band might have been caused by a similar mechanism suggested by Lewis, Cheever and Seavy(8). They have observed a lipid in pituitary fractions which caused an alteration in the mobility of aldolase on starch gel electrophoresis and caused its dissociation into subunits. It is possible that an abnormal lipid was present in the red cells of the patients in Fig. 2 and complexed with hexokinase thus increasing its anodal mobility. Digitonin would be expected to rupture the lipo-enzyme complex and therefore abolish the anomalous band.

Summary. The isoenzymes of hexokinase of several human tissues have been examined by disc electrophoresis. An infant with hemolytic anemia was found to have a band that migrated faster than hemoglobin. His father displayed the same abnormality whereas the mother's zymogram was normal.

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Escherichia coli Resistance to Ethionine.* (32484)

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In a brief report on ethionine resistance by *Escherichia coli,* Martin and Moo-Penn (1) reported a considerable delay in the onset of growth of resistant cells transferred from an ethionine medium to one without the inhibitor. When ethyl-1-C^{14}-ethionine was incorporated into the growth medium of resistant cells, labeled CO_2, acetic and formic acids were isolated. The specific activity of formic acid approximated that of C^{14}-ethionine while specific activities of acetic acid and CO_2 were low indicating considerable dilution of these compounds by carbon from other sources. Labeled acetate and CO_2 were also isolated from sensitive *E. coli* cultures grown in the presence of subinhibitory concentrations of labeled ethionine but no labeled formate was produced by sensitive cells.

Indirect evidence indicated that this *E. coli* strain was resistant to ethionine by virtue of an ability to decompose this inhibitor to a non-toxic substance, probably hemocysteine. The nature of the growth lag by ethionine resistant cells was not studied. The following report is concerned with further studies on the prolonged lag in growth exhibited by resistant cells and the relationship of this lag to ethionine resistance in *E. coli.*

Materials and methods. Microorganism. Escherichia coli ATCC 9637 was used throughout this investigation. Ethionine resistant cells were obtained by passage of sensitive cells on agar plates containing increased concentrations of ethionine. The minimum inhibitory concentration of ethionine for 100 sensitive cells inoculated into 10 ml broth was 100 μg/ml after 24 hours incubation at 37C, and 900 μg/ml on agar plates under the same conditions. Resistant cells grew in the presence of 5 mg/ml L-ethionine in broth

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and complete growth was observed after 24 hours incubation at 37C.

Growth conditions. Inocula for growth curve and isotope studies were prepared in stationary glucose salts minimal medium(2). Resistant inocula were grown in the same medium with L-ethionine added to a final concentration of 5 mg/ml. Cells were harvested by centrifugation and washed 3 times with sterile 0.85% saline. Inocula were standardized to an optical density of 0.660 with a Bausch and Lomb "Spectronic 20" spectrophotometer at 660 mμ. Suspensions at this optical density contained approximately 1.0 $\times$ 10^9 viable cells/ml. Growth curves were determined in 300 ml Bellco nephelometer flasks containing 20 ml broth. Flasks were incubated at 37C on a rotary shaker at 200 rpm and growth estimated as an increase in optical density with time.

1-C^{14}-*L*-Ethionine and 1-C^{14}-*L*-methionine were obtained from Volk Radiochemical Corp.; S^{35}-ethionine and S^{35}-methionine were supplied by Amsterdam Radiochemical Corp., Haversham, England. Radioactivity was determined with a windowless gas flow Geiger-muller counter. Appropriate corrections for absorption, background and decay were made where indicated. O-Succinyl-*L*-homoserine was synthesized according to procedures described by Flavin and Slaughter(3).

Enzyme preparations. Cells were suspended in .05 M Tris buffer pH 8.0, disrupted with a French pressure cell at 20,000 psi, and then centrifuged in a Sorval refrigerated centrifuge at 29,000 $\times$ *g*, 2C. The supernatant was dialyzed overnight in .05 M Tris buffer pH 8.0 containing 10^{-4} M β-mercaptopropionate, 10^{-5} M pyridoxalphosphate, and 10^{-4} M EDTA. Protein concentrations were estimated by the method of Lowry *et al*(4). Cystathionine synthetase was determined by assaying the rate of α-ketobutyrate formation from O-succinyl-*L*-homoserine with lactic dehydrogenase and NADH at 340 mμ with a Beckman model DU spectrophotometer; cystathionase activity was estimated by the method of Rowbury and Woods(5).

Results. Nature of E. coli *ATCC 9637 resistance to ethionine.* A total of 900 ethionine sensitive colonies, growing on 11 Petri plates of mineral agar without drug, were replica plated on two series of plates; one containing minimal media and the other minimal media plus 1 mg/ml *L*-ethionine. After 24 hours incubation, 843 of the replica plated colonies grew on minimal medium plates and 705 of these colonies developed, after 72 hours incubation, on plates containing ethionine. Thus 84% of the colonies eventually gave rise to resistant progeny. When cells resistant to 5 mg/ml l-ethionine *(in broth)* were grown for 20 hours in minimal medium without drug, resistance fell from 5 mg/ml to 1 to 2 mg/ml by pour plate assay and 1 mg/ml by broth assay. After a second passage in minimal media the cells exhibited the same ethionine sensitivity as did the original parent strain.

Growth of resistant cells. An increased lag in growth over that of sensitive cells was observed when resistant cells were cultured in minimal medium. This lag occurred when resistant cultures growing in the presence of 1.0 to 5.0 mg/ml l-ethionine were used as inocula for growth in shaker flasks without drug. The length of the lag over that of sensitive cell growth was dependent upon the inoculum size. A typical growth lag is presented in Fig. 1. A reduction of this lag to that of sensitive cells occurred if ethionine or certain metabolites known to be intermediates in methionine biosynthesis were present in the basal medium. The metabolites tested and their effect on the extended lag in growth are shown in Table I. Inocula prepared from either resistant or sensitive cells grown in broth containing 10 mM concentrations of any one of the reversing metabolites and used as inocula in basal media gave similar extended lag periods.

Cell viability during the turbidimetric lag phase. Further growth curve studies on resistant cells were conducted by determining viable cell numbers during the lag phase. For these experiments, the inoculum size was such that there was a 4 hour lag over that of sensitive cells. Aliquots were withdrawn with time, and the number of viable cells determined by quantitative plating. The results, Fig. 2, show a loss in cell viability of 60 to 70% of the initial population during

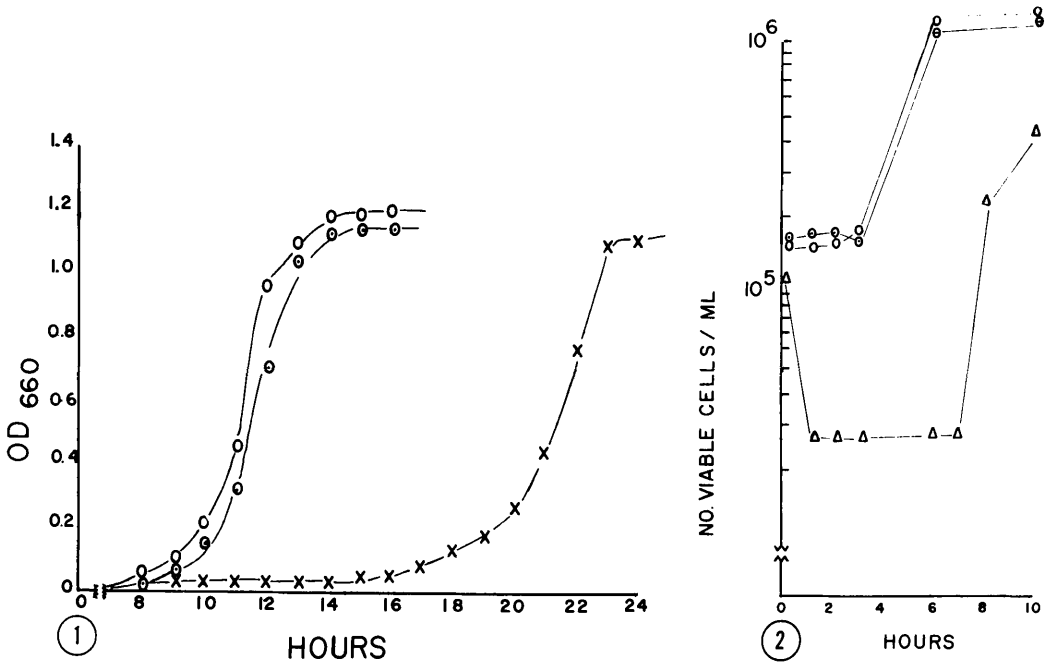

FIG. 1. Growth of resistant and sensitive cells in media by optical density O — O, sensitive cells; × — ×, ethionine resistant cells; ⊙ — ⊙, ethionine resistant cells plus 10 μmoles of lag-reducing metabolites (see Table I). Inoculum size was 10^8 cells in 20 ml broth.

FIG. 2. Growth of resistant and sensitive cells in minimal broth determined by direct viable count. O — O, sensitive cells growing in minimal media; ⊙ — ⊙, resistant cells growing in minimal media supplemented with 5 mg ethionine/ml; △ — △, resistant cells growing in minimal media. Inoculum size was 10^5 cells in 20 ml broth.

TABLE I. Effect of Metabolites on the Extended Growth Lag Exhibited by Resistant Cells Grown in Minimal Broth.

A. Metabolites Reducing the Lag:	B. Metabolites Not Reducing the Lag:
l - ethionine	homoserine
D - ethionine	succinic acid
l - methionine	homoserine and succinic acid
D - methionine	cysteine, homoserine and succinic acid
Allo - cystathionine	cysteine and succinic acid
homocysteine	cysteine
	adenine

Final concentrations were 10 mM except that of adenine which was 0.4 mM.

the first 2 hours of incubation while no detectable death occurred in drug sensitive controls or with resistant cells in media containing ethionine. The same pattern of early death followed by recovery was observed with sensitive cell inocula prepared from cultures grown in the presence of methionine, homocysteine, or cystathionine.

Incorporation of ethionine by sensitive and resistant cells. The elimination of the extended lag in growth (prevention of death) of resistant cells by ethionine suggested that the drug was being utilized by resistant cells. The incorporation of S^{35} and ethyl-1-C^{14}-*L*-ethionine into whole cells of resistant and sensitive populations was investigated. The incorporation of S^{35}-ethionine into sensitive and resistant cells is shown in Fig. 3. The pattern of incorporation of label by sensitive and resistant cells was similar while the rate and pattern of ethyl-1-C^{14}-ethionine incorporation (Fig. 4) was clearly different in

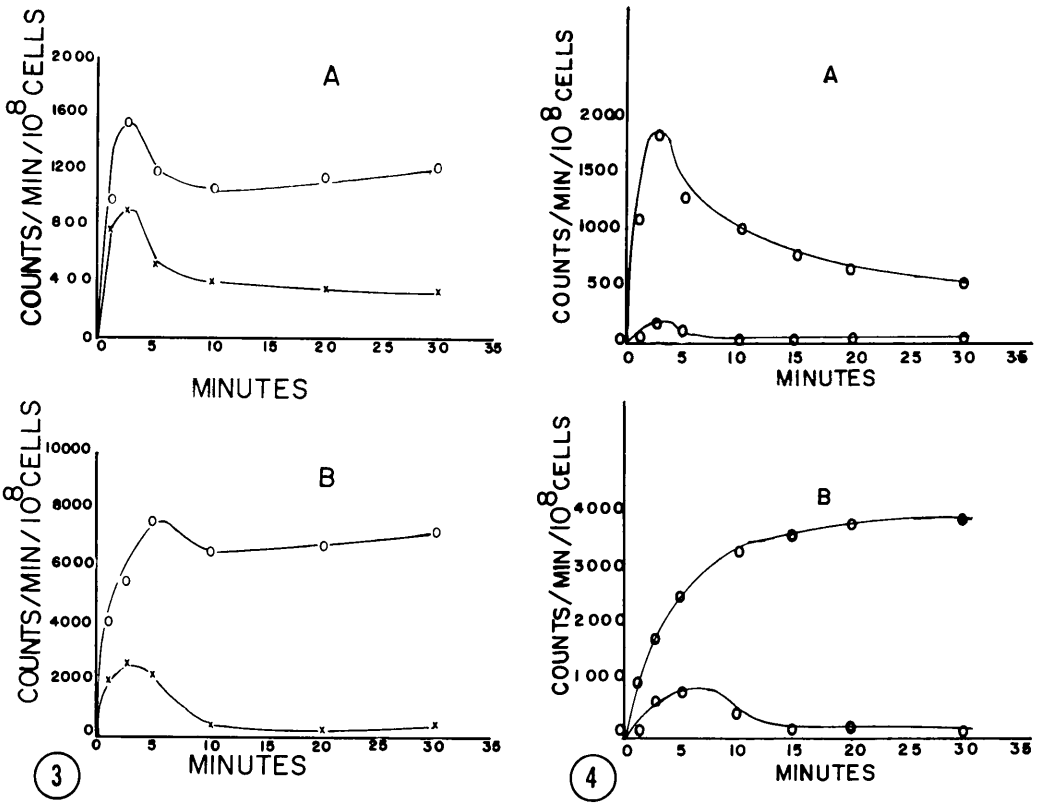

FIG. 3. S^{35}-ethionine incorporation by whole cells. A-resistant cells plus 5 mg/ml l-ethionine. B-sensitive cells. O — O, total incorporation; × — ×, incorporation into amino acid pool. Minimal broth cultures were grown with shaking to mid log-phase. (O. D. = .300), and S^{35}-ethionine 4.2 × 10^{-4} moles was added 1 × (10^6cpm).

FIG. 4. C^{11}-ethionine incorporation by whole cells. A-Resistant cells growing in 5 mg/ml L-ethionine. B-Sensitive cells growing in minimal media. The experiment was conducted as described in Figure 3. C^{11}-ethionine 7.4 × 10^{-4} moles was added (1.3 × 10^6 cpm).

sensitive and resistant cells. Resistant cells actively took up C^{14}-ethionine for a few minutes followed by a rapid loss in label. C^{14}-ethionine was taken up at a reduced rate by sensitive cells, but once incorporated, the label was not subsequently lost.

Repression of cystathionine synthetase in resistant cells. Growth curve studies of resis-

TABLE II. Cystathionine Synthetase Levels in Sensitive and Resistant Cells Under Various Growth Conditions.

Cell type	Growth conditions	Cystathionine Synthetase Activity μmoles α-ketobutyrate/hr/mg protein
Sensitive	Minimal Media	1.02
"	Minimal Media + *L*-Methionine 5 mg/ml	< .006
Resistant	Minimal Media + *L*-Ethionine 5 mg/ml	< .006
"	Minimal Media	0.9

Assay — 2.0 ml Tris buffer pH 8.0 0.01 ml LDH,* 0.1 ml Pyridoxal-P (0.2 μmoles), 0.1 ml cell extract, 0.05 ml NADH, 0.15 ml o-succinyl-*L*-homoserine (5.0 μmoles) and H_2O to 3.0 ml.
* 1/1000 dilution of muscle lactic acid dehydrogenase having an activity of 2,000 units/ml.

tant cells indicated that growth in a medium containing ethionine had created a temporary requirement for methionine or certain precursors of methionine. It appeared that ethionine could also satisfy this requirement. These data, with those obtained in labeled ethionine studies, suggested that ethionine served as a methionine source for resistant cells.

Methionine synthesis in *E. coli* occurs by the following reactions: Cysteine + o-succinylhomoserine $\xrightarrow{a}$ cystathionine $\xrightarrow{b}$ homocysteine $\xrightarrow{c}$ methionine. Since metabolites leading to cystathionine synthesis did not reduce the extended lag but compounds following cystathionine and cystathionine itself were effective reversing agents, it appeared that the metabolic block occurred at the point of reaction (a).

The activity of cystathionine synthetase in both resistant and sensitive strains under lag producing conditions was investigated. The results, Table II, show essentially no cystathionine synthetase activity in extracts from resistant cells or in extracts from sensitive cells grown in media containing methionine. Extracts of resistant cells grown in minimal media again had full cystathionine synthetase activity.

Cystathionase activity in extracts of resistant cells grown with ethionine was some 50% lower than that of sensitive cells. The same order of cystathionase activity was observed with extracts of sensitive cells grown with methionine or homocysteine. Resistant cells grown in minimal media again had full cystathionase activity, Table III.

TABLE III. Cystathionase Levels in Sensitive and Resistant Cells Grown in Different Metabolites.

Cell type	Growth conditions	Cystathionase activity in μmoles/pyruvate/mg protein/hr
Sensitive	Minimal Media	1.65
"	l - homocysteine, 1.25 mg/ml	.84
"	l - methionine, 5 mg/ml	.75
Resistant	Minimal Media	2.10
"	l - ethionine, 5 mg/ml	.76

Discussion. The data are interpreted to mean that *E. coli* becomes resistant to ethionine by virtue of an induced ability to convert this analogue to methionine. The high incidence of drug resistance observed with this microorganism and the requirement for the constant presence of ethionine to maintain resistance indicates resistance is induced by the drug rather than the drug acting as a selective agent for the expression of resistance by a mutational event

The prolonged growth lag observed when resistant cells were inoculated into basal broth was in part due to the rapid death of a large percentage of the initial inoculum, and in part to a delay in cell proliferation. The same compounds, ethionine, methionine, homocysteine, or cystathionine, that reduced the turbidimetric lag to that of control cells also prevented early death when growth was measured by direct viable cell count. Since sensitive cells also exhibited the same growth pattern when inoculated in basal broth if they were previously grown in the presence of methionine, homocysteine or cystathionine, it is evident that the observation of delayed growth by resistant cells is not peculiar to the acquisition of drug resistance by *E. coli*.

Under the conditions required to produce the extended growth lag, both sensitive and resistant cells exhibited a nutritional requirement for methionine or certain methionine precursors indicating in both cases a temporary impairment in *de novo* methionine synthesis. Resistant cells were repressed for cystathionine synthetase by ethionine to the same extent as sensitive cells when grown in methionine-containing media. Since methionine precursors satisfying the temporary methionine requirement occur in the methionine biosynthetic sequence between the point of significant enzyme repression and the final step in methionine synthesis, it is concluded that the extended growth lag was due to cystathionine synthetase repression.

Although the data are not sufficient to permit a detailed discussion of the series of events leading to the death of a large percentage of the original drug resistant inoculum, there are certain available facts which allow limited speculation. Important to this

are the physical growth conditions. The cells are incubated under a highly aerobic environment resulting in the promotion of rapid growth. If the cells are removed from the shaking flasks at any time during the period of viability loss, and incubated under slower growth conditions, those cells viable at the time the sample was removed grow. Since resistant cells are repressed for methionine synthesis, a metabolic imbalance apparently occurs during rapid growth resuling in the death of a large proportion of the initial inoculum. It is apparent from those data presented in Fig. 2 that those viable cells remaining grow only after a further lag period. It is unclear at present whether this lag is due to the time required for the synthesis of cystathionine synthetase alone or the additional synthesis of other enzymes necessary to remove toxic products produced by the proposed metabolic imbalance.

Growth of *E. coli* in a methionine containing medium has been reported to cause repression of o-succinyl transacetylase, cystathionine synthetase, cystathionase and hemocysteine methylase(5,6,7). Data presented here indicate that the point of significant repression in *E. coli* ATCC 9637 by ethionine for resistant cells and methionine for sensitive cells occurs at cystathionine synthetase; although they do not preclude the possibility that enzymes occurring prior to cystathionine synthetase may also be repressed. A limited degree of cystathionase repression (50%) was observed with the strain used in these studies, but was not as great as the methionine repression of this enzyme in *E. coli* previously reported. Balish and Shapiro(8) recently presented evidence with *E. coli* and *Aerobacter aerogenes* in support of a direct methylation of cystathionine followed by a split of the methylated product to yield serine and methionine. The cystathionase repression by methionine reported by these investigators is in good agreement with cystathionase repression by methionine and ethionine reported here. In addition, the lag reversing activity of cystathionine and homocysteine would seem to indicate that enzymes leading to methionine synthesis from the point of cystathionine synthetase are operative in the organism used for this study.

Studies with labeled ethionine indicate that resistant cells convert the drug to methionine by a dealkylation to form homocysteine. A limited degree of ethionine deethylation was always observed with sensitive populations. Label from 1-C^{14}-ethionine was incorporated into the TCA insoluble fractions of sensitive cells; however, all radioactivity could not be accounted for by ethionine incorporation into protein alone. A significant proportion of the label (C_2 units) entered into combination with TCA insoluble components. It is known that the ethyl group of ethionine may be transferred to the same acceptors which normally accept methyl groups from methionine and it may well be that this accounts, in part at least, for ethionine toxicity. The key to ethionine resistance by *E. coli* may not only be the ability to deethylate the analogue, but likely of more importance is the distinctive ability of resistant cells to remove the ethyl moiety from further combination with C-1 acceptors by rapidly converting it to CO_2, acetic and formic acids(1).

Summary. Ethionine resistance in *E. coli* ATCC 9637 occurs at a high frequency and requires the constant presence of the analogue to maintain resistance. Resistant cells exhibited a lag phase 6 to 8 hours longer than sensitive cell controls when cultured in a glucose salts medium. This extended lag was reduced to that of sensitive cell controls by ethionine, methionine, homocysteine or cystathionine. A similar prolonged lag occurred when sensitive cells, previously grown in the presence of methionine, homocysteine or cystathionine, were inoculated in basal glucose salts media. The extended lag in all cases tested was due to the rapid death of a significant portion of the initial inoculum during the first hour of incubation. Resistant cells were repressed for cystathionine synthetase to the same degree as sensitive cells grown in media containing methionine. The data indicate that ethionine resistance in this strain occurs by an induced ability to convert ethionine to methionine *via* homocysteine, which results in repression of cystathionine synthetase. The viability loss apparently occurred in inocula repressed for *de novo* methionine

synthesis due to metabolic imbalances brought about by the rapid growth conditions employed in this study.

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α-6-Deoxyoxytetracycline III. Total and Unbound Antibiotic Serum Concentrations After Oral Administration to Mice. (32485)

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The new tetracycline, α-6-deoxyoxytetracycline (DOOTC)* has demonstrated a marked chemotherapeutic advantage over other tetracyclines when administered orally to experimentally infected mice. For example, DOOTC was significantly more active than other tetracyclines against infections produced by the intraperitoneal inoculation of *Staphylococcus aureus* 5 mp, *Pasteurella multocida*, and *Streptococcus pyogenes* C 203 (1). In addition, DOOTC afforded 2 to 4 times greater protective activity than other tetracyclines after oral administration to mice in (a) pre-infection and (b) post-infection protocols(2). The studies reported in this paper suggest that the superior performance of DOOTC in such experiments depends upon the achievement of higher free-drug levels in serum and other body fluids.

Materials and methods. The antibiotics, *i.e.*, α-6-deoxyoxytetracycline, 6-methylene oxytetracycline (MOTC)‡, 6-demethylchlortetracycline (DMCT)§, and tetracycline (TC)¶ were used in these studies as the hydrochlorides and were of research quality. All tetracyclines were administered orally to mice in a diluent consisting of water and 1% carboxymethylcellulose.

At various times after antibiotic administration to a large number of 20-25 g Swiss Albino mice (Blue Spruce Farms, Altamont, N. Y.), a group (7 mice) was removed and sacrificed by either decapitation or exsanguination from the subclavian artery. Serum was separated from the pooled blood sample at refrigerator temperature. If not biologically assayed on the day collected the serum sample was kept frozen until used.

Bioassays of sera were performed using the common paper disc or cup-plate technique with *Bacillus cereus* var. *mycoides* as test organism(3). Standard curves were prepared using either pooled normal mouse serum or a solution containing sufficient bovine albumin to compensate for serum binding. Serum samples were used either undiluted, diluted with normal mouse serum, or diluted with the albumin solution.

The serum concentration studies in the mouse were replicated, dependent upon the antibiotic studied, from 6 to 12 times. Therefore, the values in the Tables usually represent the mean calculated from 6 to 12 different pooled sera samples. The standard error of the mean has also been calculated. These data

* Generic name, doxycyline. The trade mark of Chas. Pfizer and Co., Inc. is Vibramycin.

‡ Registered trade mark of Chas. Pfizer and Co., Inc., is Rondomycin.

§ Registered trade mark of Lederle Laboratories, American Cyanamid Co., Inc., is Declomycin. I wish to thank Dr. B. W. Corey, Medical Director of Lederle Laboratories, Pearl River, N. Y. for the supply of DMCT.

¶ Registered trade mark of Chas. Pfizer and Co., Inc. is Tetracyn.