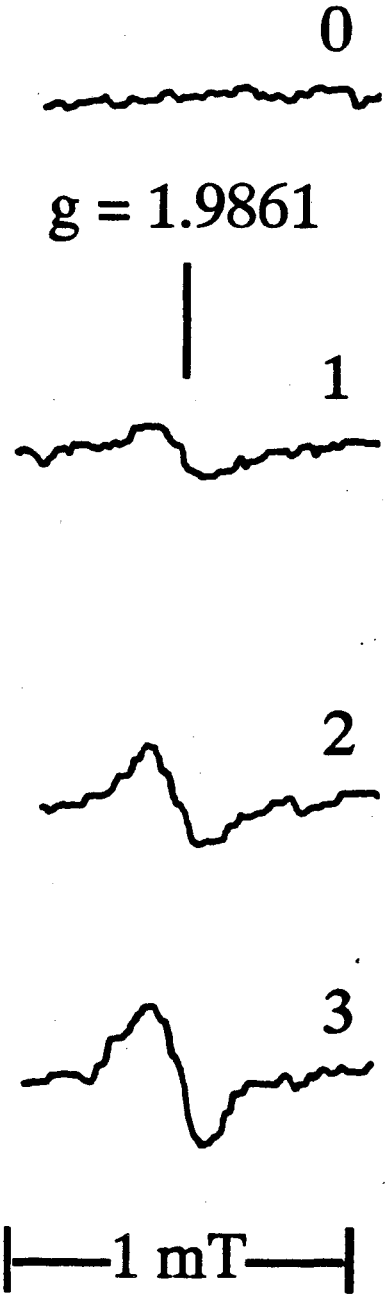

Figure 2

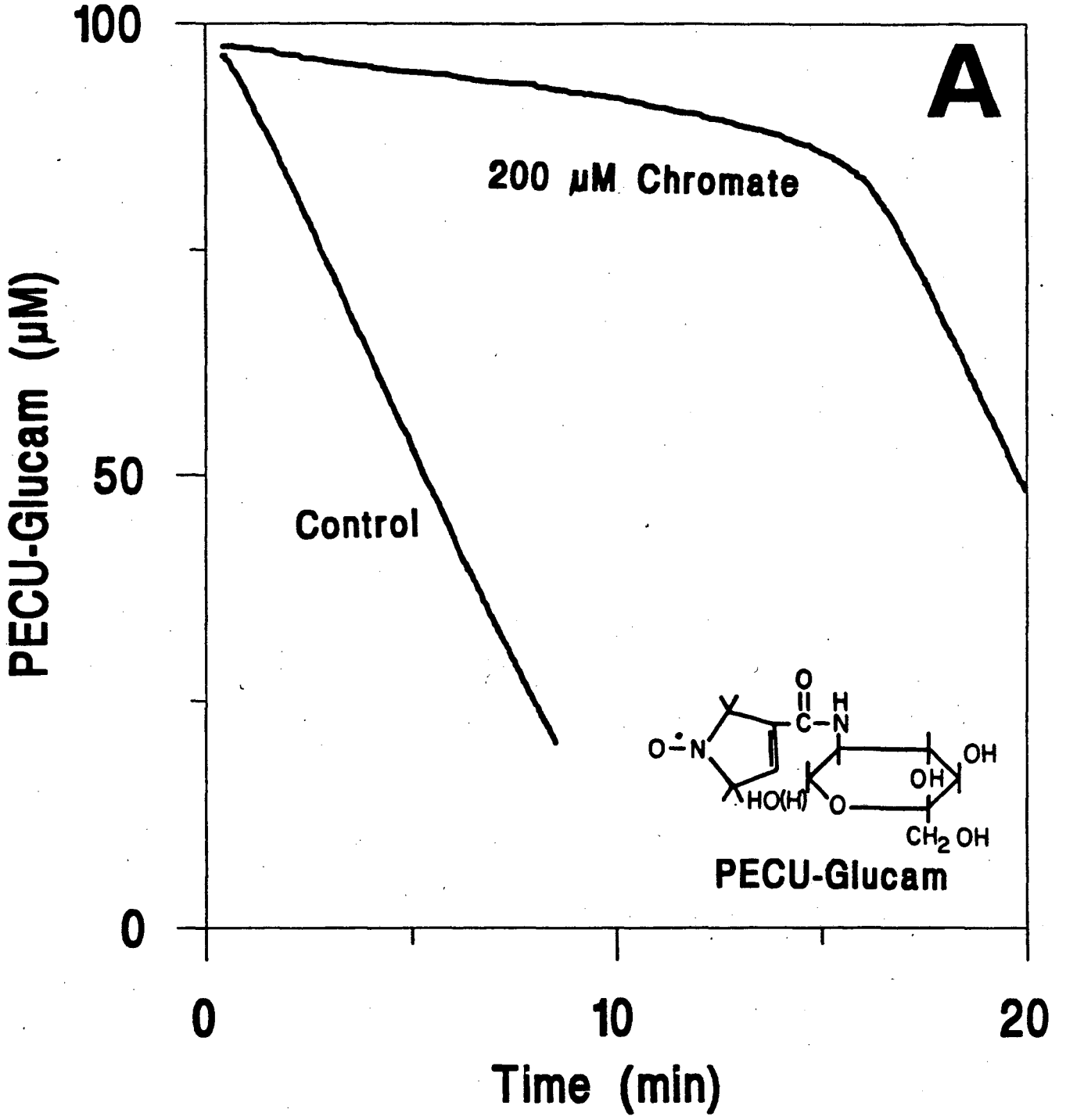
100
50
0
A
200 µM Chromate
Control
PECU-Glucam (µM)
0
10
20
Time (min)
PECU-Glucam

Figure 3A

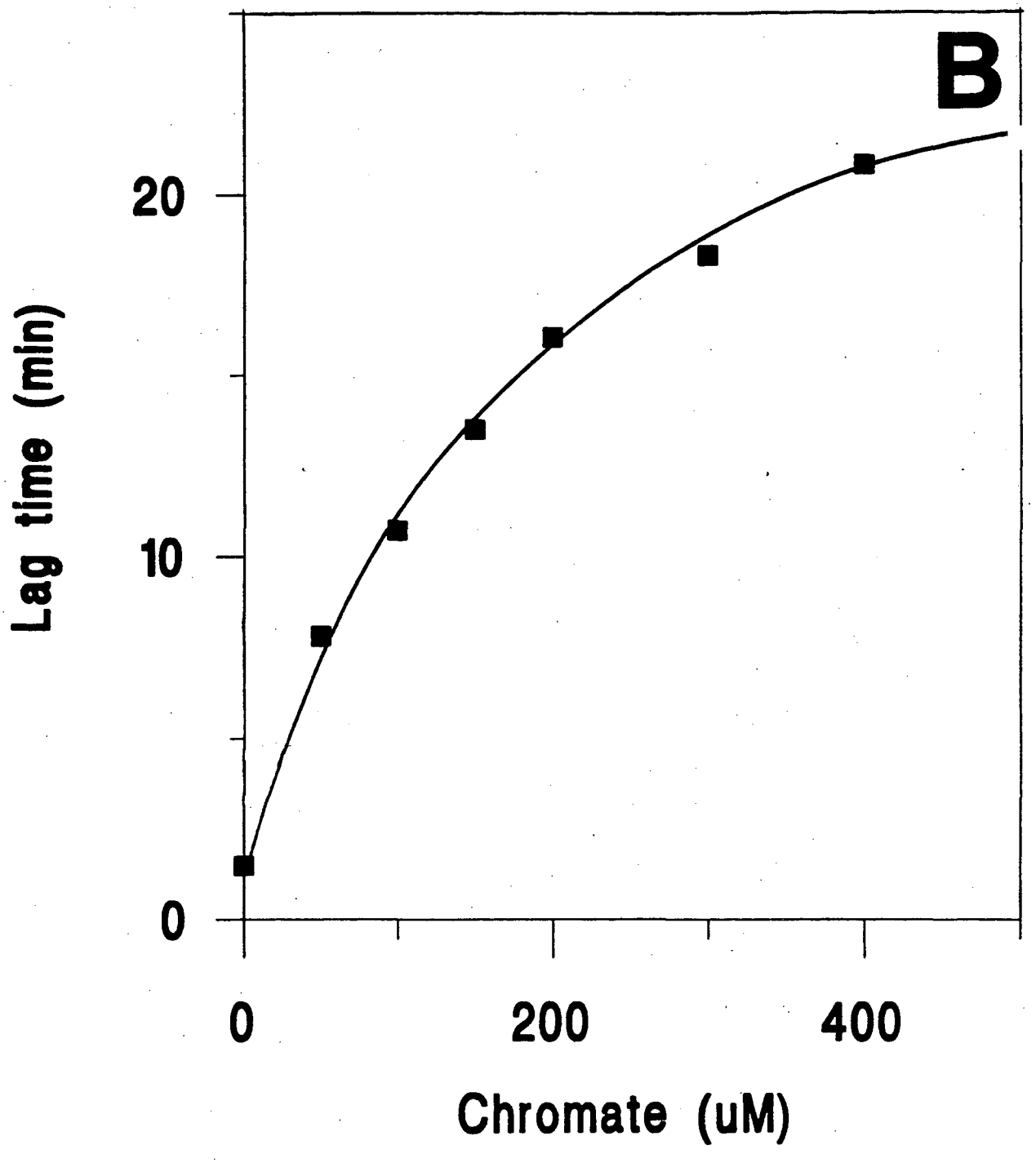

Figure 3B

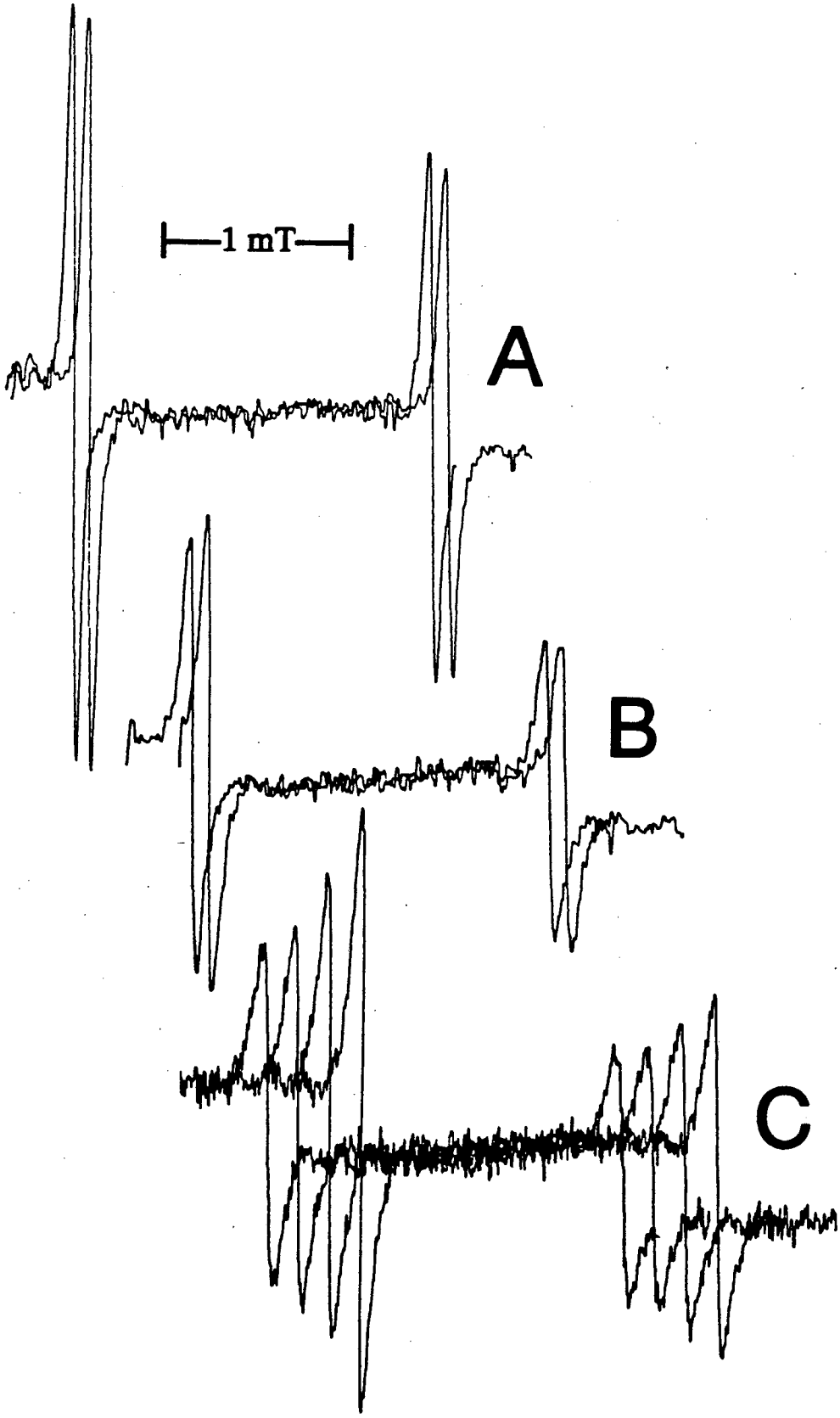

Figure 4

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