Mechanism of Growth Stimulation of L1210 Cells by 2-Mercaptoethanol

in Vitro

ROLE OF THE MIXED DISULFIDE OF 2-MERCAPTOETHANOL AND CYSTEINE

Tetsuro Ishii, Shiro Bannai, and Yoshiki Sugita

From the Division of Biochemistry, Tsukuba University School of Medicine, Sakura, Niihari, Ibaraki 305, Japan

(Received for publication, May 1, 1981)

The mechanism of the growth-promoting action of 2-mercaptoethanol on mouse lymphoma L1210 cells in vitro has been studied. The normal line of L1210 is deficient in its capacity to take up cystine, and this limits the growth of the cells in normal culture media containing cystine. In the presence of 2-mercaptoethanol, the cells obtain a high capacity to utilize cystine in the medium. The reaction of 2-mercaptoethanol with cystine produces a mixed disulfide of 2-mercaptoethanol and cysteine. The mixed disulfide is taken up by the cells mainly via the L system, a transport system for neutral α-amino acids such as leucine. The mixed disulfide within the cells is rapidly reduced to produce cysteine and 2-mercaptoethanol which escapes rapidly into the medium and reacts with cystine again. With the aid of this cyclic action of 2-mercaptoethanol, the cells are able to utilize cystine constantly.

Thiol compounds have beneficial effects on some types of mammalian cells cultured in vitro. One of the most effective and frequently used thiol compounds is 2-mercaptoethanol. In lymphocyte cultures, 2-mercaptoethanol enhances viability (1, 2), antibody formation (2), and blast transformation (3). Other reports describe enhancement by 2-mercaptoethanol of the growth or colony formation of various types of murine (1, 4-8) and human (9-11) cells in vitro. In most cases, 2-mercaptoethanol is effective at concentrations from 10 to 100 μM, and its action resembles those of macrophages or feeder layer cells (6, 12, 13). The mechanism of action of 2-mercaptoethanol has, however, not been established.

The normal line of mouse lymphoma L1210 is one of the most extensively studied cell lines with respect to its thiol-dependence in vitro (1, 4, 7, 13-15). L1210 cells require cyst(e)ine for survival and growth, because they have a low capacity to synthesize cysteine (16). Cysteine is utilized by L1210 cells, but it is easily oxidized to cystine in the culture medium. In a previous paper (17) it has been shown that L1210 cells are deficient in their capacity to take up cystine and that the cellular cysteine and glutathione contents decrease considerably in normal culture media containing cystine. A variant cell line of L1210, which adapts and grows in normal culture media, has a high capacity to take up cystine (17). The feeder layer cells promote growth of L1210 cells by supplying cysteine continuously (17). In the presence of 2-mercaptoethanol, the cells constantly utilize cystine in the medium and the cellular cysteine and glutathione levels are maintained during cell culture (17). This effect seemed to be the most crucial function of 2-mercaptoethanol in stimulating growth of the cells and was not explained by mere reduction of cystine to cysteine in the culture medium (17).

In this report, the more precise action of 2-mercaptoethanol on L1210 cells has been studied using [2-35S]mercaptoethanol and [35S]cystine. We show here that the mixed disulfide of 2-mercaptoethanol and cysteine, which is formed by the reaction of 2-mercaptoethanol with cystine in the medium, plays an important role in the action of 2-mercaptoethanol.

EXPERIMENTAL PROCEDURES

Materials—L-[35S]Cystine (125 mCi/mmol), 2-[35S]mercaptoethanol (21 mCi/mmol), and [3]H]leucine (120 Ci/nmol) were purchased from the Radiochemical Center (Amersham); calf serum and culture medium were from Grand Island Biological Co., bovine albumin (Fraction V from plasma) was from Armour Pharmaceutical Co., cellulose thin layer plates were from Funakoshi Pharmaceutical Co. (Tokyo), 2-mercaptoethanol was from Wako Chemical Co. (Tokyo), and anti-bovine albumin serum was from Fujizoki Pharmaceutical Co. (Tokyo). The mixed disulfide of 2-mercaptoethanol and cysteine was prepared as follows. Cystine (1.5 g), 2-mercaptoethanol (2 ml), and distilled water (200 ml) were mixed in a glass bottle. The pH of the mixture was adjusted to 8.0 with NaOH, and it was stirred by pumping humidified air during the reaction. After several days at room temperature, the pH of the mixture was lowered to 5 with HCl. Insoluble cystine was removed by centrifugation, and the supernatant was evaporated to dryness at 60 °C. The soluble fraction was evaporated and dissolved in 10 ml of 75% (v/v) aqueous phenol. The solution was layered on a cellulose column (2.5 × 45 cm) (Whatman CF 11) which had been equilibrated with the 75% aqueous phenol. The column was eluted with the 75% aqueous phenol and about 5 ml of fractions of the effluent were collected. The fractions were analyzed for amino acids by cellulose thin layer chromatography. The mixed disulfide was eluted from the column faster than cysteine and cystine. The fractions that contain the mixed disulfide were combined and an equal volume of distilled water was added. The phenol was removed by extraction with an equal volume of benzene, repeated three times. The mixed disulfide was then evaporated to dryness at 60 °C and dissolved in 5 ml of distilled water. The solution was mixed with 15 ml of ethanol and stored at 4 °C for 1 day to precipitate the mixed disulfide. The white precipitate was collected by filtration and the precipitation with ethanol was repeated. The product (about 100 mg) was washed with 75% ethanol, dried at 45 °C, and stored at 4 °C. The purified material gave a single spot on cellulose thin layer chromatography developed in different solvents. The result of the element analysis of the sample was as follows:

<table>
<thead>
<tr>
<th>Element</th>
<th>Calculated</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>30.44</td>
<td>30.52</td>
</tr>
<tr>
<td>H</td>
<td>5.62</td>
<td>5.50</td>
</tr>
<tr>
<td>N</td>
<td>17.0</td>
<td>7.08</td>
</tr>
<tr>
<td>S</td>
<td>32.50</td>
<td>32.43</td>
</tr>
</tbody>
</table>

* This investigation was partly supported by Grant-in-Aid for Scientific Research 577134, Ministry of Education, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
To prepare the mixed disulfide labeled at cysteine with \(^{35}\)S, \(^{35}\)S-cysteine (final concentration 1.5 mM) was mixed with 2-mercaptoethanol (final concentration, 3 mM) in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (pH 8.0) and stood for several days at 4 °C under sterile conditions. The reaction mixture was then evaporated to dryness and dissolved in a small volume of distilled water. The mixed disulfide was purified by cellulose thin layer chromatography in an n-butyl alcohol/acetic acid/water (3:2:2) solvent. The mixed disulfide-containing powder was scratched off the plate and the mixed disulfide was extracted from the powder with distilled water. The mixed disulfide labeled at 2-mercaptoethanol with \(^{35}\)S was prepared using 2-\[^{35}\]Smercaptoethanol (final concentration, 1 mM) and cystine (final concentration, 5 mM) by the same procedure as described above. Before use, the purity of the isolated mixed disulfide was checked by cellulose thin layer chromatography in a 75% aqueous phenol solvent.

**Cell Culture—** L1210 cells (National Cancer Institute, United States line) were cultured in vitro in a 2-mercaptoethanol-supplemented culture medium (17). The culture medium used in this study was Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin (50 units/ml), and streptomycin (50 \(\mu\)g/ml). Usually, 10 \(\mu\)M 2-mercaptoethanol was added at the time of cell inoculation. The cells were incubated at 37 °C in a 5% CO\(_2\) atmosphere and were counted with a hemocytometer. The viability of the cells was estimated by the nigrosine (0.05%) exclusion method.

**Uptake of Radioactive Compounds by the Cells—** The cells (2.5 × 10\(^7\)) were washed and suspended in 0.45 ml of prewarmed PBS (18) containing glucose (1 mg/ml). Uptake was started by the addition of radioactive compounds (0.05 ml) to the cell suspension. The mixture was incubated with shaking at 37 °C. Then the cells were rapidly separated from the medium with Microfuge (Beckman) using a layer of the mixture of mineral oil/dibutyl phthalate (15:85, v/v), and the radioactivity in the cells was counted (17).

**Determination of Thiol Compounds—** Total glutathione was determined by the enzymatic method (20) as described previously (17). The thiol content of the medium was determined by DTNB using cysteine as a standard (17).

**RESULTS**

**Effects of the Mixed Disulfide of 2-Mercaptoethanol and Cysteine on Growth and Cellular Glutathione Content—** An important role of 2-mercaptoethanol in stimulating the growth of L1210 cells is the promotion of the utilization of medium cysteine by the cells (17). By the reaction with 2-mercaptoethanol, cysteine is converted to cysteine, and the mixed disulfide of cysteine and 2-mercaptoethanol. The effect of cysteine on cell growth was examined previously (17). In this study, the role of the mixed disulfide in the action of 2-mercaptoethanol has been examined. The mixed disulfide was purified from the reaction mixture of cystine and 2-mercaptoethanol by cellulose thin layer chromatography as described under "Experimental Procedures." The mixed disulfide has almost the same growth-promoting activity as 2-mercaptoethanol over the initial concentration range of 2.5–200 \(\mu\)M. The growth-promoting activity of the mixed disulfide was saturated at about 10 \(\mu\)M.

In the presence of 10 \(\mu\)M 2-mercaptoethanol, the cellular glutathione level was maintained between 3 and 7 nmol/mg of cell protein during the culture (17). The mixed disulfide has almost the same effect as 2-mercaptoethanol on the glutathione content of the cells.

**Uptake of the Mixed Disulfide and 2-Mercaptoethanol—** To examine a possibility that the cells utilize the mixed disulfide as a source of cysteine, the cellular capacity to take up the mixed disulfide was measured. Fig. 1 shows the uptake of the following two kinds of \(^{35}\)S-labeled mixed disulfide versus time.

\[
\text{OH}-\text{CH}_{2}-\text{CH}_{2}-\text{S}^{35}\text{S}-\text{CH}_{2}-\text{CH(NH}_{2}\text{)}-\text{CO}_{2}\text{H (C)}
\]

\[
\text{OH}-\text{CH}_{2}-\text{CH}_{2}-^{35}\text{S}-\text{S}-\text{CH}_{2}-\text{CH(NH}_{2}\text{)}-\text{CO}_{2}\text{H (M)}
\]

The mixed disulfide (C), synthesized from \(^{35}\)S-cystine and 2-mercaptoethanol, was taken up by the cells at the initial rate of about 10 nmol/min/mg of cell protein, which is about 300-fold higher than that of cystine (17). The uptake of the radioactivity did not reach saturation within 10 min. On the other hand, the mixed disulfide (M) was taken up by the cells at an initial rate similar to that of type (C), but the radioactivity in the cells decreased after 1 min from the onset of the uptake, suggesting that radioactive compounds escaped from the cells.

Fig. 1 also shows 2-\[^{35}\]Smercaptoethanol uptake versus time. Hardly any radioactivity was taken up by the cells.

**Metabolic Conversion of the Mixed Disulfide—** The cells were incubated with the mixed disulfide (C) for 5 min in PBS containing glucose. About 9% of the total radioactivity was found within the cells and most of it was acid-soluble. The acid-soluble fraction of the cells was treated with N-ethylmaleimide and analyzed by column chromatography. Five components were detected by the chromatography. The three major peaks were identified as the N-ethylmaleimide adduct of glutathione, and two stereospecific isomers of N-ethylmaleimide adducts of cysteine (see Refs. 19 and 21). No mixed disulfide or oxidized glutathione were detected in the sample by the cellulose thin layer chromatography developed in a 75% aqueous phenol solvent. These results show that the mixed disulfide taken up by the cells is rapidly reduced to produce cysteine, and a part of the cysteine is incorporated into cellular glutathione (Table I).

Next we analyzed the radioactivity in the extracellular fraction. The cell-free medium was reacted with N-ethylmaleimide and analyzed by cellulose thin layer chromatography.
of oxidized 2-mercaptoethanol were detected by the column chromatography.

Table 1 summarizes the above results. These results show that more than 40% of the mixed disulfide is converted to other compounds by the cells within 5 min, and that cysteine and 2-mercaptoethanol rapidly appear in the incubation medium.

The formation of thiols, such as cysteine and 2-mercaptoethanol, in the medium was also observed colorimetrically when unlabeled mixed disulfide was incubated with the cells. Fig. 3 shows the thiol formation in the medium versus time. The thiol concentration in the medium increased almost linearly from 0 to 65 μM in the first 5 min. This value is roughly equivalent to the sum of the concentrations of cysteine and 2-mercaptoethanol derived from the mixed disulfide as given in Table I. When the mixed disulfide was omitted, thiols were not formed in the medium.

Characterization of the Transport System of the Mixed Disulfide—Table II describes the inhibition by several amino acids of the uptake of the mixed disulfide (C) into L1210 cells. The uptake of the radioactivity was inhibited markedly by phenylalanine and leucine, but only slightly by acidic and basic amino acids, and glycine. The table also describes the inhibition of [3H]leucine uptake by the mixed disulfide and several amino acids. The mixed disulfide considerably inhibited the uptake of the leucine. These results suggest that the mixed disulfide is transported mainly by the L system which prefers neutral amino acid with bulky side chain such as leucine and phenylalanine (23).

Fig. 4 shows a close correlation between the inhibition of the mixed disulfide uptake and inhibition of the thiol formation in the medium by some amino acids. One exceptional amino acid examined so far was serine. It inhibited the mixed disulfide uptake into the cells much more than the thiol formation in the incubation medium.

Conversion of 2-Mercaptoethanol during Cell Growth—To examine the whole change of 2-mercaptoethanol during the culture, the cells were incubated for 48 h in the normal culture medium containing 20 μM 2-[35S]mercaptoethanol. The cells were growing throughout the incubation. More than 97% of the total radioactivity remained in the culture medium. About 70% of the radioactivity in the culture medium was found in the acid-soluble fraction. Fig. 5 shows the pattern of the column chromatography of the acid-soluble fraction treated with N-ethylmaleimide. Following a small unidentified peak, three major peaks corresponding to the N-ethylmaleimide adduct of 2-mercaptoethanol, oxidized 2-mercaptoethanol, and mixed disulfide with cysteine were observed. This indicates that the mixed disulfide and the reduced form of 2-mercaptoethanol are present in the normal culture medium where cells are growing. When the culture medium was first treated with excess dithiothreitol to reduce the disulfide bonds existing then with N-ethylmaleimide, about 96% of the radioactivity was found in the acid-soluble fraction. The major component in the acid-soluble fraction was identified as the N-ethylmaleimide adduct of 2-mercaptoethanol by column chromatography. These results suggest that a part of 2-mercaptoethanol is bound to the protein(s) in the medium via disulfide bonds and that 2-mercaptoethanol is metabolically very stable during the cell culture.

It was also found that oxidized 2-mercaptoethanol prepared by air oxidation is gradually reduced during incubation with the cells in PBS containing 0.1% glucose. This cellular capacity may explain the reason why the disulfide form of 2-mercaptoethanol has almost the same growth-promoting activity as its reduced form (4).

Binding of 2-Mercaptoethanol to Serum Albumin—To
Mercaptoethanol Dependence of L1210 Cells

Fig. 3. Thiol formation in the medium versus time. The cells (5.0 x 10^6 cells/ml) were incubated with (●) or without (○) 100 μM mixed disulfide in PBS containing 0.1% glucose at 37 °C. The thiol concentration was determined by DTNB.

Inhibition by amino acids of the uptake of 35S-labeled mixed disulfide (C) and [3H]leucine

Table II

The concentration of the radioactive compound was 100 μM. Values are averages of triplicate determinations. The time for uptake was 40 s.

<table>
<thead>
<tr>
<th>Inhibitor (5 mM)</th>
<th>Inhibition of uptake of</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>35S-Mixed disulfide</td>
<td>[3H]Leucine</td>
</tr>
<tr>
<td>Mixed disulfide</td>
<td>87</td>
<td>74</td>
</tr>
<tr>
<td>Phe</td>
<td>84</td>
<td>66</td>
</tr>
<tr>
<td>Leu</td>
<td>79</td>
<td>72</td>
</tr>
<tr>
<td>Met</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>Ser</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Ala</td>
<td>47</td>
<td>24</td>
</tr>
<tr>
<td>Glu</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>Asp</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 4. Correlation between inhibitory action of amino acids on the thiol formation in the medium and that on the uptake of the mixed disulfide. The cells (5.0 x 10^6 cells/ml) were incubated with 100 μM mixed disulfide and 5 mM amino acid in PBS containing 0.1% glucose at 37 °C for 3 min. The thiol concentration of the cell-free medium was determined by DTNB. The uptake of the mixed disulfide (C) was measured as described in Table II.

Fig. 5. Separation of radioactive compounds in the acid-soluble fraction of the culture medium in which the cells were incubated with 2-[35S]mercaptoethanol for 48 h. The cells were incubated in the culture medium containing 10% calf serum at 2.0 x 10^6 cells/ml and cultured for 48 h in the presence of 20 μM 2-[35S] mercaptoethanol (2ME) (21 mCi/mmol). The medium was centrifuged and 0.5 ml of the supernatant was mixed with 0.015 ml of 0.65 M N-ethylmaleimide (NEM). After 10 min, 0.05 ml of 50% trichloroacetic acid was added to the solution. The acid-soluble fraction was diluted with 0.1 N HCl and applied to the column of the amino acid analyzer. The effluent from the column was collected every 2 min (about 2.1 ml). The radioactivity of a 0.5-ml portion of each fraction was measured in a toluene-Triton scintillation fluid.

Fig. 6. Dissociation of albumin-bound 2-[35S]mercaptoethanol. Bovine serum albumin (80 mg/ml) was incubated with 2-[35S] mercaptoethanol (2 mM) (21 mCi/mmol) in PBS at room temperature for 1 day and layered on a Sephadex G-25 column to remove the unbound fraction of 2-mercaptoethanol. About 20% of the radioactivity was bound to the albumin. The 2-mercaptoethanol-bound albumin (final concentration, 1.5 mg/ml) was incubated with 100 μM cysteine in PBS (●), with culture medium (●), with 200 μM cystine in PBS (●), or in PBS only (○), at 37 °C. At indicated times, a 0.1-ml portion was mixed with 0.025 ml of 50% trichloroacetic acid, and the radioactivity in the acid-soluble fraction was counted.

characterize the protein(s) that bound 2-mercaptopethanol, the above culture medium was chromatographed on a Sephadex G-200 column. The elution profile showed two major radioactive peaks. The eluting position of the first one corresponded...
not observed at inhibitory effect of phenylalanine and leucine, however, was on the growth-promoting activity of 2-mercaptoethanol. The concentration of the mixed disulfide formed in the culture medium.

The values are averages ± S.D. of triplicate cultures.

<table>
<thead>
<tr>
<th>Amino acid (mM)</th>
<th>Cell yield (increase in viable cell number/48 h) with initial concentrations of 2-mercaptoethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 µM</td>
</tr>
<tr>
<td>None</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>Phe</td>
<td>3.2 ± 0.5</td>
</tr>
<tr>
<td>Leu</td>
<td>5.4 ± 0.2</td>
</tr>
<tr>
<td>Met</td>
<td>7.5 ± 1.4</td>
</tr>
<tr>
<td>Ser</td>
<td>6.4 ± 0.8</td>
</tr>
<tr>
<td>Ala</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td>Glu</td>
<td>7.7 ± 0.6</td>
</tr>
<tr>
<td>Lys</td>
<td>6.9 ± 0.6</td>
</tr>
<tr>
<td>Arg</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td>Gly</td>
<td>7.1 ± 0.5</td>
</tr>
</tbody>
</table>

The results of this investigation show the manner in which 2-mercaptoethanol improves utilization of medium cystine by L1210 cells. A schematic diagram of the action of 2-mercaptoethanol is proposed in Fig. 7. In the culture medium, the mixed disulfide of 2-mercaptoethanol and cysteine is formed by the reaction of 2-mercaptoethanol with cysteine. The mixed disulfide, which is an amino acid with a noncharged long side chain containing a disulfide bond, is taken up by the cells at a high rate. The uptake of the mixed disulfide is remarkably inhibited by leucine and phenylalanine and, since the uptake of leucine is inhibited by the mixed disulfide as well, the mixed disulfide is probably taken up mainly by the L1210 cells. The mixed disulfide was not detected in the cells (Table I) and seems to be reduced rapidly in the cells to produce 2-mercaptoethanol and cysteine. The 2-mercaptoethanol does not accumulate in the cells and escapes to the medium and reacts with cystine. Thus, 2-mercaptoethanol is repeatedly taken up by the cells in the form of mixed disulfide with cysteine and returns to the medium in its reduced form. With the aid of this cyclic action of 2-mercaptoethanol, L1210 cells utilize cysteine constantly. This function of 2-mercaptoethanol seems to be essential for the cells to grow because they have a very low capacity to take up cystine.

We observed accumulation of cysteine in the medium when the mixed disulfide was incubated with the cells. The rate of the increase of cysteine in the medium appeared to depend on the rate of the mixed disulfide uptake into the cells (Fig. 4). The appearance of cysteine in the medium may be caused by the efflux of cellular cysteine which is formed by the reduction of the mixed disulfide within the cells. Cysteine is also formed in the culture medium by the direct reaction of 2-mercaptoethanol with cystine and is probably taken up into the cells via the ASC system which transports neutral α-amino acids such as alanine, serine, and cysteine (24). Cysteine in the culture medium is rapidly oxidized to cystine, but it is maintained during the culture due to the cyclic action of 2-mercaptoethanol and may serve to preserve cellular cysteine.

The metabolic stability and the cyclic action of 2-mercaptoethanol explain why it is effective at low concentrations and why its activity remains after air oxidation during preincubation with the culture medium. The effect of 2-mercaptoethanol on the utilization of medium cystine may possibly be found in various types of cells, because cysteine is usually contained in culture media and most of the culture cells have the transport system L.

The interaction of 2-mercaptoethanol with serum albumin may affect the action of 2-mercaptoethanol. It is shown that 2-mercaptoethanol readily binds to serum albumin, the most abundant serum protein present in the culture medium. The reactive thiol group of albumin in plasma is partially masked with cysteine, and to a lesser extent, with glutathione (25). During storage in the culture medium, it is usually masked with cysteine by the reaction with cystine (26). In the culture of human fibroblasts, the free thiol content of the albumin increases via thiol-disulfide exchange reactions with the cysteine (27).

Click et al. (2) first showed that 2-mercaptoethanol promotes antibody synthesis of lymphoid cells in vitro. A hypothesis that 2-mercaptoethanol activates a protein factor in fetal calf serum was proposed (28). The factor was fractionated after the serum was incubated with 2-mercaptoethanol. Recently, the factor was shown to be albumin (29). It seems clear that 2-mercaptoethanol is bound to the serum albumin and included in its assay system. From the complex, 2-mercaptoethanol may be released during the incubation, because the
present results show the release of 2-mercaptoethanol by incubation in cystine-containing medium.

Broome and Jeng (4) tested more than 40 kinds of thiol compounds for growth promotion of L1210 cells in vitro and found that only 2-mercaptoethanol, thioglycerol, and cysteamine are effective at low concentrations. The efficiency of thiol compounds in promoting cell growth probably depends on their ability to promote cyst(e)ine uptake into the cells. Our study on the action of 2-mercaptoethanol suggests that the utility to the cells of the mixed disulfide made of a thiol compound and cysteine is important. The mixed disulfide of cysteine and thioglycerol or cysteamine might also be formed in the culture medium and effectively incorporated into the cells.

Acknowledgments—We are grateful to I. Iida for the element analysis of the mixed disulfide, and to D. Fujii for reading the manuscript.

REFERENCES