process referred to as endosmosis. If specific for each other, antibody and antigen will meet between the two wells, combine and form an easily discernible precipitin line. The procedure employed, was as reported previously[2]. In this study, CIE was tested for its sensitivity in detecting antigens in the Pneumovax as well as individual PCP antigen types 3, 7, 9, 14.

Coagglutination (COAG). COAG is a technique for identifying specific microorganisms or their antigens using antibody coated staphylococci. Antibodies bind to protein A in staphylococcal cell wall via the Fc region, leaving the Fab region free to react with the appropriate antigen.

Three different staphylococcal preparations were tested in this study: Cowan 1 strain of *Staphylococcal aureus* (prepared in our lab) and Pansorbin *S. aureus* (Calbiochem-Behring, La Jolla, CA) both sensitized with Omniserum, and the Phadebact pneumococcal COAG kit (Pharmacia Diagnostic, Piscataway, NJ).

Preparation of *S. aureus* Cowan 1 strain was based on the method of Wasilauskas[3] and was briefly as follows: 1) The organisms were grown in two test tubes containing 5 ml of trypticase soy broth (TSB) at 37 °C for 8–12 h. 2) Using sterile technique, two 500 ml flasks, each containing approximately 250 ml of TSB, were inoculated by adding the contents of the test tubes. The flasks were then incubated at 37 °C for 18–24 h. 3) The bacteria were transferred to two 250 ml centrifuge bottles and spun down at 12,000 rpm for 10 min. Supernatant was poured off. 4) The organisms were washed five times with phosphate buffered saline (PBS) with NaN_3. 5) A 10% suspension of staphylococci (w/v) was made in 0.5% formaldehyde solution (in PBS with NaN_3) and left to stand for 3 h at room temperature. 6) The organisms were then washed three times with PBS. 7) A 10% suspension of staphylococci (w/v) was made in PBS and heated at 80 °C for 1 h in H_2O bath. 8) The organisms were finally washed three times with PBS and resuspended to a final concentration of 10% in PBS.

Staphylococci were sensitized by mixing 1 ml of the 10% suspension with 0.1 ml of Omniserum. The mixture was allowed to stand for 3 h at room temperature with occasional handshaking. Once sensitized, the organisms were harvested by centrifugation and then resuspended to 1 ml volume in PBS. A negative control was prepared following the above procedure but using normal rabbit serum instead of Omniserum.

Organisms in Pansorbin (7.1% w/v) were sensitized using the same procedure as for the Cowan 1 strain. Coagglutination tests for both the Cowan 1 strain and the Pansorbin were conducted on black agglutination plates by adding one drop (20 µl) of antigen solution to one drop of staphylococcal reagent. The drops were mixed with a wooden applicator and then the plate was rocked by hand for approximately 1 min. Finally, the plate was observed under high intensity light for signs of coagglutination.

The Phadebact Pneumococcus coagglutination kit was used according to the manufacturer's instructions.

LA test employing Directigen was the most sensitive, detecting *S. pneumoniae* vaccine antigens up to a dilution of 1:32,568. Next in order of sensitivity was COAG with Phadebact with an end point of 1:4096. CIE did not detect antigens beyond a dilution of 1:1024. Other agglutination tests were less sensitive: latex particles sensitized by us: 1:2048; both staph COAG preparations (Cowan 1 and Pansorbin) 1:512.

The results were similar when types 3 and 9 PCP antigen were assayed (table). CIE was, however, quite insensitive for types 7 and 14, which have poor anodal mobility. This has been reported previously[4] and is an important deficiency of CIE, because types 7 and 14 are of significance in pediatric infections. We conclude that, overall, latex agglutination with Directigen kit provided the more sensitive assay for detection of PCP antigens in vitro. Staphylococcal coagglutination, including reagents from the commercially available Phadebact kit, and counterimmunoelectrophoresis were less sensitive. CIE was particularly insensitive against types 7 and 14 which have poor anodal mobility. Agglutination tests are more rapid than CIE, but may require additional specimen manipulations to remove nonspecific reactants.

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Endogenous copper is cytotoxic to a lymphoma in primary culture which requires thiols for growth

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Summary. With the use of bathocuproine sulfonate, a copper-specific chelator as an indicator, we have demonstrated that copper ions, present as a natural medium constituent are toxic to the growth of a lymphoma in primary culture and are principally responsible for the growth requirement of mercaptoethanol and other thiols. By chelating trace copper normally present in the medium, bathocuproine sulfonate retarded the oxidation of cysteine to poorly utilized cystine, thus permitting its direct utilization by the cells for growth.

Key words. Lymphoma, primary culture; copper chelator; copper, endogenous; copper, cytotoxicity; bathocuproine sulfonate; cysteine oxidation.

A principal concern in cellular nutrition is the growth requirements of mammalian cells obtained directly from animal sources; that is, in primary culture. Unlike cells in established culture, which may have been selected as uniquely hardy specimens and then passaged continuously over generations, primary cultured cells have supplemental requirements[1]. For instance, several murine and human tumor cells in primary culture have an obligatory requirement for mercaptoethanol (ME) or other thiols, a property generally not shared by cells in established culture or by many normal cells in primary culture[2–4].

Both Ishii et al.[5] and Ohmori and Yamamoto[6] have postulated

that L1210 murine lymphoma cells in primary culture are unable to utilize cystine present in the growth medium. They present evidence that mercaptoethanol reacts with cystine by sulfhydryl-disulfide interchange to form its mixed disulfide with cysteine. This mixed disulfide is then taken up in the cell via the leucine transport carrier, and split within the cell to furnish a source of cysteine for growth. We have earlier indicated[7] that the thiol requirement for L1210 cells in primary culture can also be met by the addition of bathocuproine sulfonate (BCS) a copper-specific chelator[8-10]. In this report we describe the relationship between growth support obtained by added thiols and that observed upon the removal of endogenous copper in the growth medium.

Bathocuproine sulfonate, at micromolar concentrations, supported the growth of L1210 cells in primary culture with a cystine containing medium (fig. 1). Bathophenanthroline sulfonate[11] (BPS) a related chelator lacking the methyl groups at positions 2- and 9- and not specific for copper, did not support the growth of these cells (fig. 1). The marked decrease in cell number obtained with BPS may be the result of removal of essential ions, such as Zn^{++} or Fe^{+++} from the medium. Added Cu^{++}, but not Zn^{++} or Fe^{++} abolished the growth support of BCS (table).

Gampp and Zuberbühler[12] have reviewed the detailed mechanism of the overall reaction by which copper catalyzes the oxidation of cysteine to cystine;

$$2\ RSH + O_2 \xrightleftharpoons{Cu^{++}} RSSR + H_2O_2.$$

We have examined the effect of BCS on this oxidation by following the disappearance of cysteine in growth medium. As shown in figure 2, BCS reduced the rate of cysteine oxidation, while BPS failed to preserve cysteine and added copper sulfate markedly enhanced the rate of its oxidation.

The prevention of its oxidation permits the use of cysteine as a nutrient source (fig. 3). In the absence of BCS, cysteine is oxidized to the poorly utilized cystine as shown in figure 2 and noted by Ishii et al.[5]. Many reports have stressed the toxicity of added cysteine[15-18], which may be due to the formation of peroxide as shown in the above equation. BCS protects against such toxicity, but mercaptoethanol does not, as is apparent at the higher cysteine concentrations depicted in figure 3.

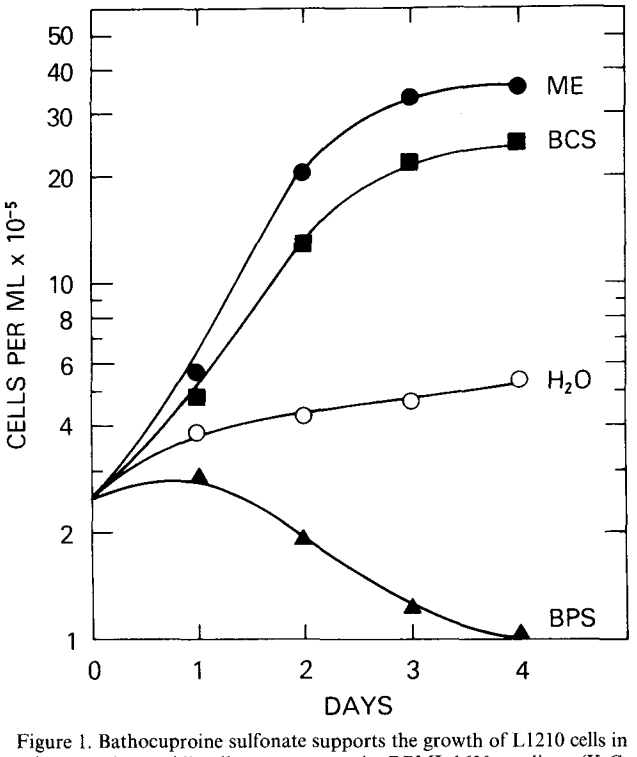

Figure 1. Bathocuproine sulfonate supports the growth of L1210 cells in primary culture. All cells were grown in RPMI 1630 medium (K.C. Biologicals, Lenexa, KA) containing 16.5% heat inactivated fetal bovine serum (Sterile Systems, Logan, UT) and 40 µg/ml Gentamicin (Schering Corp., Kenilworth, NJ). 2-Mercaptoethanol (Sigma Chemical Co., St Louis, MO) was used at a final concentration of 50 µM. The water soluble disodium salts of BCS and BPS were obtained from the G. Frederick Smith Chemical Co., Columbus, OH, and each was present at a concentration of 25 µM.

The in vivo L1210 line was routinely passaged as a free cell suspension by i.p. injection of 10^5 cells into DBA/2 female mice. Prior to use in primary culture, 10^5 cells were injected i.p. into male CDF_1 mice. These were sacrificed on days 6 or 7 and cells removed from the peritoneal cavity by flushing with 6 to 10 ml of the medium (less ME, BCS or BPS) used for growing the primary culture. The cells were washed three times by gentle centrifugation and resuspension to remove ascitic fluid and red blood cells, pooled, adjusted to a density of 2.5×10^5 cells per ml as determined with a Coulter counter, model ZBI, and 7 ml of the cell suspension were added to 25 cm^2 Corning flasks. ME, BCS and BPS were introduced in water (10 µl/ml). Cells were grown at 37 °C in tightly stoppered flasks.

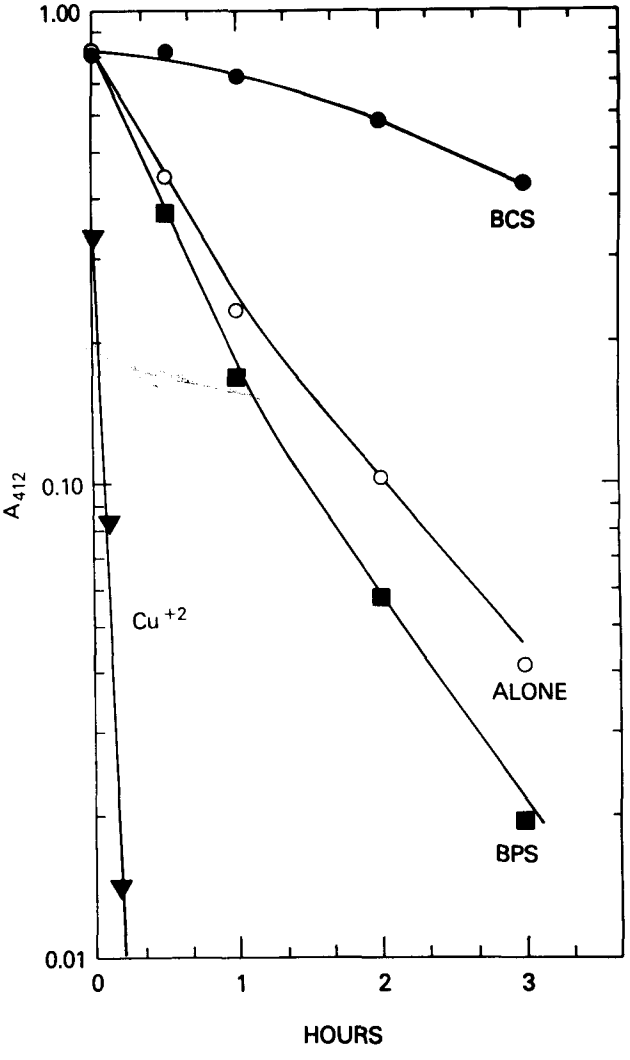

Figure 2. Protection of autooxidation of cysteine by bathocuproine sulfonate. Cysteine (50 µM) was incubated in complete growth medium at 37 °C either alone or in the presence of bathocuproine sulfonate, BCS, 25 µM, bathophenanthroline sulfonate, BPS, 25 µM, or copper sulfate, Cu^{+2}, 25 µM. At the indicated intervals aliquots were assayed for thiol groups as described by Ellman[13].

This oxidation of cysteine in growth medium may be due to the cysteine oxidase activity of the copper containing protein of serum, ceruloplasmin, as proposed by Albergoni and Cassini[19]. Frieden[20], however, argues that the oxidation of cysteine must follow its removal of copper from ceruloplasmin, since other activities of this copper protein are inhibited by azide but the oxidation of cysteine is not, an observation which we have confirmed. Trace copper contamination of other components of the incubation medium, including the salts, water and vessels have also been suggested as a possible basis for catalysis of cysteine oxidation[21]. The relationship between the thiol requirement of cells in primary culture and their sensitivity to the cytotoxic activity of trace copper is summarized in figure 4.

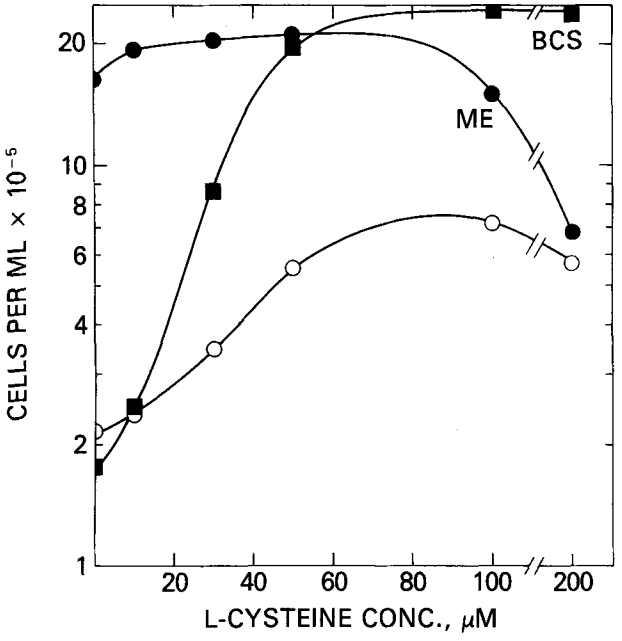

Figure 3. Chelation of endogenous copper by bathocuproine sulfonate permits growth with cysteine as a nutrient source. Cell suspensions were prepared as described in figure 1, and incubated at 37°C at 45 h in RPMI 1630 medium from which cystine was omitted. The ordinate indicates the number of cells present at the end of this incubation period. Freshly prepared solutions of L-cysteine were kept in ice prior to addition to flasks to give the indicated concentrations. The bathocuproine sulfonate (BCS) concentration was 25 μM and the mercaptoethanol (ME) concentration was 50 μM. The open circles indicate the poor growth obtained with cysteine alone. Mercaptoethanol promoted cell growth in the absence of added cystine or cysteine by making cysteine available from its mixed disulfide with proteins of the fetal bovine serum, an observation recognized by Eagle and coworkers over two decades ago[14].

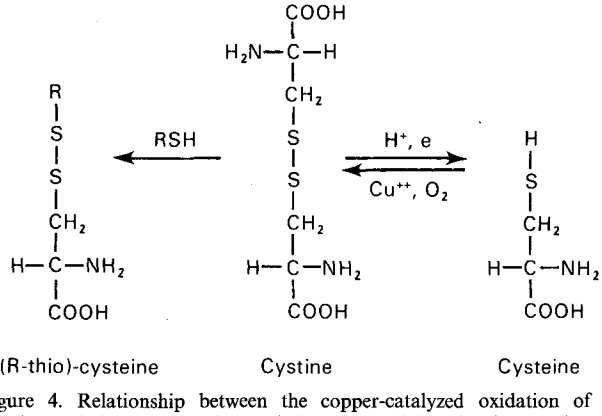

Figure 4. Relationship between the copper-catalyzed oxidation of cysteine and the support of growth by added thiols. The three amino acids indicated above enter cells through different transport systems. The S-(R-thio)-cysteine is taken up via the leucine preferring system[5], cystine by the cystine-glutamate system[22-24] and cysteine by the alanine-serine-cysteine preferring system[25,26]. The cystine-glutamate transport system is deficient in the tumor cells which are sensitive to copper or require added thiols.

Added copper ion selectively interferes with cell growth supported by bathocuproine sulfonate

Metal ion 10 μM	Growth supported by Bathocuproine sulfonate (25 μM), percent of control	Hydroxyethyldisulfide (25 μM), percent of control
Cu**	0	90
Zn**	92	96
Fe**	56	74

The cells were grown as described in figure 1 for 45 h in the presence of bathocuproine sulfonate or hydroxyethyldisulfide, the oxidized form of mercaptoethanol, with the additions as indicated in the table.

Note added in proof: Dr Alan L. Epstein of the Department of Medicine and Cancer Center, Northwestern University Medical School, Chicago, I11 (USA) has informed us that the BCS-cysteine supplement will support the establishment of 3 human malignant lymphoma cell lines with chromosome 14 translocations.

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