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Title: INTRACELLULAR OXIDATION OF GLUTATHIONE IN L-5178Y LYMPHOMA CELLS BY PROCARBAZINE AND DIAMIDE: PARALLEL STUDIES

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Abstract approved: _____
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A moderate but prolonged increase in the intracellular ratio of oxidized to reduced glutathione was observed in L-5178Y lymphoma cells, following treatment of tumor-bearing mice with procarbazine (300 mg/kg body wt.). Evidence obtained with an *in vitro* model system utilizing the glutathione-oxidizing reagent, diamide, indicated the extent of oxidation of glutathione observed with procarbazine treatment could be significant in terms of a mechanism of cytotoxicity.

A steady, controlled oxidative challenge by diamide was achieved by incubation of L-5178Y cell suspensions in Bio-Fiber 50^{R} Minibeakers perfused with Fischer's medium with or without diamide. A linear relationship between glutathione oxidation and level of diamide in the perfusate was seen, and this effect persisted after a 60 minute recovery period during which cells were treated with control medium. Diamide was administered for three hours. Viability did not appear to be impaired by incubation in the hollow fiber devices or

by treatment with low levels of diamide, although mice implanted with diamide treated cells survived significantly longer than controls, and the increase in survival time appeared to be correlated with effects on the glutathione redox state.

Protein synthesis, as measured by ^{14}C-leucine incorporation, was inhibited in L-5178Y cells treated with diamide by hollow fiber perfusion. The extent of inhibition was correlated with the level of diamide in the perfusate. Over 50% inhibition was seen at percent oxidized gluthatione values that were only slightly higher than normal (2.5% compared to 1.6% of total gluthathione).

A rapid and accurate method for the determination of the intracellular specific activity of ^{14}C-leucine was developed, based upon a popular technique which consists of dinitrophenylation of the amino acids with ^{3}H-labeled Sanger's reagent, followed by measurement of the $^{3}H/^{14}C$ ratio in the isolated leucine derivative after methylation. High performance liquid chromatography was utilized to isolate methyl-DNP-leucine. Resolution of the leucine derivative from the derivatives of all other amino acids was achieved in six minutes elution time, using small particle (5μ) silica packings.

Utilizing this procedure, and chosing leucine levels that produced linear incorporation kinetics, inhibition of protein synthesis by procarbazine was seen to occur after as little as one hour of treatment of tumor bearing mice. Reported values for inhibition of protein

synthesis in L-5178Y cells by procarbazine were shown to be erroneous, because of inconsistencies in labeling kinetics between controls and treated mice. This procedure further revealed that diethylmaleate, which has been used as a specific reagent for decreasing total glutathione levels in vivo, has a profound influence on the rate of protein synthesis in L-5178Y cells treated in vivo. It also appeared that diethylmaleate treatment might be leading to glutathione oxidation as well.

A rapid, sensitive and specific method for the determination of protein-glutathione mixed disulfides was developed. Reduced Ellman's reagent was used to cleave the mixed disulfides, and the released glutathione was determined by an established enzymatic method utilizing glutathione reductase and Ellman's reagent in a cyclic reduction catalyzed by NADPH.

Fetal bovine serum was found to be a convenient standard for mixed disulfide determination. The observation that fetal bovine serum appears to be uniquely rich in protein-glutathione mixed disulfides is also interesting in connection with reports that this serum is unique in preventing or delaying neoplastic transformation in tissue culture.

Intracellular Oxidation of Glutathione in L-5178Y Lymphoma Cells by Procarbazine and Diamide: Parallel Studies

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS USED IN THE TEXT

Ci	-	Curie
DNP	-	dinitrophenyl
DTNB	-	dithio bis-2(nitrobenzoic acid); Ellman's reagent
DTT	-	dithiothreitol; Cleland's reagent
EtOH	-	ethanol
FCS	-	fetal calf serum
FDNB	-	fluorodinitrobenzene; Sanger's reagent
GDW	-	glass distilled water
GSH	-	reduced glutathione
GSSG	-	oxidized glutathione
LDH	-	lactate dehydrogenase
NBS	-	reduced Ellman's reagent
NEM	-	N-ethylmaleimide
PCV	-	packed cell volume
PSH	-	protein thiol
PSSG	-	protein-glutathione mixed disulfide
SH	-	thiol
TCA	-	trichloroacetic acid

INTRACELLULAR OXIDATION OF GLUTATHIONE IN L-5178Y LYMPHOMA CELLS BY PROCARBAZINE AND DIAMIDE: PARALLEL STUDIES

INTRODUCTION

Procarbazine

Clinical Use

Procarbazine (Matulan[R], natulan, ibenzmethyzin, N-isopropyl-(2-methylhydrazino)-p-toluamide hydrochloride; Appendix I), is a carcinostatic drug which has been used clinically in the treatment of Hodgkin's Disease for over ten years. Its effectiveness in cases where tumors have become resistant to other drugs (135) and in combination chemotherapy (39) suggests it may have a unique mechanism of action. Clinical effectiveness has been observed in the treatment of a variety of lymphomas (205), polycythemia vera (188), malignant melanoma (132), multiple myeloma (141), bronchogenic carcinoma (181), and, most recently, in the treatment of brain tumors (129).

Fifty to seventy percent of all patients treated with procarbazine experience side effects, which include nausea, vomiting, leukocytopenia and thrombocytopenia. Central nervous effects can occur at higher doses, but are seldom experienced at therapeutic doses, which are of the order of 200 mg per square meter body surface area

per day (200). Other biological effects include immuno-suppression (133), carcinogenesis (105), teratogenesis (31), and depression of spermatogenesis (128).

Pharmacology

Oral LD_{50}'s reported by Roche Laboratories (172) for mice, rats and rabbits are 1320 mg/kg, 785 mg/kg, and 145 mg/kg, respectively. Miller (135) reports the i.p. LD_{50} in mice to be 800 mg/kg.

The half-life of procarbazine in blood ranges from seven minutes in man to twenty-four minutes in rats (164), and is mainly a reflection of its conversion to azo procarbazine (Figure 1). Within 24 hours after administration, 70% of the administered dose appears

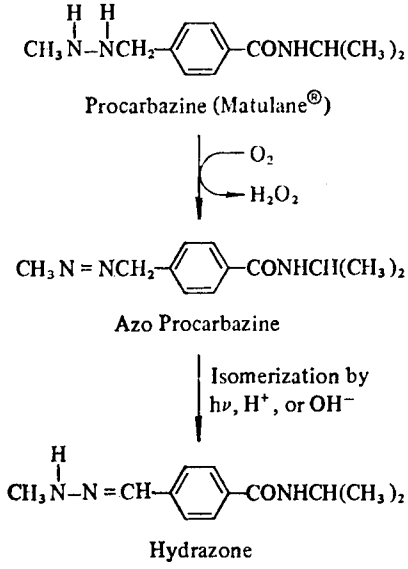


Figure 1. Chemical degradation of procarbazine through the hydrazone. Reactions occur in vivo, as well as in vitro. From: Reed, 1975 (158).

in the urine as isopropylterephthalamic acid (Figure 2) and 40% of the administered dose is expired, mainly as CO_2 and H_2O (189). In man, peak concentrations in the cerebrospinal fluid are achieved within 30 to 90 minutes after oral or intravenous administration (153). Little residual radioactivity can be found in experimental animals 24 hours after administration of either carbonyl or N-methyl labeled procarbazine (189).

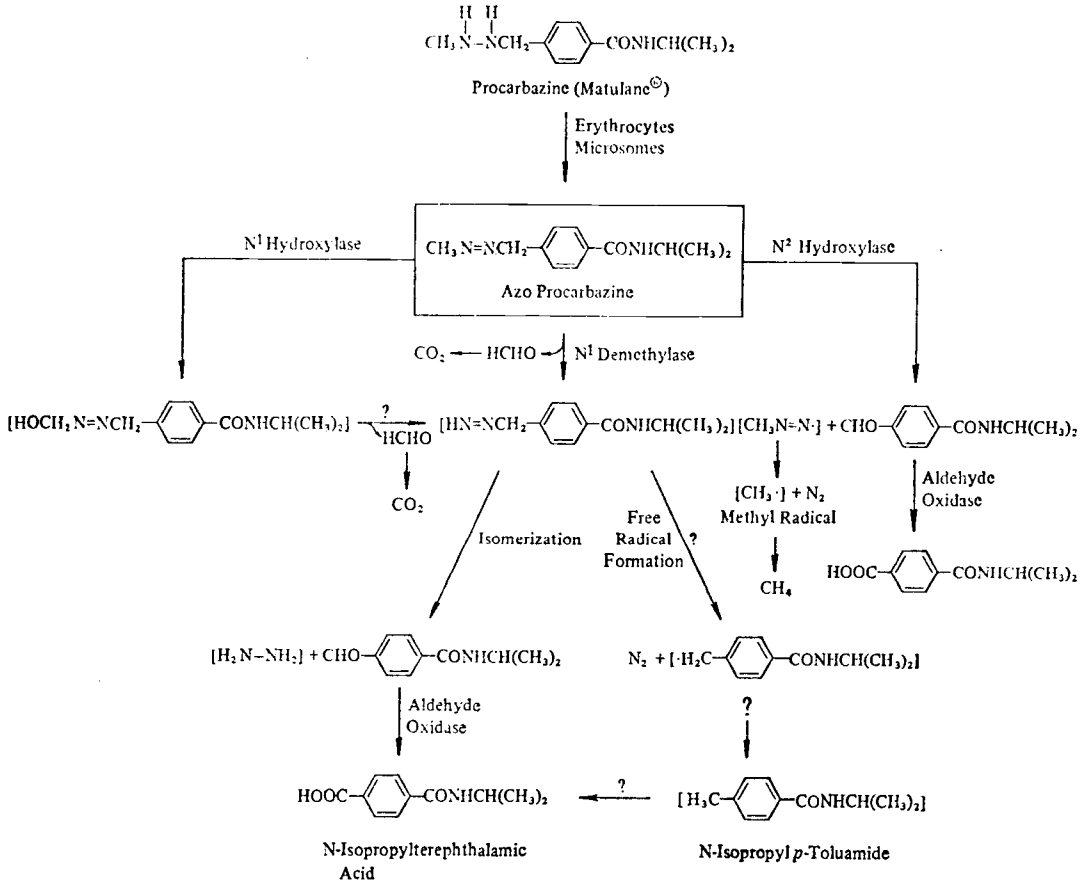


Figure 2. Other possible pathways of procarbazine metabolism. Brackets denote postulated but unidentified intermediates (168). From Reed, D. J., in Handbook of Experimental Pharmacology, Springer-Verlag (1975).

Chemistry and Metabolism

A number of disubstituted hydrazines or azo compounds have potent biological effects, ranging from general antibiotic action to carcinogenesis, but only methylhydrazine derivatives with the general structure $CH_3NHNHCH_2ArR$ have been found to have antitumor activity (19). Procarbazine is the most promising of these methylhydrazine derivatives.

Oxidation to azo procarbazine, a process which is catalyzed by hemoglobin (3), whole erythrocytes (2, 142) and liver microsomes (162), appears to be essential for biological activity. No effect is seen when Ehrlich ascites cells are treated *in vitro* with freshly prepared solutions of procarbazine, whereas aged solutions cause inhibition of protein and DNA synthesis (64, 73). Azo procarbazine administered *in vivo* is biologically active (20).

Conversion of azo procarbazine to the hydrazone (Figure 1) does not appear to be a significant pathway *in vivo*, on the basis of CO_2 and methane formation from the hydrazone, procarbazine, and azo procarbazine (168), but it does occur (28).

Hydrogen peroxide is formed during the oxidation of procarbazine to azo procarbazine (Figure 1), and hydrogen peroxide itself has anti-tumor activity (123). However, aged solutions of procarbazine are equally inhibitory in the presence of catalase (64), and the

observation that azo procarbazine is biologically active indicates that hydrogen peroxide does not play an essential role in vivo. It may explain the degradation of DNA that is seen in vitro (13) which cannot be duplicated in vivo (122).

Free radicals are extensively implicated in the metabolism of procarbazine (Figure 2). It may be of interest in this regard that ionizing radiation has a synergistic effect on DNA degradation in vitro (14), although only an additive effect is seen in vivo (166). Numerous chromatid breaks are characteristic of procarbazine treatment (176). Nucleic acid alkylation might be expected, particularly from the methyldiazene moiety. However, the extent of alkylation does not appear to be sufficient to account for the observed cytotoxicity of procarbazine (25). No significant physicochemical changes in DNA have been observed after in vivo treatment with procarbazine (113).

Free radicals could have an accelerating effect on lipid peroxidation. Hemolysis of erythrocytes by phenyldiazenecarboxylate has been ascribed to the generation of free radicals during its degradation (118). It may therefore be significant that procarbazine has been found to facilitate erythrocyte hemolysis (142).

Selective methylation of t-RNA by procarbazine and interference with normal patterns of methylation have been observed (122).

However, the level of methylation is quite low, and t-RNA from procarbazine treated perfused rat liver does not differ from control t-RNA in activity (113). Nevertheless, Kreis (122) has found indications that methionine, which would be expected to compete with procarbazine for t-RNA methylation, may be capable of abolishing the cytotoxic effects of procarbazine.

Cytotoxic Effects

Suppression of mitosis is perhaps the most striking effect of procarbazine at the cellular level (176). Rao (166) observed maximal suppression of mitosis five hours after treatment of tumor bearing mice with procarbazine, although a sudden, transient increase in mitotic activity was seen at first. Gutterman et al. (73) report maximal inhibition after eight hours of treatment, with the mitotic index remaining well below 10% of that of controls throughout the first 48 hours. Even after 72 hours of treatment, when protein and nucleic acid synthesis had fully recovered, mitotic activity remained suppressed. Cell death occurs mostly after complete recovery of protein and nucleic acid synthesis (184).

Figure 3 shows effects on protein, RNA and DNA synthesis, as reported by different authors. Sartorelli and Tsunamura (184) determined leucine, thymidine and uracil incorporation in L5178Y lymphoma cells, grown as ascites tumors in C57 BL X $DBAF_1$ mice,

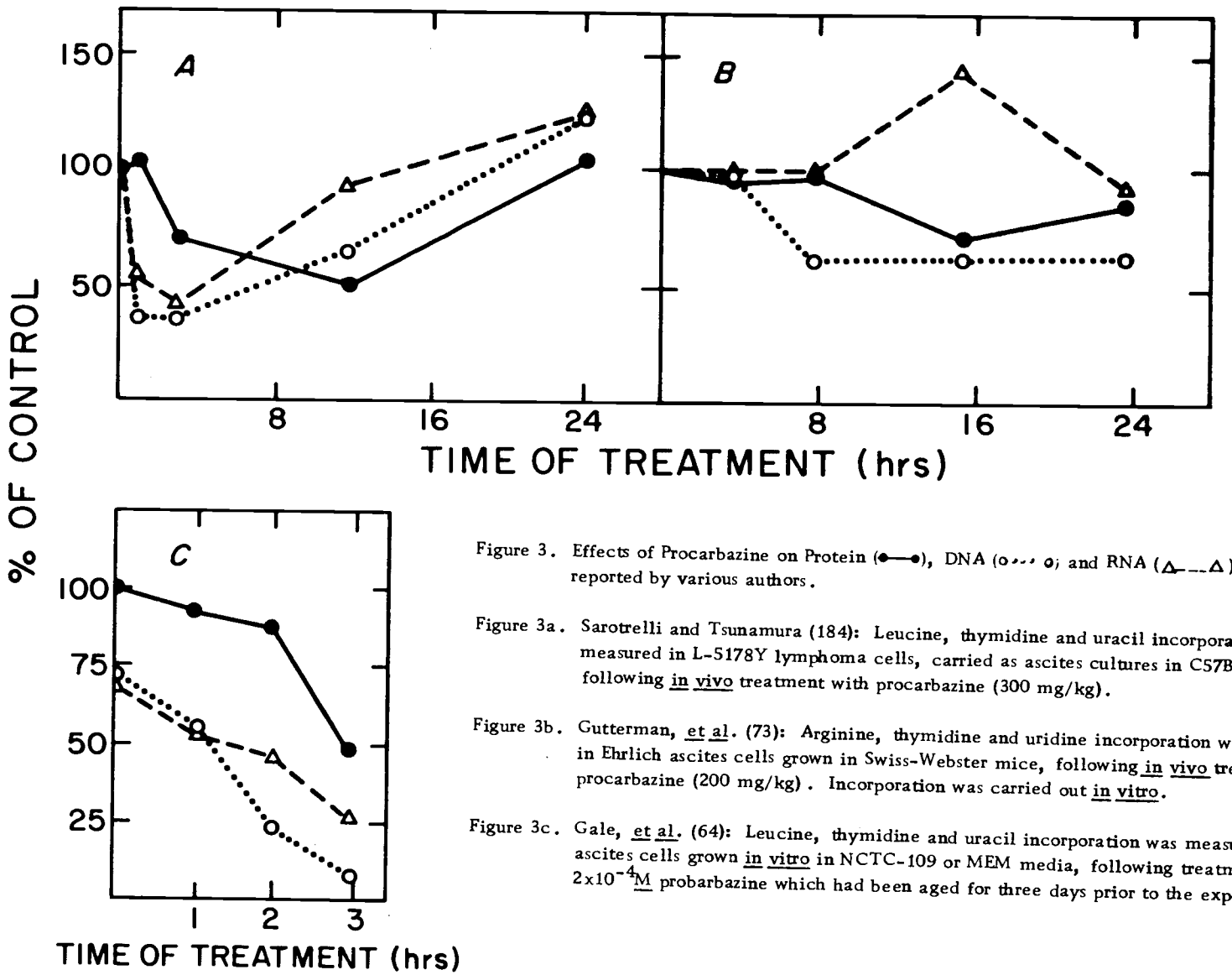


Figure 3. Effects of Procarbazine on Protein (●—●), DNA (o···o) and RNA (Δ---Δ) Synthesis as reported by various authors.

Figure 3a. Sarotrelli and Tsunamura (184): Leucine, thymidine and uracil incorporation were measured in L-5178Y lymphoma cells, carried as ascites cultures in C57BL x $DBAF_1$ mice, following in vivo treatment with procarbazine (300 mg/kg).

Figure 3b. Gutterman, et al. (73): Arginine, thymidine and uridine incorporation were measured in Ehrlich ascites cells grown in Swiss-Webster mice, following in vivo treatment with procarbazine (200 mg/kg). Incorporation was carried out in vitro.

Figure 3c. Gale, et al. (64): Leucine, thymidine and uracil incorporation was measured in Ehrlich a ascites cells grown in vitro in NCTC-109 or MEM media, following treatment with 2×10^{-4}M probarbazine which had been aged for three days prior to the experiment.

following in vivo treatment with 300 mg/kg procarbazine on the 6th day after implantation (Figure 3a). Radioactive precursors were administered in vivo, one hour prior to sacrifice. DNA and RNA synthesis were maximally inhibited three hours after treatment with procarbazine, and protein synthesis was maximally inhibited by 12 hours. The extent of inhibition was, in all cases, of the order of 50%, but all activities returned to normal or even higher levels, by 24 hours. It was demonstrated that the decrease in incorporation was not due to increased degradation of macromolecules, and that the activities of the enzymes thymidine kinase, thymidine monophosphate kinase, and DNA nucleotidyl-transferase were not affected.

Gutterman et al. (73) measured arginine, thymidine and uridine incorporation in Ehrlich ascites cells grown in male albino Swiss-Webster mice, following in vivo treatment with 200 mg/kg procarbazine six to eight days after implantation (Figure 3b). However, radioactive precursor incorporation was carried out in vitro with washed cells, resuspended in Krebs-Ringer medium containing 100 mg glucose per 100 ml. Cells were incubated at 37°C and 30 minutes were allowed for incorporation. An enhancement, rather than an inhibition, of RNA synthesis was observed. Protein and DNA synthesis showed a more modest but more prolongued inhibition than that observed by Sartorelli and Tsunamura (184).

Gale et al. (64) have used aged solutions of procarbazine to study effects on protein and nucleic acid synthesis in Ehrlich ascites cells cultured in vitro (Figure 3c). Inhibition was more pronounced than that observed in in vivo studies, and was found to be linearly related to drug concentration. Catalase did not affect the extent of inhibition to a significant extent. They also observed that the inhibition of DNA synthesis could be reversed by washing the cells free of the drug.

Koblet and Diggelmann (113) observed almost complete cessation of protein synthesis in perfused livers obtained from rats pretreated in vivo with procarbazine. The extent of ribosome aggregation and the ability to load t-RNA appeared normal. However, the rate of incorporation of leucine by polysomes from pretreated animals was significantly lower than with polysomes from untreated rats.

Sartorelli and Tsunamura (184) note that procarbazine treatment of tumor bearing mice leads to an accumulation of RNA and protein, and an increase in cell volume in L-5178Y lymphoma cells, at about the time when cell death is maximal.

Obrecht and Fusenig (150) have reported a decrease in NAD^+ levels and inhibition of glycolysis and respiration by procarbazine, although tumor cells were less sensitive in this regard than the intestinal mucosa.

Gutterman et al. (73) found no effect on oxygen uptake or on the production of $^{14}CO_2$ from either glucose-1-^{14}C or glucose-6-^{14}C in Ehrlich ascites cells harvested from Swiss-Webster mice which had been treated for eight hr. with 200 mg/kg procarbazine, though aerobic glycolysis was depressed by 25%. No change in the rate of glucose utilization was observed in Ehrlich ascites cells treated in vitro with aged procarbazine solutions (64).

On the basis of experiments with formaldehyde, 4-formyl-N-isopropyl-benzamide and 4-hydroxymethyl-N-isopropylamide, Weitzel et al. (229) have suggested that the therapeutic effect of procarbazine might be due to specific inhibition of nucleoside transport.

Evidence of possible membrane damage by procarbazine was obtained by Zangger (237) using Ehrlich ascites cells treated in vitro. Distinct morphological changes, characterized by stalagmosis and stalagmoptysis, were observed, but only at levels of procarbazine much higher than required for tumor inhibition.

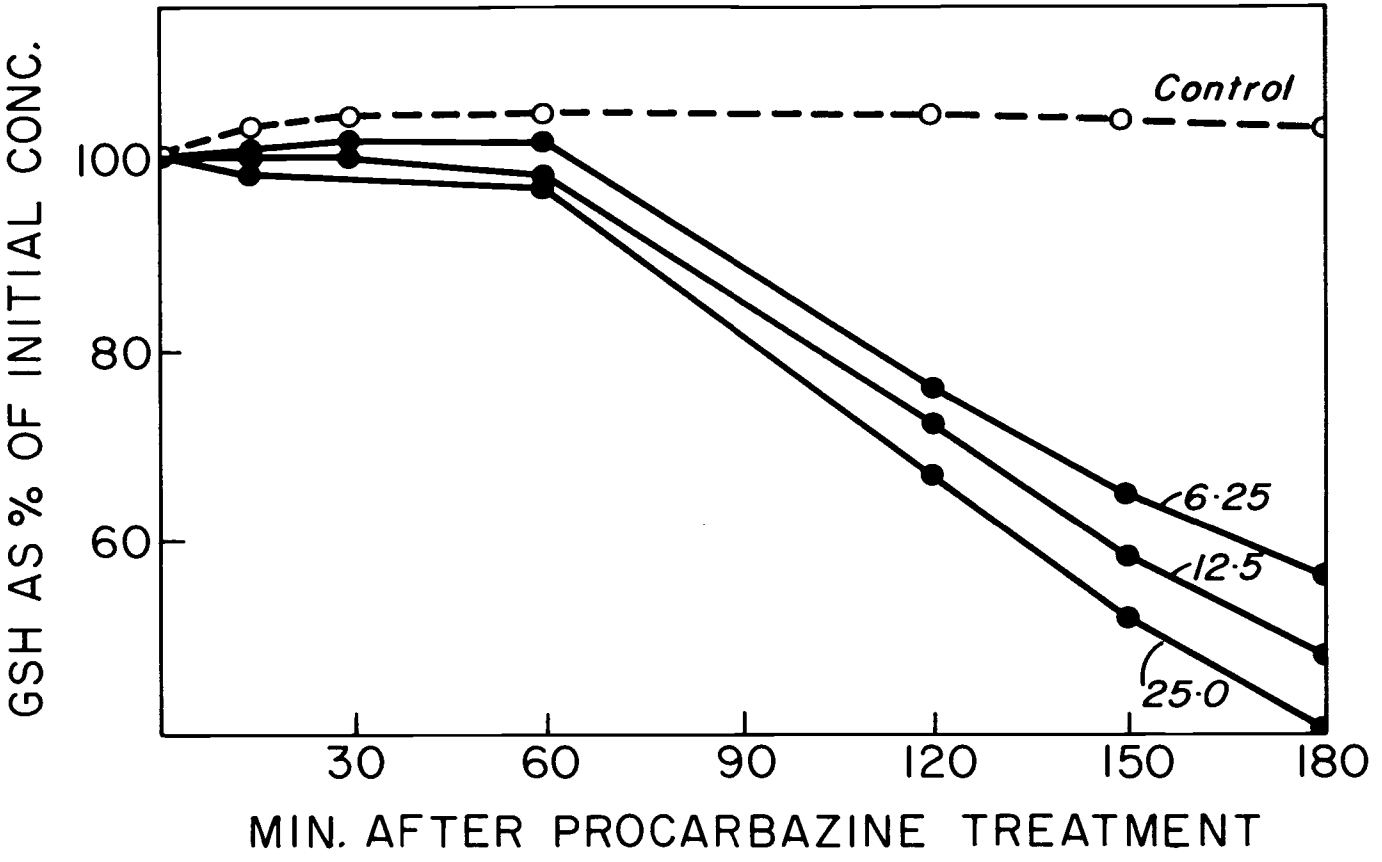
Preliminary Indications of Effects on Thiols

Kosower and Miyadera (114) have suggested that the antibiotic action of diazene antibiotics, and the corresponding hydrazides, may involve the intracellular oxidation of glutathione. In fact, specific reagents for the intracellular oxidation of glutathione belong to this class of compounds (116, 117, 119). Azo procarbazine might be

expected to behave similarly, and free radical formation during the metabolism of procarbazine might also be expected to result in GSH oxidation.

Mortensen (142) examined the reaction between procarbazine and reduced glutathione (GSH), and could not detect any oxidation. This is not surprising, since they are both reducing agents, and it is azo procarbazine that would be expected to react with GSH. He was equally unsuccessful in producing a decrease in GSH levels in erythrocytes, in the presence of glucose. Glucose can be utilized to generate reducing equivalents for reduction of oxidized glutathione (GSSG). After incubation with procarbazine 60 minutes in the absence of glucose (Figure 4), GSH levels dropped precipitously. When erythrocytes were washed prior to addition of procarbazine, the drop in GSH levels was immediate. Addition of glucose resulted in regeneration of GSH.

Preliminary experiments in this laboratory (169), revealed a significant decrease in rat liver GSH levels after treatment for two hours with procarbazine in vivo (200 mg/kg). A rebound to 150% of control was seen by five hours. Other hydrazines also caused a decrease in GSH levels, but with a different time-course (Table 1).



Mortensen; Scand. J. Haemat. 2 288 (1965).

Figure 4. Effect of procarbazine on erythrocyte GSH levels. Washed erythrocytes were incubated with various levels of procarbazine (6.25, 12.5 and 25.0 μmoles/ml) in saline. From: Mortensen; Scand. J. Haemat. 2 288 (1965).

Table 1. Changes in the total glutathione content of rat liver after *in vivo* treatment with various hydrazines.

Hydrazine	Dose (mmole/kg body wt., admin. i.p.)	Total Gluthathione (percent of control) Time of Treatment (hrs prior to sacrifice)					
		1	2	3	4	5	6
Procarbazine	0.8	81	70	90	130	150	
Monomethylhydrazine	0.4	71	95	104	102		
Ethylhydrazine	0.2	103	95	75	88	98	
Hydrazine	0.4	115	90	90	75	76	100

Unpublished observations, Reed and Wittkop (159); average GSH level in control rat liver was 5.1 ± 0.8 μmoles/gram wet wt, determined by GSH reductase method (214).

Diamide

Chemistry

Diamide (Diazenedicarboxylic acid bis (N, N-dimethylamide); Appendix 1) is one of a number of disubstituted azo compounds which have been introduced over the past few years as specific reagents for glutathione oxidation (116, 117, 119). It has been the reagent of choice in studies concerned with the biological significance of GSH oxidation.

The mechanism of oxidation involves a nucleophilic attack by GSH on the N=N double bond, followed by reductive cleavage of the S-N bond to release GSSG and the hydrazide:

$$GSH + RN{=}NR \longrightarrow GS\text{-}\overset{R\ H}{N\text{-}N}R \xrightarrow{+GS^-,\,H^+} GSSG + \overset{H\ H}{RN\text{-}NR}$$

The half-time for the reaction between diamide and GSH is of the order of a few seconds, and permeation across the cell membrane is virtually instantaneous (119). Considering that the half-time for regeneration of GSH following oxidation by diamide is also of the order of a few seconds in actively metabolizing cells (17), it becomes apparent that partial oxidation of the GSH pool in actively metabolizing cells would be quite difficult to achieve. The hollow fiber perfusion technique that is presented in this thesis satisfactorily copes with this difficulty.

Diamide will also oxidize flavins and pyridine nucleotides, as well as other thiols, including lipoic acid and coenzyme A (26, 80, 115, 151). The rate of reaction with these electron donors, however, is much lower than with GSH, and the enzymatic linkage between these redox couples in intact cells would lessen the impact of non-specific reactions to the extent that claims of specificity may be valid. In intact cells, pyridine nucleotides and protein thiols become oxidized after treatment with diamide (80), but it is not clear whether this is a primary or secondary effect.

Biological Effects

It is interesting to compare the biological effects of procarbazine with those of other related compounds, such as diamide, which have been found to react with glutathione. A number of diazene compounds have antifungal activity, and this activity appears to be correlated with their ability to oxidize glutathione (114). Methyl phenyldiazenecarboxylate, the first of the series of reagents introduced by Kosower et al. as specific reagents for glutathione oxidation (117), has been found to inhibit germination, growth and sporulation of the fungus Trichoderma viride (228), and to inhibit reversibly protein and nucleic acid synthesis in nucleated cells (79).

Diamide has been found to cause inhibition of protein synthesis in rabbit reticulocytes (238), inhibition of RNA synthesis in E. coli

(239), inhibition of growth in E. coli (227), and reversible inhibition of DNA, RNA and protein synthesis in nucleated mammalian cells (81). Harris et al. (81) also observed mitotic arrest, but assigned this effect to oxidation of protein thiols. Interference with oxidative phosphorylation (191) and amino acid transport (84) have also been noted.

The Intracellular Glutathione Redox Potential

Glutathione (GSH; Appendix 1) is present in cells at ten times the concentration of all other low molecular weight thiols combined (98) and is linked enzymatically with a number of other important redox couples in the cell, thus allowing it to exert a regulatory influence on these couples. As a parameter of cell function, the glutathione redox potential may be considered to be analogous to the adenylate energy charge. Both of these parameters depend upon the relative levels of different forms of the same substance rather than the combined total level. They also appear to be closely regulated in vivo, with a rapid recovery rate, and are capable of directing metabolic events (i.e., the hexose monophosphate shunt; 88) toward maintaining their proper state.

Redox Control of Enzyme Activity

Any enzyme containing an essential thiol group which is accessible

to GSH and GSSG, is subject to redox control. If the redox potential in the cell becomes oxidizing, the enzyme thiol will become oxidized by the reaction:

$$GSSG + PSH \rightleftharpoons GSH + GSSP$$

which is enzymatically catalyzed in intact cells (37, 215) and occurs at appreciable rates without enzymatic catalysis (23, 94, 198, 233).

Substantial amounts of protein-glutathione mixed disulfides (PSSG) have been found in a variety of tissues (96, 138, 233) and can be induced by administration of low molecular weight disulfides. Cysteamine or cystamine administered to mice appears bound to tissue proteins as mixed disulfides, with maximal binding occurring 30 minutes following administration (47). Cystine administered in vivo appears bound to tissue proteins as mixed disulfides, and the degree of binding and turnover rate differ between normal and wound tissue (232). Mixed disulfides have been found in serum albumin (108). It has been noted that the extent of mixed disulfide formation with serum proteins in vivo can be affected by administration of glutamate (234), although the role of glutamate appears to be to bind divalent cations which catalyze the reaction (231). The mixed disulfide between hemoglobin and GSH has also been found to be naturally occurring (236).

The extent to which a given protein thiol will become oxidized at a given GSSG/GSH ratio will depend on the microenvironment of that

particular protein thiol, but on the average, it can be calculated on the basis of reports by Modig (138, 139) that such enzymes occur normally about 30% as mixed disulfides in vivo, and, if this is true, a change from 1.5 to 3% GSSG would lead to 65% inhibition of enzyme activity.

Close to one hundred enzymes with essential thiol groups have been identified (98), and mixed disulfide formation has been demonstrated to lead to inhibition, at least in the cases of rat liver glycogen synthetase (50), bovine brain hexokinase (167), rabbit muscle glyceraldehyde-3-P dehydrogenase (18), Clostridum formyltetrahydrofolate synthetase (149), Pseudomonas histidine ammonia lyase (59), bovine liver rhodanase (223), papain (10), streptococcal proteinase (52), rat thymus nucleoside pyrimidine dexyribosyltransferase (56), and RNA synthetase (206). In the case of rat thymus nucleoside pyrimidine deoxyribosyltransferase, mixed disulfide formation and inhibition were achieved in vivo by cystamine administration (56).

Rabbit liver fructose diphosphatase (161), and glutathione reductase (137), appear to be activated by mixed disulfide formation. Hemoglobin is neither activated nor inactivated by mixed disulfide formation, although thiol blocking reagents can affect its oxygen transport properties and solubility (98).

Another mechanism of control of enzyme activity by the redox potential of the medium involves the breakage and formation of intramolecular protein disulfide bonds. Such a mechanism is involved in the formation of insulin from proinsulin, and its inactivation by scrambling (219) as well as in the reactivation of reduced lysozyme (185). In such cases, there is an optimal GSSG/GSH ratio, above or below which, inactivation results.

Redox Control of Protein Synthesis

Complete oxidation of glutathione by diamide causes instantaneous cessation of protein synthesis in rabbit reticulocytes (238). Although translation recovers upon partial regeneration of GSH, initiation does not recover until regeneration is complete.

When a small amount of GSSG is added to rabbit reticulocyte lysate, in the presence of normal amounts of GSH, initiation is inhibited, but translation is unaffected (120). As little as 5% GSSG causes 90% inhibition of protein synthesis in this system. If GSSG is allowed to act for more than 12 minutes, the inhibition is not reversed upon regeneration of GSH, and is accompanied by the degradation of polysomes to monosomes (121). These experiments suggest several mechanism of inhibition may be involved. Specific mechanisms have been investigated in some detail and a few of these studies are outlined below.

A cytoplasmic factor, which has been identified as a nucleoside diphosphate phosphohydrolase, inhibits protein synthesis in vitro. This inhibition is accentuated by GSSG and abolished by reduced thiols or GTP (175).

Sea urchin eggs, which exhibit a redox cycle during mitosis, also exhibit a corresponding protein synthesis cycle. In fact, the same KCl and TCA soluble protein which is involved in the SH/SS fluctuation during the cell cycle, was found to have a stimulatory effect on protein synthesis, and this stimulatory effect depended upon the SH content of the protein, and involved an interaction between its thiols and those of other proteins, resulting in association of monosomes to polysomes (134).

When rat liver polysomes are treated with thiol reagents, they rapidly dissociate into monosomes, and more slowly, into ribosomal subunits (45). When ribosomes are dissociated into 30S and 50S subunits by incubation with GSSG, they become completely inactive toward poly-U directed poly-Phe synthesis. Poly-U binding is not affected, but the ability to bind Phe-t-RNA is completely lost. This inhibition is accompanied by the binding of three glutathione residues per subunit, presumably as mixed disulfides (62).

The SH groups of the 60S subunit of human ribosomes have been found to play a role in the binding of Phe-t-RNA. When 60s subunits are preincubated with NEM and assayed with untreated 40s subunits,

the binding of Phe-t-RNA is only 17% of control and synthesis of poly-Phe is 35% of control. However, NEM treated 40s subunits assayed with untreated 60s subunits display 77% activity in Phe-t-RNA binding and 95% activity in poly-Phe synthesis (12). The mechanism of NEM deactivation of E. coli ribosomes may be different, because sulfhydryl reagents have no effect on E. coli 50s subunits, which can only be demonstrated to react with a fraction of a molecule of SH reagent per subunit, compared to 2.7 for the 30s subunit (195).

It has been possible to assign specific functions to particular ribosomal proteins in E. coli 30s subunits, by correlating loss of activity with reaction with NEM, at different NEM concentration. Using this method, the ability to bind 50s subunits and initiation complex formation have been assigned to protein S18, and Phe-t-RNA binding has been assigned to S21 (66).

Hultin has identified one site on the mammalian ribosome at which reversible disulfide linkage between ribosomal proteins can occur. Unmasking of protein 10 by high salt concentrations allows it to react with thiol reagents. When ribosomes are dialyzed against thiol-free media prior to unmasking, the unmasking becomes irreversible, and a new protein factor appears upon degradation of the ribosome, which presumably corresponds to the disulfide between protein 10 and protein IIIA, which is situated adjacent to it on the ribosome. Addition of mercaptoethanol either before or after

unmasking prevented the appearance of this new fraction. Reaction with NEM after unmasking resulted in three times more inhibition of protein synthesis than reaction with NEM before unmasking (91).

Two soluble protein factors (EF_1 and EF_2) are required for peptide chain elongation in mammalian ribosomes. EF_1 is required for the binding of incoming amino-acyl-t-RNA to the acceptor site on the ribosome, by forming a complex with the amino-acyl-t-RNA, GTP, and the 40s subunit. After peptide bond formation at the acceptor site, peptidyl-t-RNA requires EF_2 for its transportation to the donor site, a process which requires GTP and reduced thiols. EF_2 binding to the ribosomes is enhanced by preincubation of EF_2 with reduced glutathione. Oxidized glutathione can completely inhibit the amino-acyl transfer reaction (207). Formation of the EF_2-GTP complex does not appear to involve the SH group of EF_2 (9).

Cycloheximide inhibits the translocation of peptidyl-t-RNA from the donor to the acceptor site. This effect is prevented by raising the sulfhydryl concentration of the incubation mixture. EF_2 which has been preincubated with GSSG reduces the inhibition by cycloheximide (10). However, cycloheximide has no effect on bacterial or mitochondrial protein synthesis, suggesting differences in mechanism (194).

Tylocrebrine inhibits protein synthesis in E. coli and mitochondria, as well as in mammalian endoplasmic reticulum (82).

Its mechanism of action appears to involve the inhibition of breakdown of polysomes and release of nascent peptides. This inhibition is overcome by increasing the concentration of dithiothreitol, GSH, or mercaptoethanol (89).

Microsomes from regenerating rat liver have about twice the protein synthesis activity as microsomes from normal liver. Treatment of normal liver microsomes with thiols or excess GTP increases their activity by a factor of two (146). However, polysomes which are detached from the membrane by deoxycholate treatment show the same activity and the same response to thiols, whether they come from normal or regenerating liver. Reconstituted rough endoplasmic reticulum displays a sulfhydryl requirement which does not exist in the absence of the membrane, regardless of whether the ribosomes were originally membrane bound or free (147).

Nuclear Thiols and Disulfides

Intracellular oxidation of glutathione with diamide, in a relaxed mutant of *E. coli* which does not require active protein synthesis for RNA synthesis, results in complete cessation of RNA synthesis in about 20 minutes (239). The time requirement suggests the effect is achieved through oxidation of protein thiols.

RNA polymerase is inhibited by thiol reagents (76, 143), and it has been demonstrated that mixed disulfide formation with cystamine

leads to reversible inhibition (206). Modig (139) has demonstrated that cystamine can cause disulfide formation with nucleoproteins in vivo. Purine nucleoside phosphorylase (4), nucleoside-pyrimidine deoxyribosyl transferase (56) and leucyl-t-RNA synthetase (174) are also inhibited by thiol reagents. Essential thiol groups are common to all the amino-acyl-t-RNA synthetases, except lysyl-t-RNA synthetase (124).

It is interesting to note that both poly-ADPR synthetase and nucleoprotein phosphokinase, which catalyze functionally significant modifications of nucleoproteins, require reduced thiols for maximal activity (235, 93). Increased phosphate incorporation into chromatin was achieved by treatment of rats with the thiol-reducing reagent, dithiothreitol. This implies that these enzymes may be responsive to the thiol/disulfide redox state of the cell. It is therefore possible that even if enzymatic modifications of histones or non-histone proteins are involved in the transition between resting and dividing cells, as was recently proposed in the case of phosphorylation of histone F-1 (22), such processes could be primarily controlled by the glutathione redox potential.

Intracellular oxidation of glutathione by diamide leads to inhibition of mitosis in Chinese Hamster ovary cells (81), and this effect has been ascribed to the oxidation of protein thiol groups. Addition of thiols to non-dividing yeast cells induces cell division (144), and

thiols have been found to stimulate cell division in Tubiflex, Chilomonas, fibroblasts and mouse skin (140).

The concentration of SH groups in sea urchin eggs has been observed to increase sharply at the beginning of mitosis, and to fade shortly after anaphase (103). This SH cycle has been found to be largely due to a TCA soluble protein, which is apparently the microtubule protein of the mitotic spindle (36). The same pattern has been observed in synchronous HeLa cells (182).

A thiol-disulfide exchange reaction between microtubule protein and a ribonucleoprotein has been observed in sea urchin eggs (180). It was also observed that this exchange is accompanied by contraction of microtubule protein fibers, reassembled in vitro (179). This exchange reaction also occurs with sperm flagella microtubules (107). It is conceivable that other specific thiol-disulfide interchange enzymes might exist, enabling selective effects on protein-DNA interactions to occur via a redox mechanism.

Metabolically active chromatin contains a higher proportion of reduced to oxidized thiols than metabolically inactive chromatin (154). The levels of reduced thiols in nucleoproteins are elevated during liver regeneration (104). It is apparent that the degree of protein-DNA binding is subject to redox control. Reduction of chromatin with dithiothreitol results in loss of protein (5). Effects on protein-DNA binding caused by cytostatic alkylating agents have been found to

be correlated with the degree of inhibition of cell multiplication (72). It was observed that these effects could be mimicked by treatment with thiol reagents (72).

In Chinese Hamster cell culture, histone thiol levels were found to be highest during periods of maximal DNA synthesis (216). In sea urchin eggs the proportion of histone thiol to disulfide was observed to increase from less than 10% to more than 50%, 140, 180, and 275 minutes after fertilization, at which times, DNA synthesis was also maximal. A sharp increase at 30 minutes was also observed, although it did not occur in subsequent cycles. The phosphate content of the proteins remained constant for 10-90 minutes after fertilization, and then appeared to vary reciprocally with the proportion of reduced thiol (155).

When histone F-3 thiols are oxidized, these histones become less effective in decreasing the template activity of DNA in vitro (85). Histone F-3 occurs mainly in the reduced, monomeric form in interphase chromosomes of HeLa cells, but is polymerized or complexed with non-histone protein through disulfide bonds in metaphase chromosomes (178). A similar pattern is seen in sea urchin eggs (156).

The histone fraction normally referred to as histone F-1 has been shown to contain a non-histone protein component (100). The non-histone component (P-1) has been purified and characterized. It was found to contain about one mole percent cysteine, and it was

therefore suggested that although histone F-1 itself contains no cysteine, it might be subject to redox control (as is histone F-3), through protein P-1 (196).

Cortisol, a hormone which induces RNA synthesis in rat liver, causes an increase in the thiol content of nuclear proteins, which coincides with the time-course of activation of RNA synthesis. The increase in thiol content has been demonstrated to be due to the cleavage of disulfide bridges between the nucleoproteins (42).

Other Processes Controlled by the Redox State

Cell adhesion is sensitive to sulfhydryl reagents (71). Macrophage spreading can be induced by dithiothreitol in the presence of divalent cations (165). Phagocytosis is found to be accompanied by increased glutathione reductase activity (204). Rh antigen activity of red blood cells is altered with sulfhydryl reagents (68).

It has been suggested that autoimmunity in rheumatoid arthritis could result from protein denaturation due to accelerated formation of disulfide bonds (130). Serum sulfhydryl levels are seen to increase with clinical improvement and decrease with relapsing disease activity (131), and a number of drugs that are beneficial in the treatment of rheumatoid arthritis alter serum protein sulfhydryl reactivity and accelerate thiol-disulfide interchange (222). Cataracts have been correlated with decreased GSH levels (201) and increased protein

disulfide levels (75). Cholesterol synthesis appears to be subject to redox control, as well (87).

Aging has been correlated with an increasingly oxidizing glutathione redox potential (78), and an accumulation of products of lipid peroxidation (41). The glutathione redox state in L5178Y cells appears to undergo a diurnal variation, which does not appear to be due to dietary effects (thesis work). Glutathione levels can be influenced by dietary factors (211), but a diurnal variation in rat liver GSH levels was found to be largely abolished by adrenalectomy, and non-protein SH levels were found to decrease markedly in mice subjected to trauma or exposed to cold (11). Such effects could be due to hormonal influences.

There is evidence that both synthesis and reduction of glutathione are affected by hormones in normal situations (77). Growth hormone causes an increase in non-protein thiol levels in rat liver (77). It has also been found that thiol reagents increase the rate of release of this hormone from pituitary slices (186). The release of insulin also appears to be subject to redox control (83). Glutathione appears to play a role in steroid hydroxylation, thus exerting control over the rate of synthesis of steroid hormones (230). Thiols react directly with insulin, vasopressin, oxytocin, hydrocortisone, oestradiol-17-β thyroxin and oxidation products of adrenalin (98). The extent to which such reactivity is significant in vivo is unclear.

In the case of vasopressin and oxytosin, antagonism by thiols in vivo has been found to be due to changes in membrane thiols, rather than to direct reaction with the hormones (74). Adrenalin can cause a rise, fall or no change in thiol levels, depending on the animal, the tissue and the dose of hormone used (127).

A number of thiol reagents and oxidizing agents, including GSSG, have been found to increase islet tissue permeability (225), whereas various thiols protect from such effects and can reverse them (226). Many reports have appeared on the effects of thiol reagents on the transport of glucose and cations, and the mechanisms thereof. Smith and Ellman (197) have recently reviewed the subject. Thiols have been shown to alter neural activity by affecting the transport of cations across the neural membrane (43).

Orlowski and Meister (157) have proposed that glutathione may be involved in a general mechanism for amino acid transport, via the γ-glutamyl cycle. The enzyme γ-glutamyl transpeptidase catalyzes the transfer of the γ-glutamyl residue from glutathione to a variety of amino acids. The amino acid is believed to be transported in this form and then degraded by γ-glutamyl cyclotransferase to release the free amino acid. Oxidation of glutathione with diamide results in inhibition of amino acid transport (84).

The ratio of reduced to oxidized glutathione has been found to control the rate of the hexose monophosphate pathway (97), and the

high HMP shunt activity observed in Ehrlich Ascites tumor cells has been traced to glutathione metabolism in these cells (88).

An increase in SH concentrations upon addition of ADP to mitochondria in state 4 has been observed, and it was noted that this increase is abolished by uncouplers of oxidative phosphorylation (177). However, this difference appears to be due to decreased accessibility of protein thiols, rather than to differences in absolute amounts (34). Nevertheless, addition of diamide to rat liver mitochondria results in a decrease in the respiratory index which is reversed upon treatment with dithiothreitol (191). Deficiency in mitochondrial ion transport following diamide treatment was observed (191).

Carbonylcyanide m-chlorophenylhydrazone, a well known uncoupling agent, inhibits lactose and amino acid transport by isolated membrane vesicles. Its inhibitory effects are not alleviated by dilution and washing, but the addition of a variety of thiol reagents dramatically blocks and reverses its inhibitory activity (101).

Role of Glutathione in Neoplasia

Processes which are aberrant in the neoplastic state, such as cell adhesion, membrane surface antigenicity (126), membrane transport (171), aerobic glycolysis (224), charge transfer (209), hormone responsiveness and cell division, appear to be subject to redox control. Redox mechanisms have also been implicated in the

process of viral infection (60, 217), and it is possible that redox changes within the cell are directed by the invading parasite (193).

A number of carcinogens have been found to interact with thiol groups, and to cause pronounced changes in the thiol and disulfide levels in cells (77). Production of free radicals, which has been correlated with carcinogenic activity (159), would be expected to result in an oxidative challenge. Free radical inhibitory activity has been correlated with carcinostatic activity (65).

Glutathione reductase and glutathione peroxidase activities were found to be abnormal in hepatomous rat liver induced by diethylnitrosamine (160). The ratio of activities of glutathione peroxidase to glutathione reductase in rat liver is seen to increase with age. Both fetal liver and 4-dimethylaminobenzene-induced primary hepatoma had ratios between 1.5 and 2.0, whereas adult rat liver has ratios in the range of 2.5 - 5.9, suggesting that azo-dye tumor induction involves a reversion to fetal glutathione metabolism (210).

It is interesting that the activity of γ-glutamyl transpeptidase is found to be as much as two orders of magnitude higher in certain hepatomas and in neonatal rat liver than in normal adult rat liver (54), and is increased upon treatment with carcinogens (55).

Cytotoxic effects of carcinogenic N-nitrosodialkylamines are prevented by cysteamine pretreatment in vivo (49). It may be relevant to note that mercaptoethanol, cysteine and cysteamine are capable of

inducing polyploidy in cultured human lymphocytes (95). Cysteine has been found to be effective in treating transplanted malignant murine thyomas (27). Hydrogen peroxide, which is used as a reagent for intracellular oxidation of GSH, has antitumor activity (123). Iodine, which could act by oxidizing glutathione, has also been found to be an effective carcinostatic agent (221). In fact, thiol reagents have long been used in the clinical treatment of cancer (112).

MATERIALS AND METHODS

Cell Culture Techniques

L-5178Y Ascites Culture

L-5178Y lymphoma cells, obtained from Professor Alan Sartorelli (Yale University School of Medicine, New Haven, Connecticut) were grown in male BDF_1 mice (Texas Inbred Mice Co., Houston, Texas). Transplantation was achieved by collecting ascites fluid seven days after implantation, separating cells by centrifugation for two minutes at 1500 g in a refrigerated Sorvall RC-2B centrifuge, and inoculating new mice with about 10^6 cells resuspended in saline (about 0.1 ml of a 1/10 dilution of packed cells). BDF_1 mice were used only to carry the cell line. For experimental work, cells were implanted as above in male DBA/2J mice (Jackson Laboratory, Bar Harbor, Maine), and harvested on the 6th day after implantation. Mice were sacrificed by cervical dislocation, and an abdominal incision was made in order to remove ascites fluid. Unless otherwise specified, the animals were anaesthetized with ether prior to drug administration.

Drug Administration

All drugs were administered intraperitoneally. Procarbazine

was dissolved in isotonic saline (37.5 mg/ml) just prior to use, and was given at a dosage of 300 mg/kg. Diethylmaleate was diluted 1/10 in glycerolformal and given at a dosage of 720 mg/kg (31). Time of treatment is defined as the time between drug administration and sacrifice.

Cell Preparation

All cell preparation procedures were carried out at 0°C. In experiments requiring the use of whole ascites fluid or the first supernatant thereof, heparin was added to the centrifuge tube to be used for collection of ascites fluid. No heparin was used in other experiments. The basic cell preparation procedure which will be referred to throughout this thesis, is described below. All centrifugations were at 1500 g for two minutes in a refrigerated Sorvall RC-2B centrifuge. Fluid volumes were scaled according to the number of mice used for each pool of ascites fluid. The values given below correspond to one mouse per pool:

1) Centrifuge ascites fluid. Discard supernatant.

2) Resuspend cells in 1 ml distilled H_2O. After 30 seconds of vigorous mixing, add 1.5 ml isotonic saline and mix quickly. Centrifuge. Discard supernatant.

3) Repeat step 2.

4) Resuspend cells in 2.5 ml isotonic saline. Centrifuge. Discard supernatant.

5) Repeat step 4.

6) Resuspend cells in 1.25 ml isotonic saline or other medium to be used.

Packed cell volume was used as a measure of the amount of cell material per unit volume of final suspension. Microscopic analysis revealed the absence of cell debris in the final suspension when the above procedure was followed. For packed cell volume determination, cell suspension was drawn into four capillary tubes (1.6 - 1.8 mm i.d.) and these were sealed with Seal-ease^{R}. Syringe needle caps were found to be effective in preventing breakage of the capillary tubes during centrifugation. Centrifugation was at 1500g for three minutes. This procedure gave very reproducible values which correlated well with cell counts, and correlated better than cell counts with glutathione concentration. For cell counts the final cell suspension was diluted 1/100 with isotonic saline in an RBC pipette, and counted on a Spencer^{R} hemocytometer.

Spinner Flask Culture

L-5178Y lymphoma cells were also cultured in vitro. Cells were obtained from BDF_1 mice bearing 6-day tumors. Mice were sacrificed without the use of ether, by cervical dislocation. After dousing the ventral side with 70% ethanol and wiping away excess ethanol, a 1 1/2 inch longitudinal incision was made through the outer

skin, with the blunt side of the scissors facing the peritoneal membrane. The skin was pulled back, and 2 ml warm Fischer's medium were injected into the peritoneal cavity. The ascites fluid was removed with the same syringe, without removing the needle. All instruments were sterilized prior to use. Cells were kept on ice in capped syringes until transferred into spinner flasks.

The cells were grown in Fischer's medium supplemented with 10% horse serum (58) at an initial concentration of 1-4 X 10^5 cells/ml. Once the culture was established, cells were diluted to less than 10^5 cells/ml with growth medium every 48 hours. For experiments, cells were grown to a concentration of 3-4 X 10^5 cells/ml and used 24 hours after addition of fresh medium. Cells were concentrated by centrifugation at 500g for 10 minutes at 37°C in an International centrifuge, and resuspension in a small volume of medium.

Hollow Fiber Perfusion

In order to simulate conditions which might exist in vivo, L-5178Y Lymphoma cells were placed in Bio-Fiber 50 minibeakers (Bio-Rad Laboratories, Richmond, California), and medium, with or without drug, was pumped through the hollow fibers (Figure 5). Cell culture in hollow fiber devices has been described (187), but the use of Bio-Fiber 50 minibeakers is unprecedented. These units were selected because they allow easy removal of aliquots of cell suspension

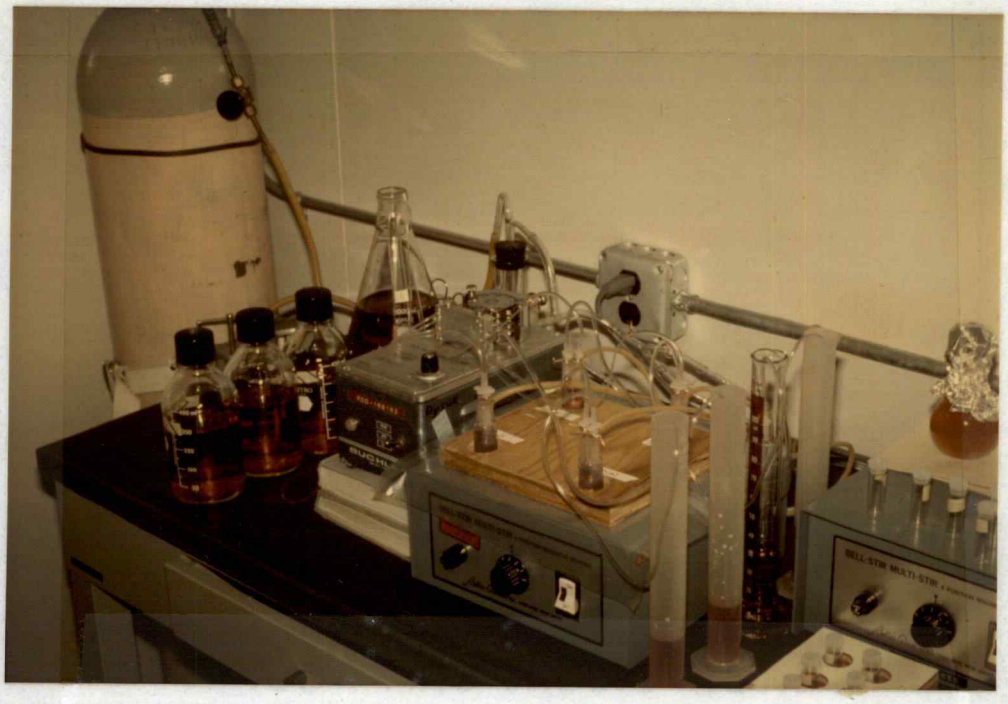


Figure 5. Photograph of hollow fiber apparatus.

without disturbing the perfusion.

For short-term storage (up to 3 weeks), hollow fiber devices were kept immersed in 75% ETOH at 0-5°C. At least 3 hours, and no more than 24 hours prior to each experiment, the dismantled devices were rinsed with glass distilled water (GDW), assembled, and filled and perfused with sterile GDW containing antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), at room temperature, for at least two hours. The apparatus was then transferred to a 37°C constant-temperature room, and the inside fluid and perfusing fluid were replaced with Fischer's medium, containing antibiotics (as above) and 0.005 M HEPES buffer. After at least 30 minutes of perfusion with medium, the inside fluid was replaced with cell suspension, and 5% CO_2 in air (water saturated) was passed through the sampling ports, at a minimal flow rate, to insure aerobic conditions and efficient bicarbonate buffering. Medium was pumped through each minibeaker at a rate of 90-100 ml/hr with a Buchler 4 channel polystatic® pump. Cells were maintained in a homogeneous state by stirring with a micro-spinbar® (15 mm X 1.5 mm; Bel Art Products, Pequannock, N. J.), on a 4-position magnetic stirrer. For removal of cell suspension aliquots, a transfer pipette was introduced through the open sampling port, taking care not to touch the fibers.

Cells were obtained from DBA/2J mice bearing 6-day tumors. Since several mice were required, heparin was added to the centrifuge

tube used for collection of ascites fluid. The cell preparation procedure described on page 35 was followed through step 4, at which time the cells were resuspended in Fischer's medium containing 10% horse serum (as described above). About 8 ml of cell suspension were required to fill each minibeaker. Aliquots of cell suspension that were taken during the course of perfusion were centrifuged at 1500 g for two minutes and resuspended in 1 ml isotonic saline. The entire amount was transferred to a homogenizing tube for homogenization. The final sample was removed from the apparatus by disconnecting the gas line, swirling the suspension, and pouring the cells out through the sampling port.

The minibeakers were rinsed with saline immediately after removal of the cell suspension. They were dismantled and immersed in saline until further cleaning could be done, by the following procedure:

1. Wash with saline until the wash fluid no longer is turbid
2. Rinse interior of minibeaker with GDW for two minutes. Clear hollow fibers by displacing medium with GDW.
3. Allow to stand overnight in aqueous 1% Triton X 100, at 0-5^{o}C
4. Rinse with GDW until no debris remains attached to fibers. If necessary, return to 1% Triton X-100 for additional soaking
5. Immerse in 80% EtOH or 1.5% Formalin. Note that final EtOH will be lower than 80% because of water inside the hollow fibers.

After prolonged storage in formalin , the minibeakers were again run through the cleaning procedure outlined above (except for the saline rinse), and then treated as described on page 39 in preparation for an experiment.

Vital Staining

Cells were diluted 1/100 with a solution of 0.1% trypan blue in saline, using an RBC pipette (213). Values obtained using 0.2% nigrosin (102) compared well with trypan blue values. Stained cells were counted after the stain had been in contact with the cells for five minutes, but no longer than ten minutes.

Plating Efficiency

Cell suspensions were diluted with Fischer's medium, containing 10% horse serum and antibiotics, to a final concentration of 20 cells per ml. The diluted cell suspension was then mixed in equal proportions with the agar medium described below, in a stoppered test tube. Final concentrations were 0.12% agar and 15% horse serum (57), in a final volume of 5 ml. The test tubes were placed in ice for exactly four minutes, within twenty minutes of mixing, to harden the agar (57), and then incubated at 37°C in a temperature-controlled room. Colonies were counted after eight to thirteen days incubation.

Agar Medium:

Fischer's medium:	Final concentration as recommended for cell culture
Antibiotics:	100 units penicillin per ml 100 μg streptomycin per ml
Agar:	0.24% final concentration. Diluted from 2% sterile solution
Horse serum:	20% final concentration
pH:	6.8

All reagents were from Grand Island Biological Company, Grand Island, New York.

Metabolic Studies

Several metabolic parameters were monitored during hollow fiber perfusion experiments. Cell suspension aliquots were taken as described on page 39, centrifuged for two minutes at 1500g and resuspended in an equal volume of saline. Perfusate samples (2 ml) were collected at regular intervals, and stored at -85°C until assayed. The following determinations were made.

Lactate Production

Lactate levels in the perfusate were measured by the method of Hohorst (86), with a few modifications. Lactic dehydrogenase, lactic acid standard solution, and NAD, were obtained from Sigma Chemical Co., St. Louis, Mo., and hydrazine sulfate was obtained from Matheson, Coleman and Bell, Norwood, Ohio. All solutions were

prepared fresh daily, as described below:

A) Hydrazine-glycine buffer: 0.4 M hydrazine sulfate; 0.005 M EDTA; 1 M glycine; pH 9.5

B) 5×10^{-2} M NAD in GDW

C) Lactic dehydrogenase: 1/5 dilution of Sigma LDH suspension (Cat. No. 826-6); approximately 200 units per ml, in GDW

To 0.5 ml samples (diluted with GDW) were added 0.4 ml solution B, the contents were mixed, and the absorbance at 340 nm was followed until it became constant (about six minutes). Solution C (0.05 ml) was then added, and the absorbance was again followed until constant (about 20 minutes). The blank contained all reagents, but no lactate. Linearity was demonstrated, and standards were included each time.

Oxygen Uptake

Cell suspension aliquots were diluted 1:3 with Fischer's medium containing 10% horse serum and antibiotics. Oxygen consumption was measured using a Gilson oxygraph equipped with a jacketed cell which was maintained at 37°C throughout the reading, and utilizing a Clark electrode for detection.

Leucine Incorporation

Cells were diluted as for oxygen uptake measurements in media containing ^{14}C-labeled leucine. Incorporation was allowed to proceed

for 20 minutes at 37°C. Protein was precipitated with 10% TCA after centrifugation to remove extracellular fluid. TCA precipitates were washed three times with 10% TCA, heated to 90°C for 15 minutes, and washed twice more with TCA, once with EtOH and once with acetone. The final precipitates were prepared for counting either by combustion with a Packard 306 Tri-Carb^{R} sample oxidizer or by digestion with Unisol^{R} (Isolab, Inc., Akron, Ohio). Amino acid specific activity was determined as described in the following section.

Leucine Incorporation Studies

Leucine Incorporation

Sartorelli and Tsunamura (184) examined leucine incorporation in L-5178Y lymphoma cells following *in vivo* treatment with procarbazine, as an indication of the rate of protein synthesis. Initial studies in this laboratory were done in the same manner. Each mouse received 0.2 ml of a solution of leucine-1-^{14}C in saline (5 μCi/5mg/10ml), and one hour was allowed for incorporation. Results obtained in this system were consistent with results reported by Sartorelli and Tsunamura, and further indicated there might be a synergistic effect on protein synthesis when procarbazine was administered in combination with diethylmaleate (a drug which lowers glutathione levels). However, variation of the level of leucine administered, and the time

allowed for incorporation, cast considered doubt on the significance of these values (see results section). It was therefore decided that a determination of the specific activity of leucine in the cells at various times after leucine administration would be helpful in the interpretation of incorporation values obtained in this system.

Extraction of Leucine from Lymphoma Cells

The location of the amino acid pool that is drawn upon for protein synthesis has been the object of considerable controversy. Fern and Garlick (53) looked at the ratio of the specific radioactivities of glycine and serine incorporated into protein in various rat tissues, and found it most closely resembled the ratio of the specific activities of the free amino acids in the tissues than the ratio seen in plasma, suggesting the immediate source for protein synthesis is intracellular, rather than extracellular. However, other authors (218) have obtained evidence that suggests the opposite, and Ilan and Singer (92) suggest that neither is the case, and that a separate intracellular pool exists, which can only be assayed accurately by looking at amino acids bound to t-RNA or incorporated into nascent peptides.

In the present work the intracellular specific activity of leucine was found to rise very rapidly to a steady state, and then to decrease over the period of incorporation. Under these conditions it would be expected that the overall intracellular specific activity should

correspond closely to the specific activity in the pool which is utilized for protein synthesis. Determinations were made on intracellular, extracellular, and t-RNA-bound amino acids for comparison.

Cells were washed free of extracellular fluid by the procedure described on page 35. However, since the final saline wash does not result in additional removal of extracellular fluid (Table 2), it was omitted in amino acid incorporation studies. As shown in Table 3, washing does not result in appreciable loss of intracellular leucine to the medium.

Table 2. Contamination of lymphoma cell preparations with extracellular fluid.

Number of washings (see pg. 35, cell preparation)	% Contamination ± Std. dev.
1X H_2O/saline	4.98 ± .70%
2X H_2O/saline	1.24 ± .23%
2X H_2O/saline, 1X saline	0.22 ± .05%
2X H_2O/saline, 2X saline	0.17 ± .02%

^{3}H-inulin was added to ascites fluid prior to first centrifugation
The % contamination (V/V) was determined from ^{3}H counts in wash supernatants
Each value is the average of four determinations on pools of 3 mice

The first two washes remove extracellular leucine and leucine from erythrocyte lysate. The third and fourth supernatants reflect leaching

Table 3. Radioactivity lost from ^{14}C-leucine-treated lymphoma cells during washing.

Time of sacrifice	Treatment	Time allowed for incorp.[a]	Leucine in supernatant 1st	2nd	3rd	4th	cells	Leucine in protein[b]
AM	Control	15 min	275	65	24	22	630	136
PM	Control	15 min	275	174	20	17	580	109
AM	Control	30 min	112	47	13	13	490	141
PM	Control	30 min	145	42	13	9	590	160
AM	Procarbazine[c]	15 min	315	92	43	47	600	80
AM	Procarbazine[c]	30 min	280	60	26	21	560	114
PM	Diethylmaleate[d]	15 min	310	140	40	25	540	56
PM	Diethylmaleate[d]	30 min	450	112	50	43	690	90

[a] Leucine-1-^{14}C was administered i.p. (0.2 ml of a 5 μcurie/5mg/10 ml solution in saline)

[b] DPM x 10^{-3} per ml packed cell volume. See cell preparation procedure, page 35

[c] 300 mg/kg administered i.p. 50 minutes prior to leucine administration

[d] 720 mg/kg as 1/10 dilution in glycerolformal, 2 hrs and 50 min prior to leucine

of low molecular weight radioactivity from the cells, but it is not clear how much of this radioactivity is due to leucine.

The final cell suspension was homogenized in 10% TCA/0.01 N HCl, and the supernatant was kept frozen at -85°C until used for specific activity determinations. The precipitate was washed three times with 10% TCA and amino acids were released from t-RNA by digestion at 90°C for 15 minutes. The precipitate was washed twice more with TCA, once with ethanol and once with acetone, and air dried for several hours. Protein counts were obtained following either combustion with a Packard Model 306 sample oxidizer, or by digestion in Unisol^{R} (Isolab, Inc., Akron, Ohio).

Preparation of DNP Derivatives

Regier and Kafatos (170) have described a method for the determination of the intracellular specific activity of leucine which utilizes radioactive FDNB (Sanger's reagent) to double-label the amino acids, allowing specific activity to be determined from the $^{14}C/^{3}H$ ratio in DNP-leucine, isolated by TLC. The method is attractive because it can be as sensitive as desired, it does not require quantitative recovery of the DNP-amino acid, and it can be applied to biological samples without tedious purification. However, analysis by two-dimensional TLC can be tedious and somewhat inaccurate, particularly when the ratio of leucine to isoleucine varies

widely from sample to sample. We therefore have investigated the use of high performance liquid chromatography in isolating the derivative.

TCA and other contaminants were removed from cell extracts by five extractions with equal volumes of ether. A 2 ml aliquot of the aqueous phase was combined with 1 ml of 0.1 M dibasic potassium phosphate, and adjusted to pH 6.8. Tritiated FDNB (Biochemical and Nuclear Corp., Berkeley, California) was then added in 1 ml EtOH, and the mixture was incubated 3 hours at 40°C. The amount and specific activity of FDNB were varied to determine optimal conditions. Figure 6 shows the kinetics of formation of DNP leucine at two levels of FDNB in a sample containing Fischer's medium and radioactive leucine. At the higher FDNB level (1.0 mg/ml) the reaction is seen to go to completion in 3 hours and less than 10% of the radioactivity is non-extractable (not DNP-leu) at that point.

After derivatization, unreacted FDNB and DNP-amines were removed by three extractions with equal volumes of ether. The solution was then acidified with 0.2 ml 6 N HCl, and the DNP-amino acids were extracted with two equal volumes of ether.

Methylation

Approximately a 10X molar excess of diazomethane, prepared from Diazald® (Aldrich Chemical Co., Milwaukee, Wisconsin), was

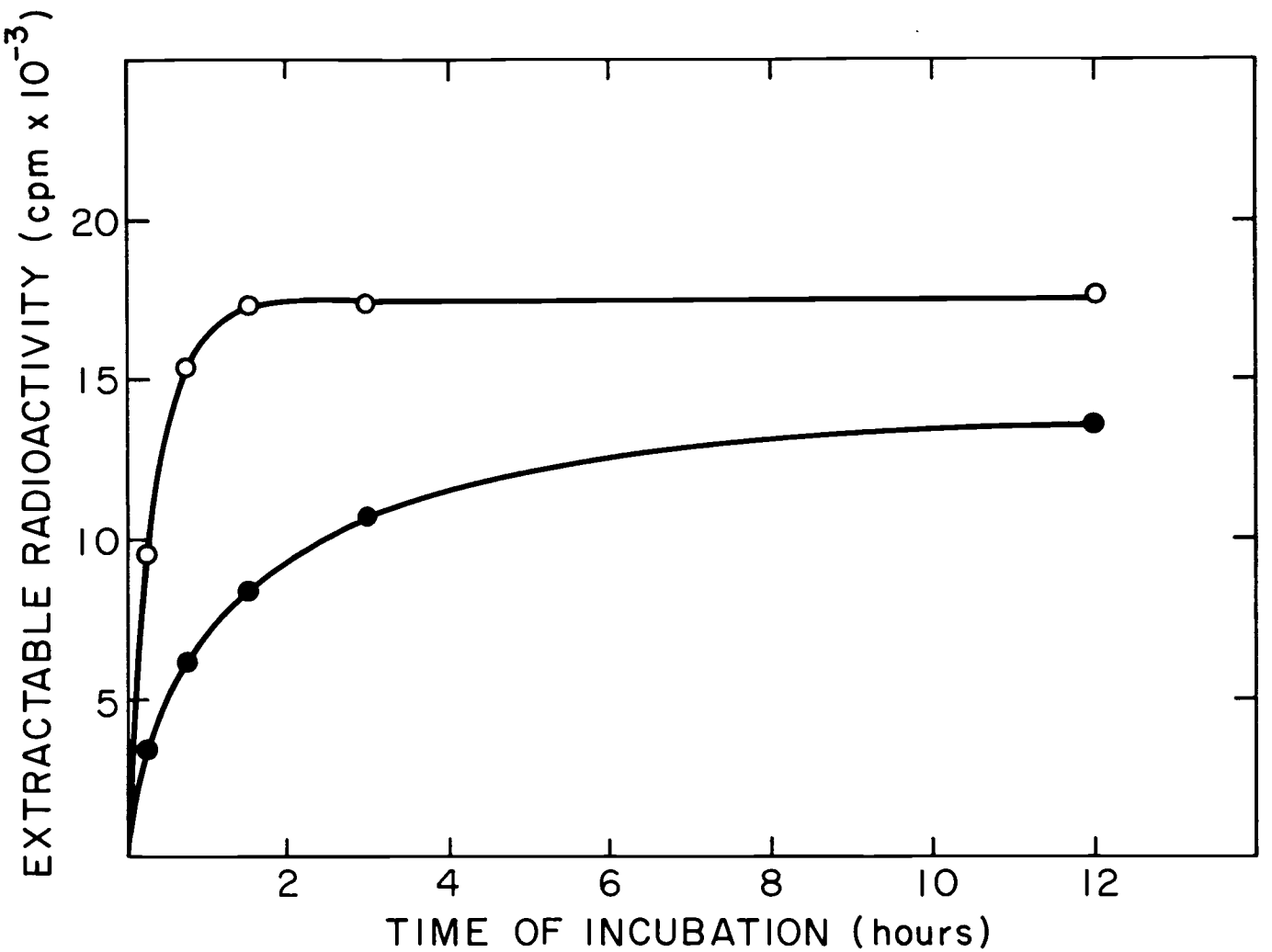


Figure 6. Kinetics of DNP-leucine formation with two FDNB levels. Samples containing 25 μg leucine/ml in Fischer's media were incubated with FDNB at 37°C in 25% EtOH, pH 8.8 phosphate. DNP leucine was extracted with ether.

added in about 1 ml ether to the ether extract containing the DNP-amino acids, and the mixture was incubated 40 minutes at room temperature in a stoppered test tube. Excess diazomethane was evaporated off, along with the ether, in a stream of nitrogen. The residue was dissolved in a minimal volume of isooctane. Diazomethane was stored in ether solution, over sodium sulfate, below 0°C.

Chromatography

Kesner, et al. (106) experienced difficulty in resolving DNP-leucine from DNP-isoleucine in silica column chromatography. This is also the case in the high pressure liquid chromatography of the methyl esters. Large particle silica packings (Biosil A^{R}, Vydac^{R}) proved ineffective in separating either DNP-leucine from DNP-isoleucine or MeDNP-leucine from MeDNP-isoleucine. However, quite satisfactory resolution was achieved using packings with particle sizes in the five micron diameter range. Lichrosorb^{R} (Altex, Berkeley, California), Spherisorb^{R} (Spectrophysics, Santa Clara, California) and Partisil^{R} (Whatman, Inc., Clifton, N. J.) all proved effective in 20 cm columns. Elution time required for optimal resolution varied with column age and condition, but Spherisorb^{R} produced the best results when columns in equal condition were compared. Satisfactory resolution of MeDNP-leucine from MeDNP-isoleucine was achieved in six minutes elution time on a 20 cm x 2.1 mm Spherisorb^{R} column with a solvent system composed of isooctane and isopropanol (400:1.25), at a flow rate of 1.2 ml/min. Figure 7 shows the

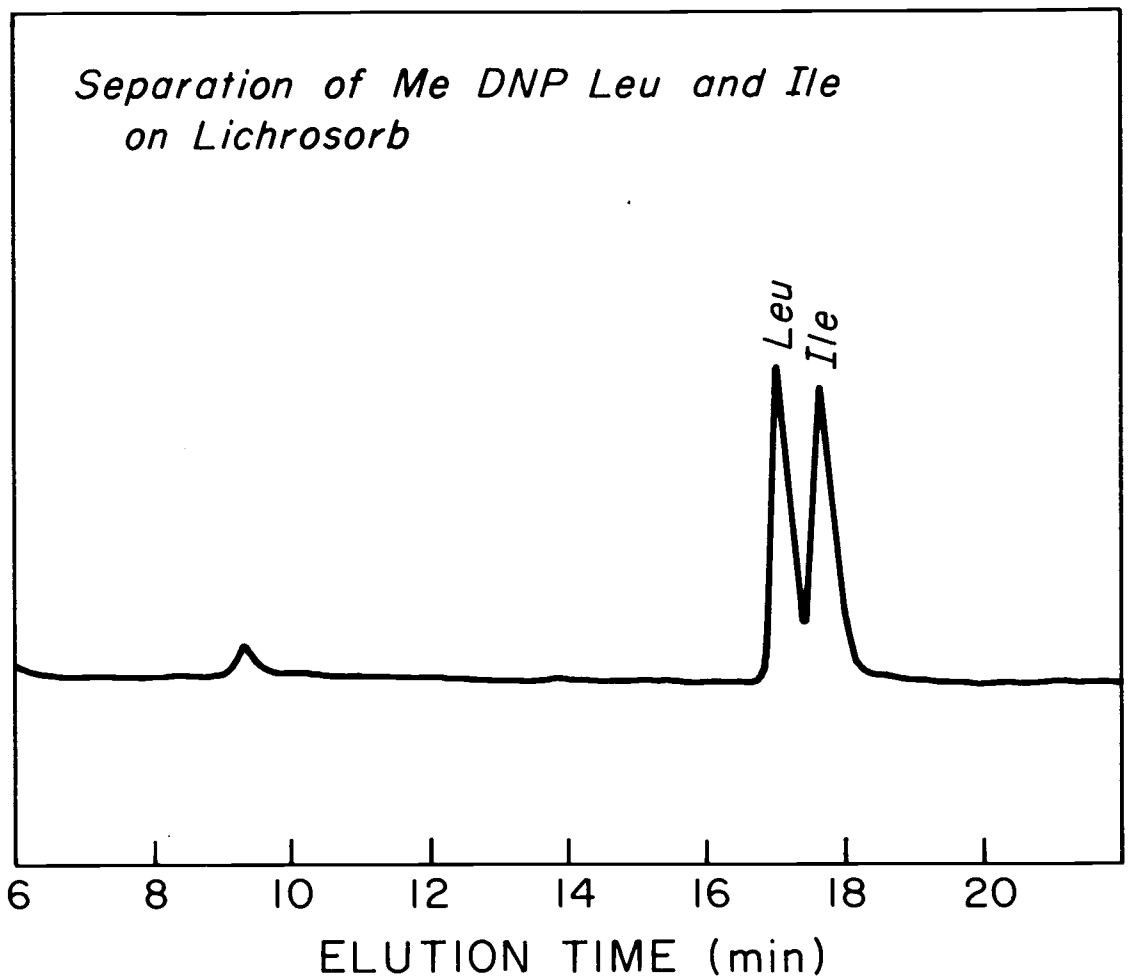


Figure 7. Chromatography of Me-DNP-leucine and Me-DNP isoleucine on lichrosorb. Solvent: isooctane isopropanol (400:1). Flow rate: 0.8 ml/min. Column dimensions: 3.2 x 250 mm.

resolution achieved on a Lichrosorb^{R} column (3.2 x 250 mm) in 20 minutes at a flow rate of 0.8 ml/min., with a solvent composition of 400:1.

Table 4 lists the elution times for various MeDNP-amino acids under different solvent conditions. Because of the wide range of polarities, gradient elution is particularly appropriate for these compounds. For gradient elution a Chromatronix Model 3500 liquid chromatograph was used. Starting solvent was isooctane:isoproapanol (400:1). The second solvent was isooctane:methylene chloride:isopropanol (400:27:400). Leu, Ille and Val were eluted in the starting solvent. The other nonpolar amino acids were eluted at 5% solvent B, and a 5-20% Solvent B gradient brought out the more polar derivatives. Figure 8 shows the gradient elution of MeDNP-amino acids derived from a lymphoma cell hydrolysate. Cells had been treated with leucine, and the MeDNP-leu peak was therefore much higher than other peaks. It is interesting to note that the MeDNP-Ile peak was still well resolved from the MeDNP-leu peak even though it was less than 5% the magnitude of the latter.

Application to analysis of all the amino acids is conceivable, but there are problems to overcome. Most of the amino acid derivatives are only slightly soluble in the starting solvent. A quantitative assay might require starting with a more polar solvent, at the expense of resolution of leucine from isoleucine. Also, several of the

Table 4. Elution volumes for various DNP amino acid methyl esters in high pressure liquid chromatography under different solvent conditions.

Amino Acid (DNP methyl ester)	Solvent Composition (Isooctane:Methylene Chloride:Isopropanol)								
	0:9:1	400:27:50	400:9:100	400:27:15	400:27:3	400:50:5	400:9:5	400:9:2	400:9:1
Leu	2.5[u]	2.6[u]	2.8[u]	3.3[u]	4.3[u]		4.3[i]	7.9[i]	14.7
Ile	2.5[u]	2.6[u]	2.8[u]	3.3[u]	4.4[u]		4.4[i]	8.4[i]	15.7
Val	2.5[u]		2.8[u]	3.4[u]			5.7	11.3	19.5
Phe	2.5[u]		2.8[u]	4.0[i]		4.8			
Met	2.5[u]		3.1[i]	4.5[i]		5.7			
Ala	2.5[u]	4.3	3.5[i]	5.6			12.8	27.6	49.7
Pro	2.8[i]		4.1	6.5		7.7			
Trp	3.3[i]		4.8	8.5					
Gly	4.2[i]	8.5	5.6	13.2			41.6	95.0	
Tyr	5.6[i]		7.7	20.6					
Glu			4.9						
Ser	5.9[i]	16.3	11.2	68.6		100+*			
Asp	2.6[i]		6.0						
Asn	5.9[i]		46.0						

Chromatography on a 3.2 x 250 mm lichrosorb column; Flow rate: 0.8 ml/min; elution volumes are in ml

* never eluted u: unresolved from closest peak i: incompletely resolved from closest peak

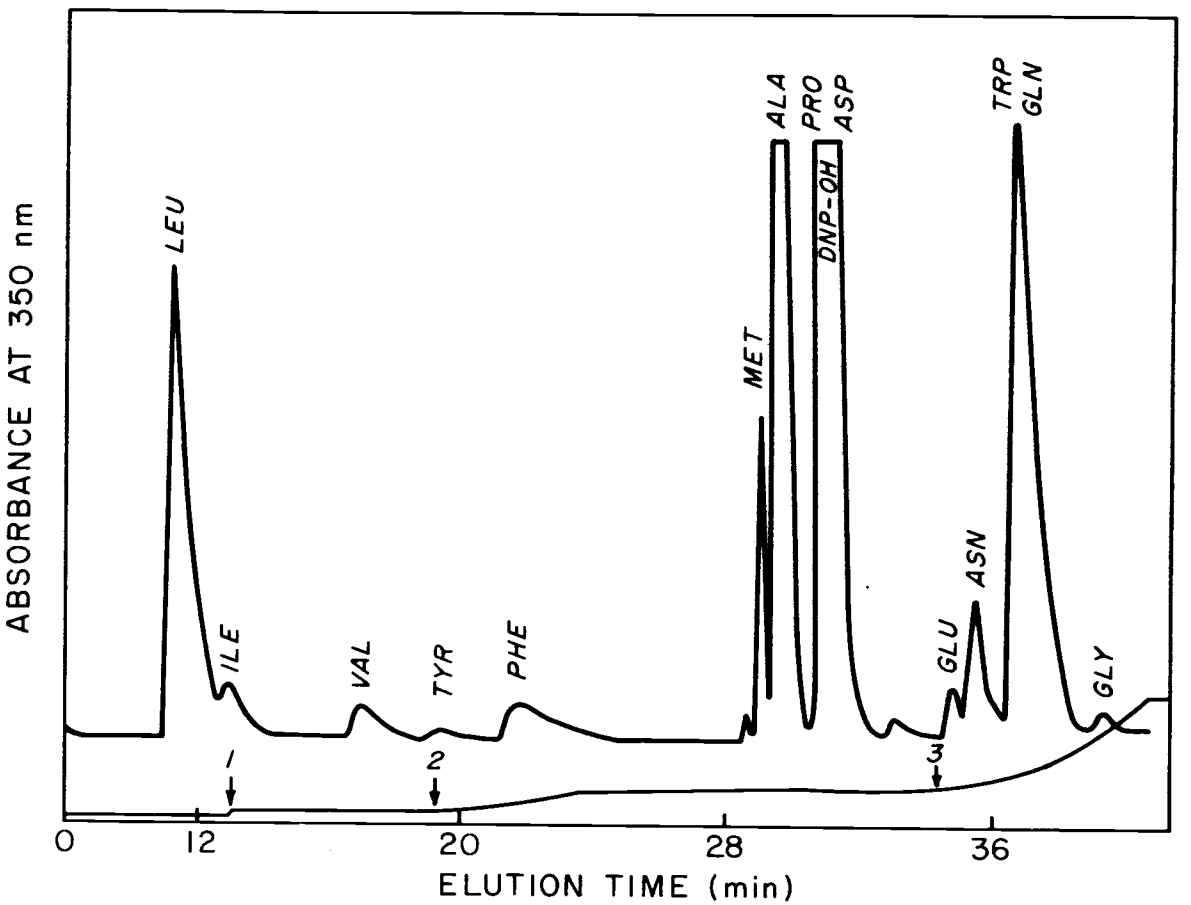


Figure 8. Gradient elution of Me DNP amino acids derived from L-5178Y lymphoma cell extracts. Lymphoma cells were extracted with 10% TCA, reacted with ^{3}H FDNB at pH 8.8 for 3 hours. DNP-amino acids were extracted from the acidified mixture, methylated with diazomethane and dissolved in isooctane. Chromatography was in isooctane: isopropanol (400:1) with isooctane methylene chloride isopropanol (solvent B) (400:27:400) blended in as follows: (1) 2% B (2) 2-10% gradient in 5 min (3) 10-25% B gradient in 10 min.

peaks are obscured by a large DNP-OH peak which is difficult to avoid.

For the analysis of leucine specific activity, isocratic elution in isooctane:isopropanol (400:0.75) served to resolve the MeDNP-leu peak from all others. Fractions were collected for counting during the first half of the peak. Only leucine needs to be eluted with starting solvent. The other amino acids can be quickly eluted with isooctane: methylene chloride:isopropanol (400:27:400) as shown in Figure 9.

Calculations

The isolated derivative was counted in a toluene based fluor containing 6.0 g/l PPO and 50 mg/ml POPOP, utilizing the $^{3}H/^{14}C$ counting mode of a Model 2425 Packard liquid scintillation counter. Calculations were simplified by the use of a Hewlett-Packard Model 9821A programmable calculator. The program used to calculate specific activity is listed in Appendix II.

Leucine Standard Curve

The linearity and reproducibility of the method was tested using leucine-1-^{14}C, alone and in combination with other amino acids. When leucine alone is carried through the procedure (Figure 10), the observed specific activity agrees very well with the expected value, although the first half of the peak has a higher $^{14}C/^{3}H$ ratio than the second half. This may be due to an isotope effect, considering that

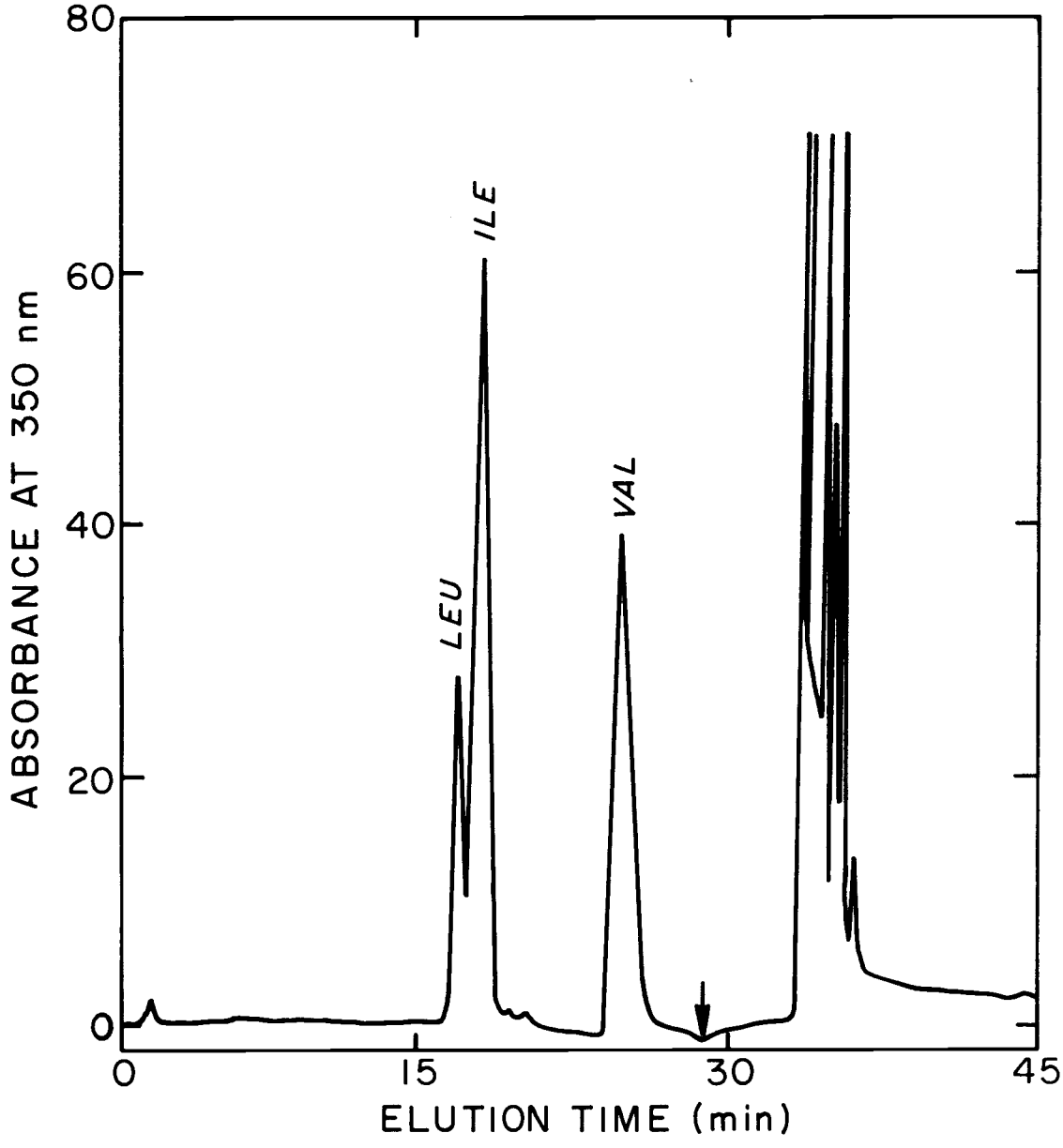


Figure 9. Isolation of MeDNP leucine by high pressure liquid chromatography. L-5178Y cells were extracted with 10% TCA following incubation in ^{14}C-leucine containing medium (Fischer's). The TCA extracts were reacted with ^{3}H FDNB and the DNP-amino acids extracted into ether, methylated and dissolved in isooctane. Chromatography was in isooctane isopropanol (400:0.15) followed by isooctane methylene chloride: isopropanol (400:27.400) as indicated. The first half of the leucine peak was collected for $^{3}H/^{14}C$ counting.

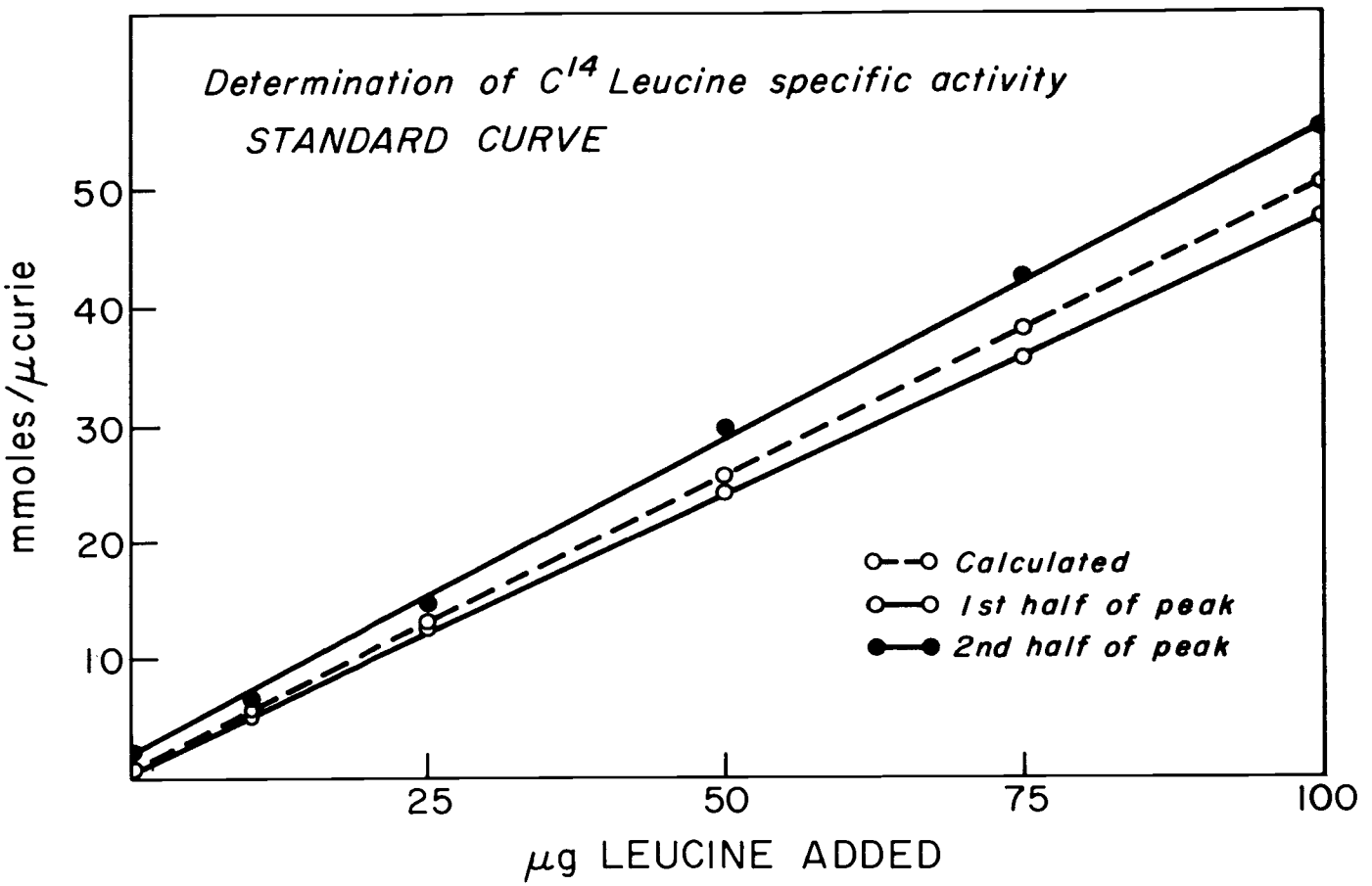


Figure 10. Determination of leucine specific activity: standard curve. Leucine (10, 25, 50, 75 and 100 μg) was added to a solution of ^{14}C-leucine in 0.05 M phosphate, pH 8.8 and reacted with ^{3}H-FDNB in 25% ethanol. The derivatives were extracted with ether from the acidified reaction mixture, and methylated with diazomethane. Chromatography was on a Lichrosorb^{R} column with isooctane:2-propanol (400:0.75). The eluate from the first and second halves of the leucine peak was collected, and specific activity was determined from the $^{14}C/^{3}H$ ratio.

the average of the first and second halves agrees better than either alone with the expected value. Isotope effects in amino acid chromatography have been reported (109). It can also be seen that the first half of the peak contains no tritiated impurity, because the intercept is zero. The second half does contain a minor contaminant, and, in fact, using very high FDNB levels, an impurity can be chromatographically detected as a shoulder on the trailing edge of the leucine peak. This evidence would argue against using any more FDNB than is needed (Figure 6). It also stresses the need for the resolving power of liquid chromatography, since such impurities could easily go undetected in TLC. It is interesting to note that the valine peak also has a higher $^{14}C/^{3}H$ ratio in the first half than in the second half. This would support the hypothesis that an isotope effect is taking place, since it would be difficult to imagine identical contamination with tritiated impurities in both cases.

Linearity of the assay in the presence of other amino acids is demonstrated in Figure 11. It would be advisable to construct such a curve when utilizing different conditions, or investigating different systems, to insure accuracy of specific activity values. However, comparisons between test and control systems, or kinetic studies of specific activity changes, can be made without reference to such a standard.

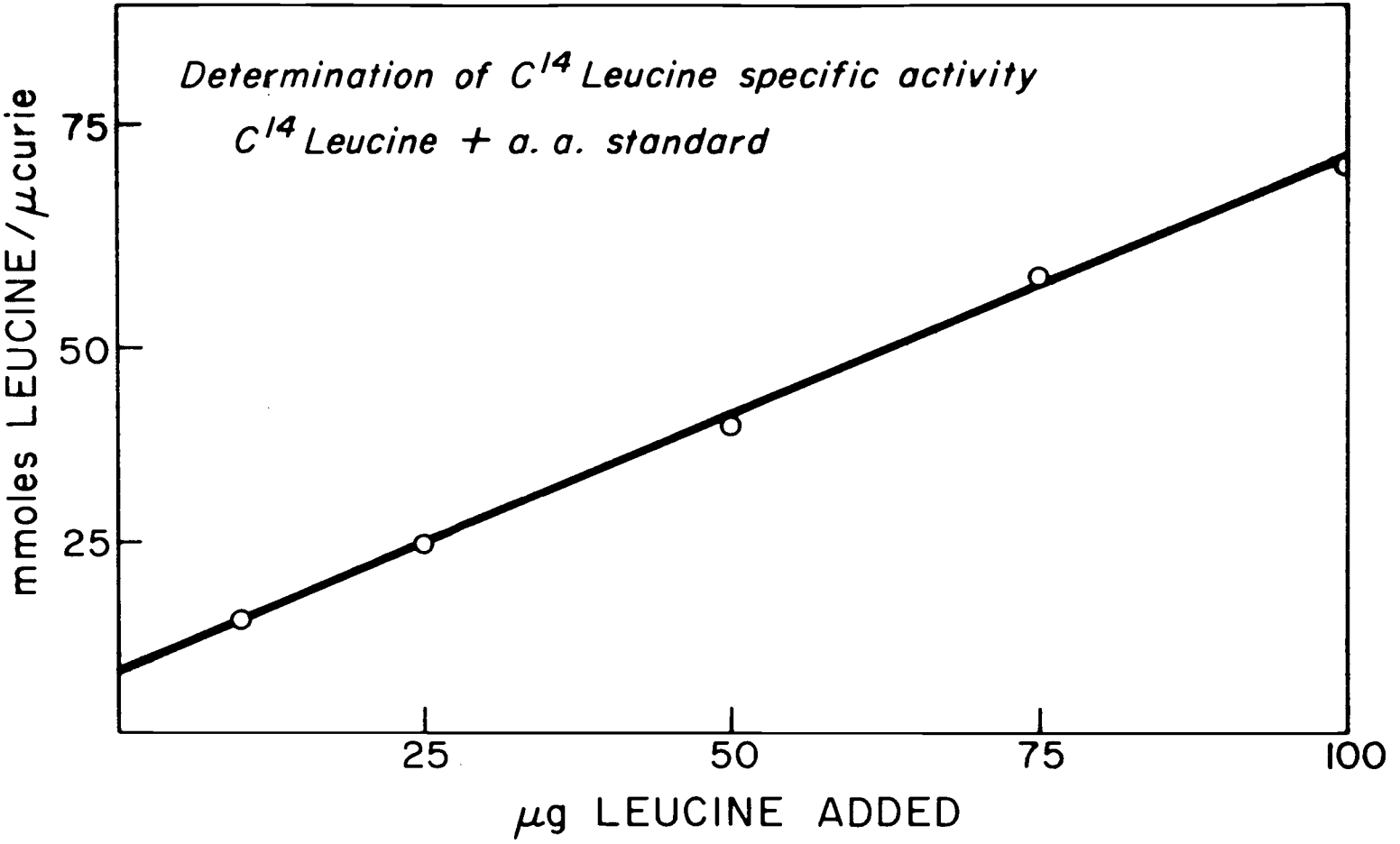


Figure 11. Determination of ^{14}C leucine specific activity: ^{14}C-leucine + amino acid standard. Leucine was added to an amino acid standard solution containing a fixed amount of ^{14}C-leucine, and the MeDNP derivatives were prepared using ^{3}H-FDNB. The first half of the leucine peak from chromatography of the derivatives by liquid chromatography was collected and specific activity was determined from the $^{14}C/^{3}H$ ratio.

Analysis of Thiols and Disulfides

Enzymatic Assay for Glutathione

GSH and GSSG were determined by an enzymatic assay (214) in which glutathione is continually reduced by NADPH and glutathione reductase, as it, in turn, reduces DTNB (Ellman's reagent) to NBS^-, which is quantitated by absorbance at 412 nm.

Glutathione reductase (Sigma type III, highly purified; 200 units per ml) was diluted 1:5 in 0.1 M phosphate buffer, pH 7.5, containing 0.005 M EDTA (buffer G), within one or two days of use. NADPH and DTNB concentrations were 2 and 3 μmoles/ml, respectively. NADPH and GSH standards were prepared fresh daily. Samples were diluted with buffer G to contain between 5 and 100 ng GSH in a final volume of 0.75 ml. Samples were allowed to equilibrate at least 10 minutes in the Cary 15 sample compartment which was maintained at 20°C, before adding reagents in the following order: 0.1 ml DTNB; 0.05 ml reductase; 0.1 ml NADPH. The blank contained all reagents, but no GSH.

For determination of GSSG, GSH was alkylated by reaction with 0.02 M NEM (N-ethylmaleimide) at pH 7.5 for one hour, at 25°C. Excess NEM was removed by ten extractions with equal volumes of ether.

Linearity of the assay is demonstrated in Figure 12. The rate of color formation reaches a maximum after a few minutes incubation, and remains constant for about 20 minutes. It is the linear portion of the recording that is used for calculations. Duplicate blanks were included in the sample compartment each time, as the assay is extremely sensitive to differences in temperature between the sample and reference compartments, and the rate of color formation in the blank is quite high.

N-Ethylmaleimide

GSH and other thiols were also determined by reaction with ^{14}C-NEM and chromatography of the adducts by TLC (203). ^{14}C-NEM was obtained from New England Nuclear (Boston, Mass.) at a specific activity of 88 μCi/mg. It was stored in pentane solution at 0-5°C, as it is extremely unstable in aqueous solution or as a dry powder. Just before use, an aliquot was taken to dryness in a stream of N_2, keeping the vessel on ice. Only plastic labware was used, since there were indications NEM might bind to glass surfaces. The NEM was dissolved, at a concentration of 10 ng/10 μl in 0.3 M potassium phosphate, pH 7.0, containing 0.005 M EDTA. Samples were adjusted to pH 7.0 and 50 μl aliquots were added to 10 μl NEM, and incubated 20 minutes at room temperature. Gregory (70) has shown that the reaction with glutathione goes to completion under these conditions,

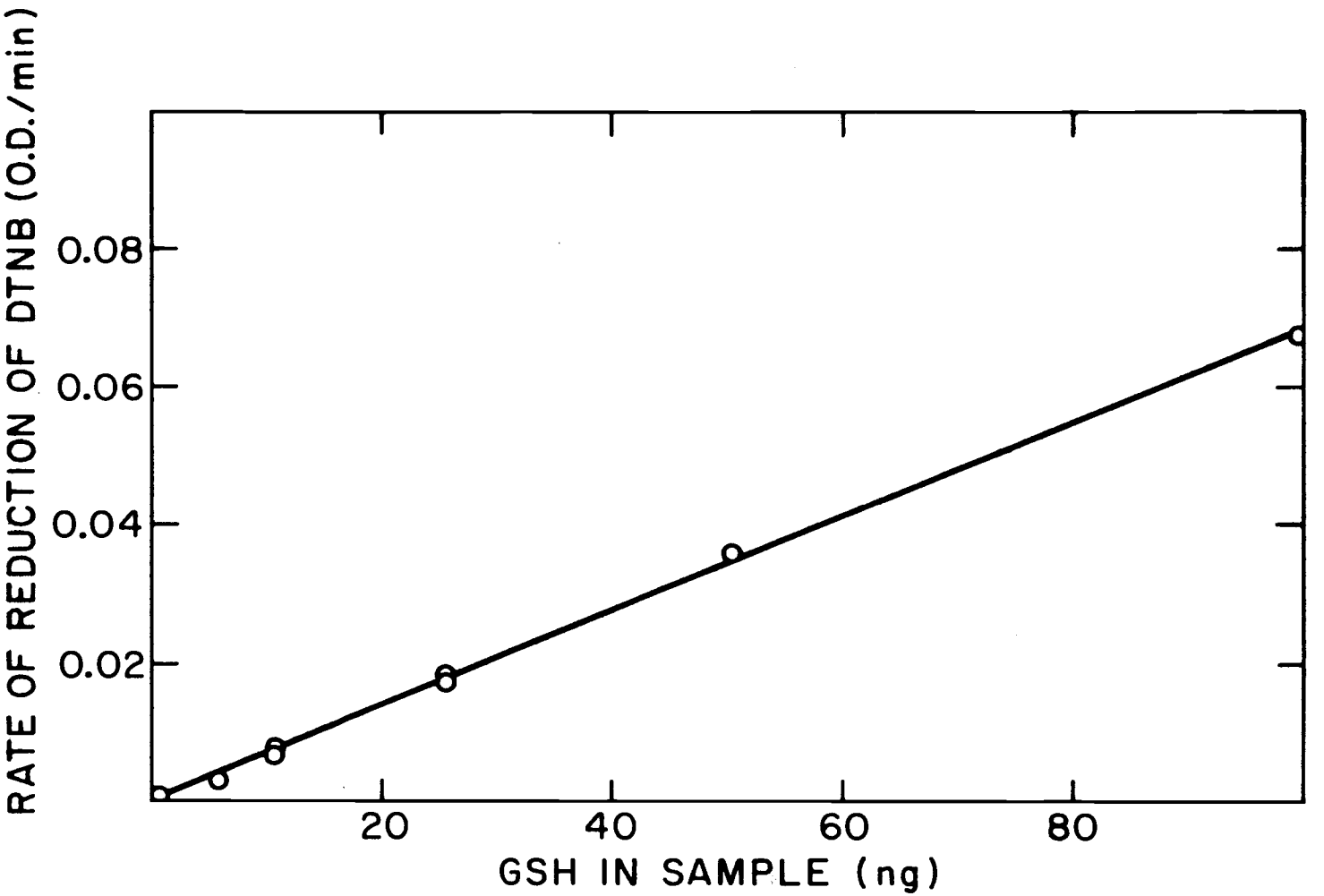


Figure 12. Enzymatic assay for glutathione: standard curve. The reaction mixture contained 2 units of glutathione reductase, 60 nmoles of DTNB, and 20 nmoles NADPH in a final volume of 1 ml. The rate of NBS^- formation was determined by following absorbance at 412 nm. Slope was calculated by least squares analysis.

while NEM degradation is minimal.

Samples were streaked on Avicel^{R} precoated cellulose TLC plates (Analtech, Inc., Newark, Del.) and eluted with butanol: pyridine:acetic acid:water (3:2:0.6:1 V/V). Table 5 shows the R_f values obtained for a number of NEM adducts. NEM itself and the adduct formed with dithiothreitol were found to run much further toward the solvent front than any adducts of interest, but tailing was a problem (Figure 13), and a number of degradation products co-chromatograph with compounds of interest. Decomposition of NEM adducts has been noted (16), and has been studied in some detail (61). Polymerization and non-specific alkylation have been observed (199). Aside from hydrolysis of the imide bond to produce N-ethylmaleamic acid, a number of other reactions must occur, as evidenced by liquid chromatography and other chromatographic methods in this laboratory. Ethylamine was identified with the aid of mass spectrometry as a product of degradation of GS-NEM, after incubation, for several days at 0-5^{o}C in acid solution.

Developed plates were scanned on a Packard Model 7200 Radio-chromatogram scanner, and the radioactive bands were eluted with GDW. Samples were counted in a xylene-Triton N-101 based fluor. Figure 14 shows the relationship between GSH concentration and the area of the GSH peak on the radiochromatogram.

Table 5. R_f values for various n-ethyl maleimide derivatives chromatographed on Avicel; solvent system: butanol/pyridine/HOAc/H_2O (3:2:0.6:1 V/V).

Derivative	R_f
NEM degradation product	0.00
Glutathione-NEM adduct	0.06
Cysteine-NEM adduct	0.29
NEM degradation product	0.46
Thionitrobenzoate-NEM adduct (NBS^--NEM)	0.78
N-ethyl maleimide (NEM)	0.82
Dithiothreitol-NEM adduct	0.91
NEM degradation product	0.96

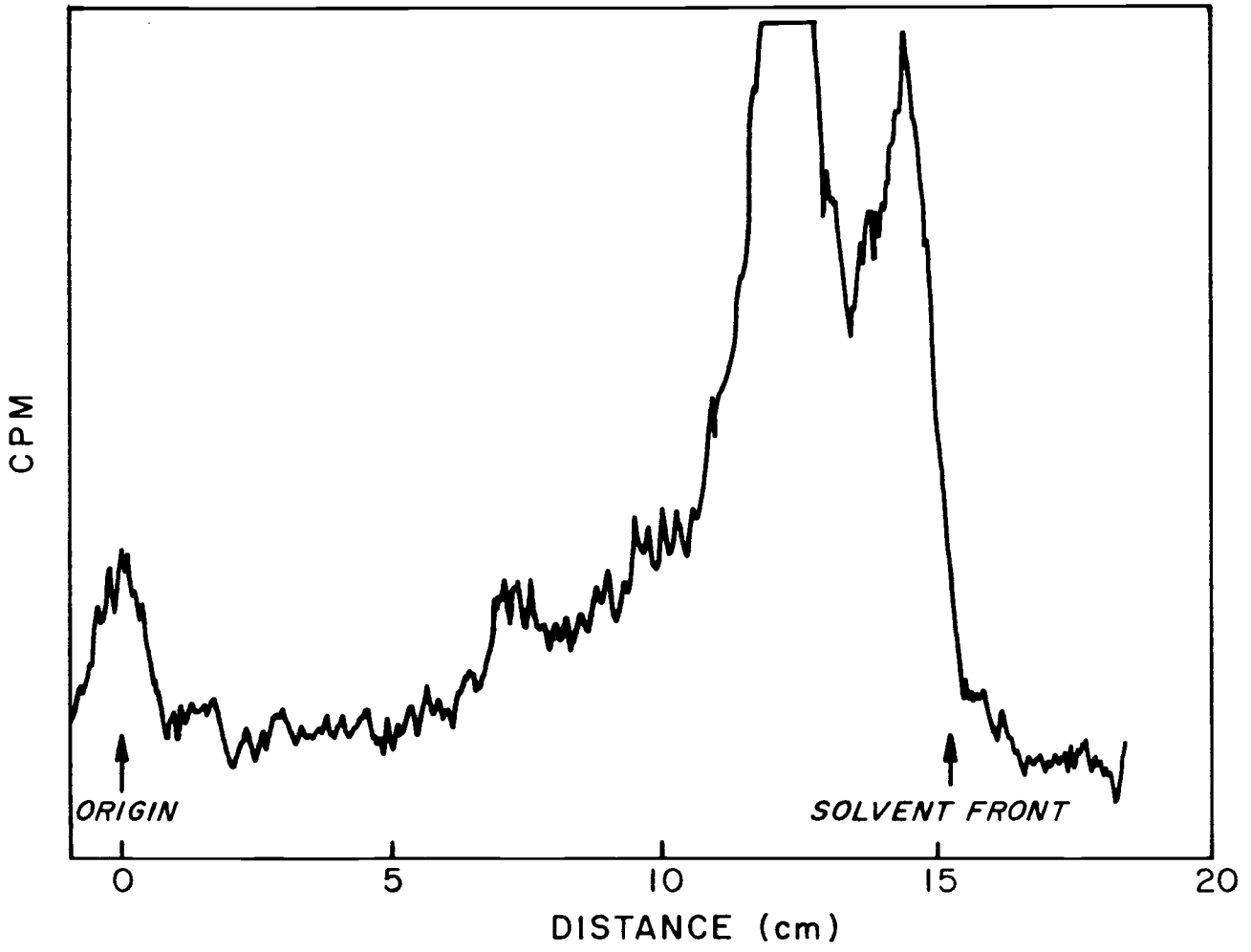


Figure 13. Radiochromatogram of N-ethylmaleimide standard. ^{14}N-ethylmaleimide (20 nmoles) was incubated in 50 µl phosphate buffer, pH 7.5 for 20 min. at room temperature. The entire amount was streaked on an Avicel^{R} precoated cellulose T.L.C plate, and chromatographed in butanol:pyridine:acetic acid:water (3:2:0.6:1 V/V).

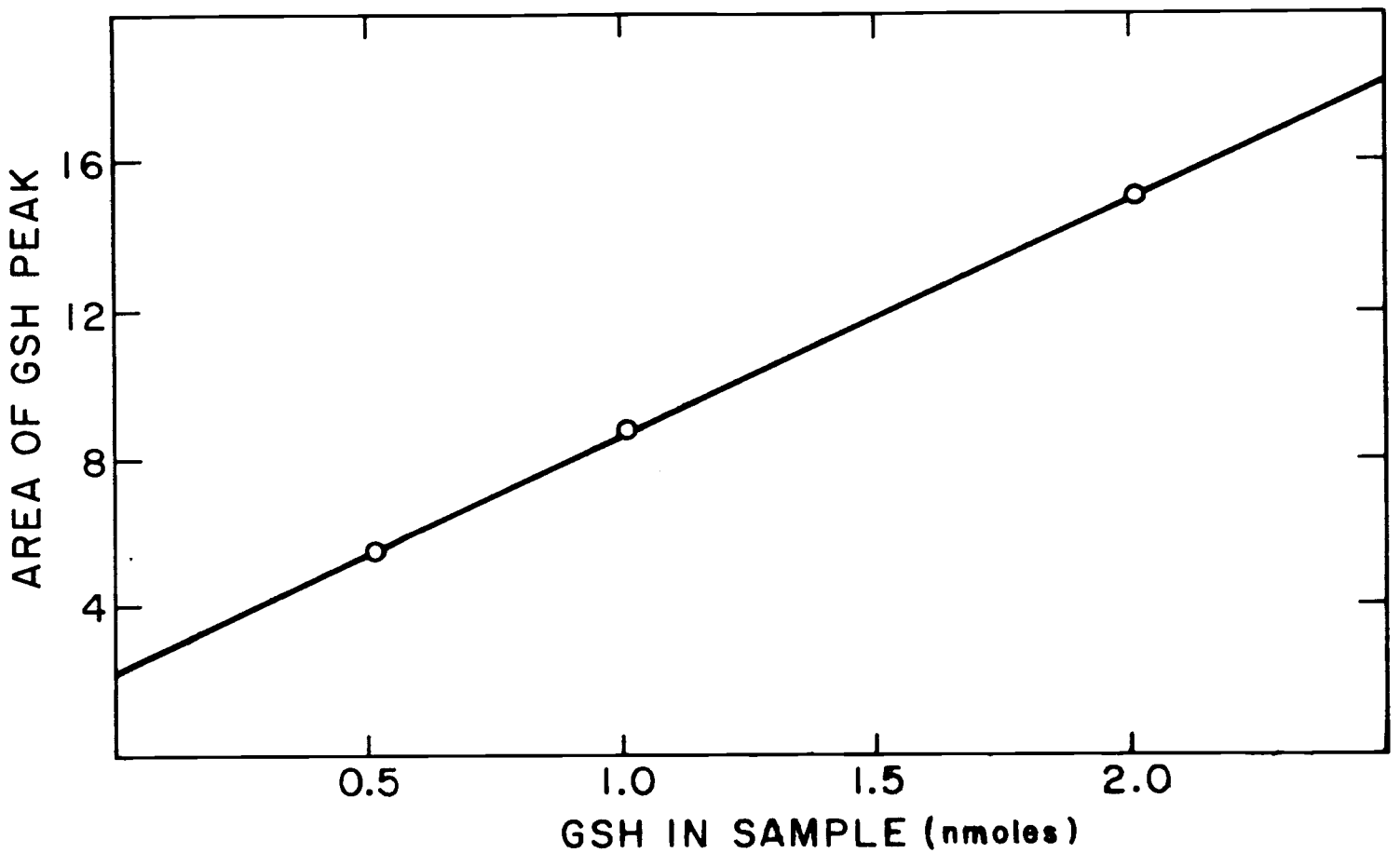


Figure 14. ^{14}C-N-ethylmaleimide assay for GSH: standard curve. GSH (0.5, 1.0 and 2.0 ng) was incubated with ^{14}C-NEM at pH 7.5 for 20 min., and the product was applied to an Avicel^{R} TLC plate and chromatographed in butanol:pyridine:acetic acid:water (3:2:0.6:1 V/V). The area of the GSH peak on the radiochromatogram was calculated by weight. Slope and intercept were calculated by least squares analysis.

Sample Preparation

For non-protein thiol analysis, cell suspensions prepared as described on page 35 were homogenized in 10% TCA containing 0.01 N HCl (214). TCA was extracted with five equal volumes of ether. All samples were stored at -85°C until analyzed.

Analysis of intra- and extracellular glutathione at the end of each step of the cell washing procedure revealed that extracellular ascites fluid contains as much as 10% of the total glutathione in whole ascites fluid, and that this glutathione is usually more than 50% oxidized. This explains the observation (Table 6) that values for %GSSG in whole ascites fluid are much higher than in washed cells. Whole ascites fluid glutathione was generally between 6 and 12% oxidized. In the case illustrated this value was 6.9%. Washing away extracellular fluid is not sufficient, however, because contaminating erythrocytes appear to have a much more oxidizing potential than the lymphoma cells. Washing with isotonic saline or KCl decreased %GSSG values to between 3 and 7%, but these values were also erratic, apparently depending on the degree of contamination with red blood cells. The erythrocyte lysate produced by 30 second exposure to GDW contained glutathione which was more than 10% oxidized. Omission of any of the steps in the washing procedure described on page 35 resulted in variable %GSSG values.

Table 6. Effect of washing and erythrocyte lysis on %GSSG in L-5178Y lymphoma cells.

Treatment	Number of Washings			%GSSG
	H_2O-Saline	Saline	KCl	
None	0	0	0	6.9
None	2	0	0	3.3
None	2	1	0	1.4
None	2	2	0	2.2
Inc. 50 min, 0°C	2	2	0	2.9
None	0	4	0	4.7
None	0	0	4	5.8

L-5178Y cells were harvested 6 days after implantation in DBA/2J mice, and washed according to the procedure described on page 35. H_2O-saline = 30 second exposure to GDW, followed by addition of 1 1/2 volumes saline. All treatments were at 0°C.

Sephadex G-25 filtration was used to separate protein from low molecular weight thiols, whenever possible. It was observed that freshly prepared rat liver microsomes could be filtered through Sephadex G-25 with a protein recovery of 80% and baseline resolution from GSH, although a very small amount of free GSH remained associated with the protein fraction and could not be removed by further chromatography. Similar success was achieved in gel filtration of the supernatant derived from lymphoma cells by homogenization in GDW followed by centrifugation at 4500g for 15 min., although this method did not allow determination of total mixed disulfide levels, since most of the protein was associated with the particulate fraction.

Lymphoma cells were therefore homogenized in 1% Triton X-100 containing 0.1 M acetate, pH 5.0, to prevent oxidation during dialysis. Samples were dialyzed against this Triton/acetate buffer (20 volume) through two buffer changes, and then dialyzed against pH 7.5 phosphate buffer (1/10 dilution of buffer G), until all traces of detergent were removed, with a final dialysis against undiluted buffer G. Fetal bovine serum, which was used as a standard for mixed disulfide analysis, did not lose any mixed disulfide during dialysis under these conditions.

Mixed Disulfide Analysis

Several reducing agents were evaluated. Dithiothreitol and sodium borohydride require conditions which tend to accelerate degradation of thiols and leave non-extractable traces which can interfere with thiol analysis. Tributylphosphine (208) was found to be highly effective and easily extractable. Mercaptoethanol was used in preparing samples for amino acid analysis of hydrolyzed samples.

Reduced Ellman's reagent (NBS^-) was found to be effective in reducing mixed disulfides by a thiol-disulfide exchange. The product, a mixed disulfide between glutathione and NBS^- (in the case of GSSP), is an intermediate in the enzymatic assay for glutathione, and excess NBSH can be removed by ether extraction following precipitation of the protein with TCA. Ackerman and Robyt (1) have noted that an exchange reaction occurs between NBS^- and protein-protein unmixed disulfides which is favorable enough in the presence of DTNB to constitute the basis for an assay for protein disulfides. We have found that the reaction with PSSG mixed disulfides is complete and stoichiometric in 20 minutes at 37°C in the presence of a large excess of NBS^- (2000X molar excess, NBS^- to GSH as GSSP), even without added DTNB (Figure 15). Linearity is demonstrated in Figure 16.

NBS^- was prepared by reaction of DTNB with a slight molar excess of DTT at pH 7.5. The oily sediment that forms upon

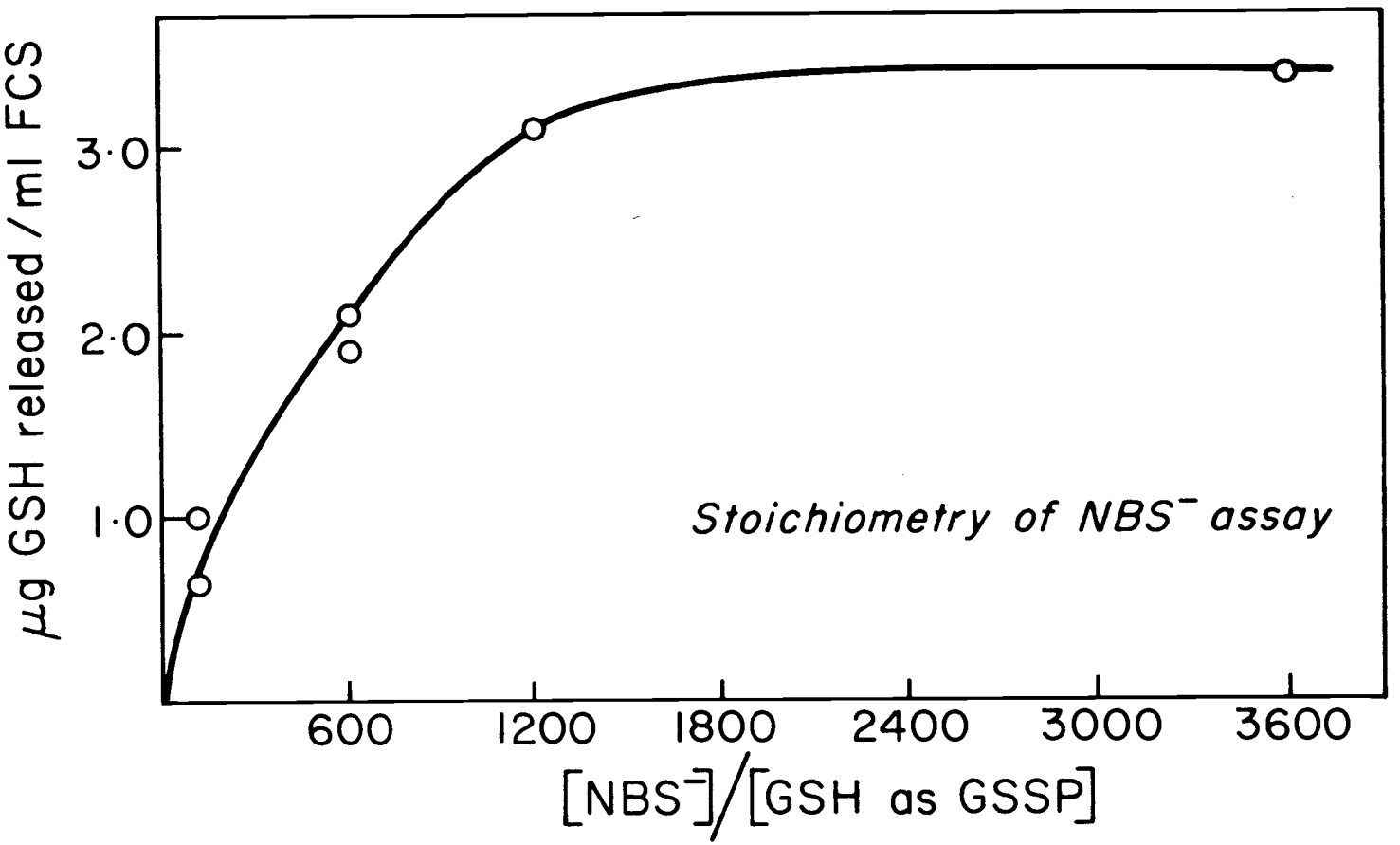


Figure 15. Stoichiometry of the NBS^- assay. Fetal calf serum was incubated with NBS^- in phosphate buffer, pH 7.5, for 20 min. at 37°C. Protein was precipitated with TCA. TCA and excess NBSH were extracted with ether, and the released GSH was assayed by the enzymatic method described on page 61.

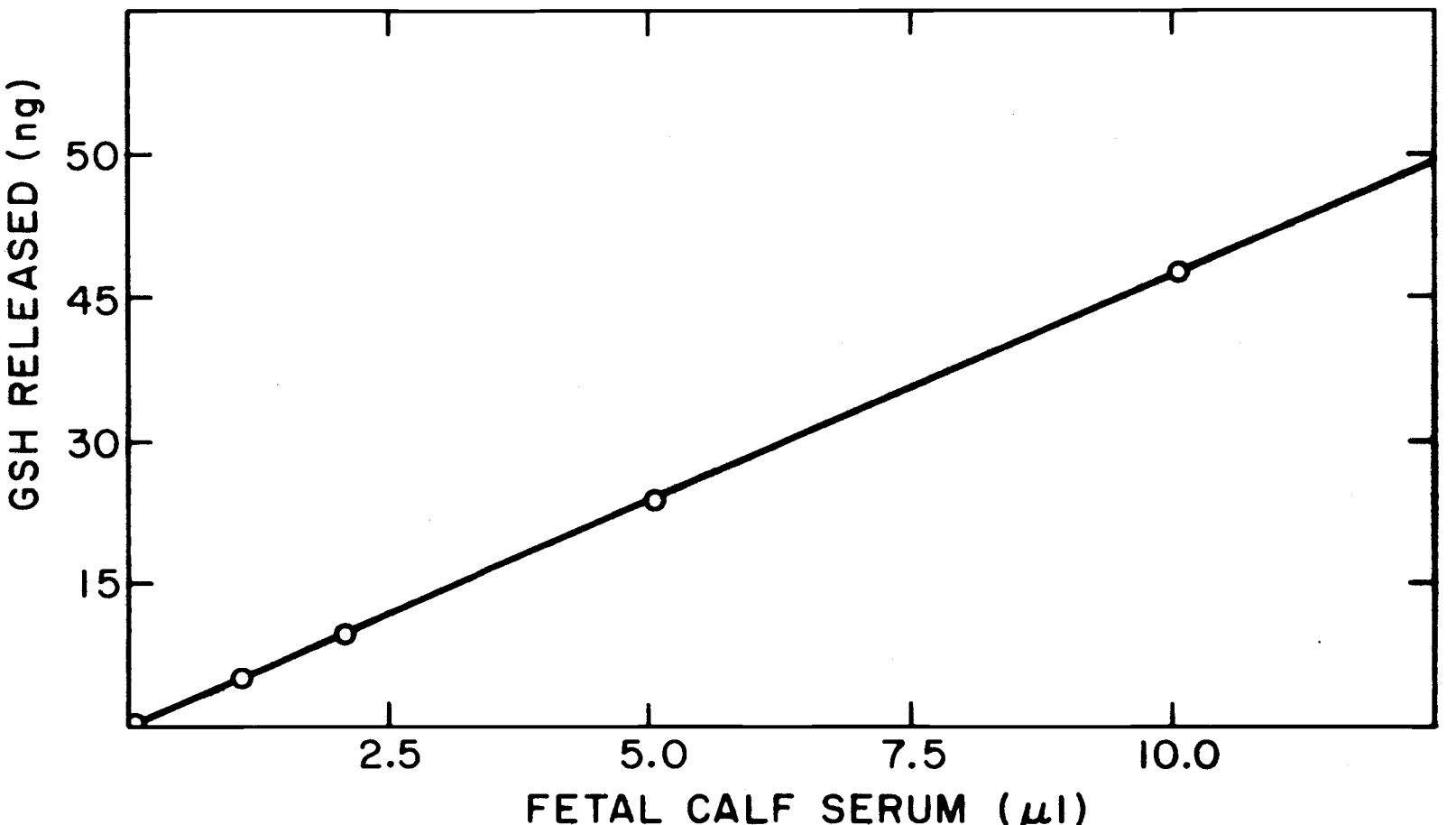


Figure 16. Linearity of NBS^- assay. Fetal calf serum was incubated with NBS^- in phosphate buffer, pH 7.5 for 20 min. at 37°C. Protein was precipitated with 10% TCA. TCA and excess NBSH were extracted with ether, and the released GSH was assayed by the enzymatic method described on page 61. Slope was calculated by least squares analysis.

acidification was collected, dissolved in ether, and washed with 0.1 N HCl. NBS^- was finally extracted into buffer G and stored under N_2. Under such conditions NBS^- is stable for several weeks.

Conditions for mixed disulfide reduction were as follows:

$NaBH_4$ and DTT:	1000X molar excess $NaBH_4$ or 10X molar excess DTT Tris buffer, pH 9.0 Incubate 90 minutes at 37°C
Mercaptoethanol:	50 µl mercaptoethanol per sample (2 ml) Tris buffer, pH 9.0 Incubate 40 minutes at 37°C
Tributylphosphine:	50 µl tributylphosphine per sample (2 ml) PO_4 buffer, pH 7.5, N_2 atmosphere Incubate 20 minutes at 37°C shaking vigorously
NBS^-:	2000 molar excess (NBS^- to GSH as GSSP) PO_4 buffer, pH 7.5 Incubate 20 minutes at 37°C

Following reduction, 1/5 volumes 70% TCA/0.05 N HCl were added and the supernatant was extracted 5 times with ether, followed in the case of tributylphosphine by two extractions with equal volumes of a saturated sulfur solution in chloroform. Sodium borohydride is allowed to decompose in acid prior to extraction with ether.

Tributylphosphine was obtained from Matheson, Coleman and Bell, Norwood, Ohio. All other reagents are from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Effects of Procarbazine on L-5178Y Cells In Vivo

Glutathione Oxidation

Lymphoma cells were harvested from procarbazine treated mice and prepared as described on page 35. Reduced and oxidized glutathione were determined, using the reductase method (page 61). The overall average values are given in Table 7 and illustrated in Figure 17. Cells from untreated mice had an average GSH content of 2.33 $\pm$ 0.77 μmoles per ml PCV, which was 1.6% oxidized (standard deviation: 0.3%).

Procarbazine treatment resulted in a prolonged increase in the ratio of oxidized to reduced glutathione with little effect on total GSH levels. Standard deviations were considerably larger than with controls, reflecting perhaps a combination of individual variability in the mice and the instability of a challenged redox system. Despite the variability the standard errors of the means (Table 7) were small because of the large number of determinations, and a T-test showed the difference to be significant at the 99% confidence level.

Diethylmaleate has been used as a specific reagent for lowering total glutathione levels in vivo (31). In the present study the interaction between diethylmaleate and procarbazine on the glutathione

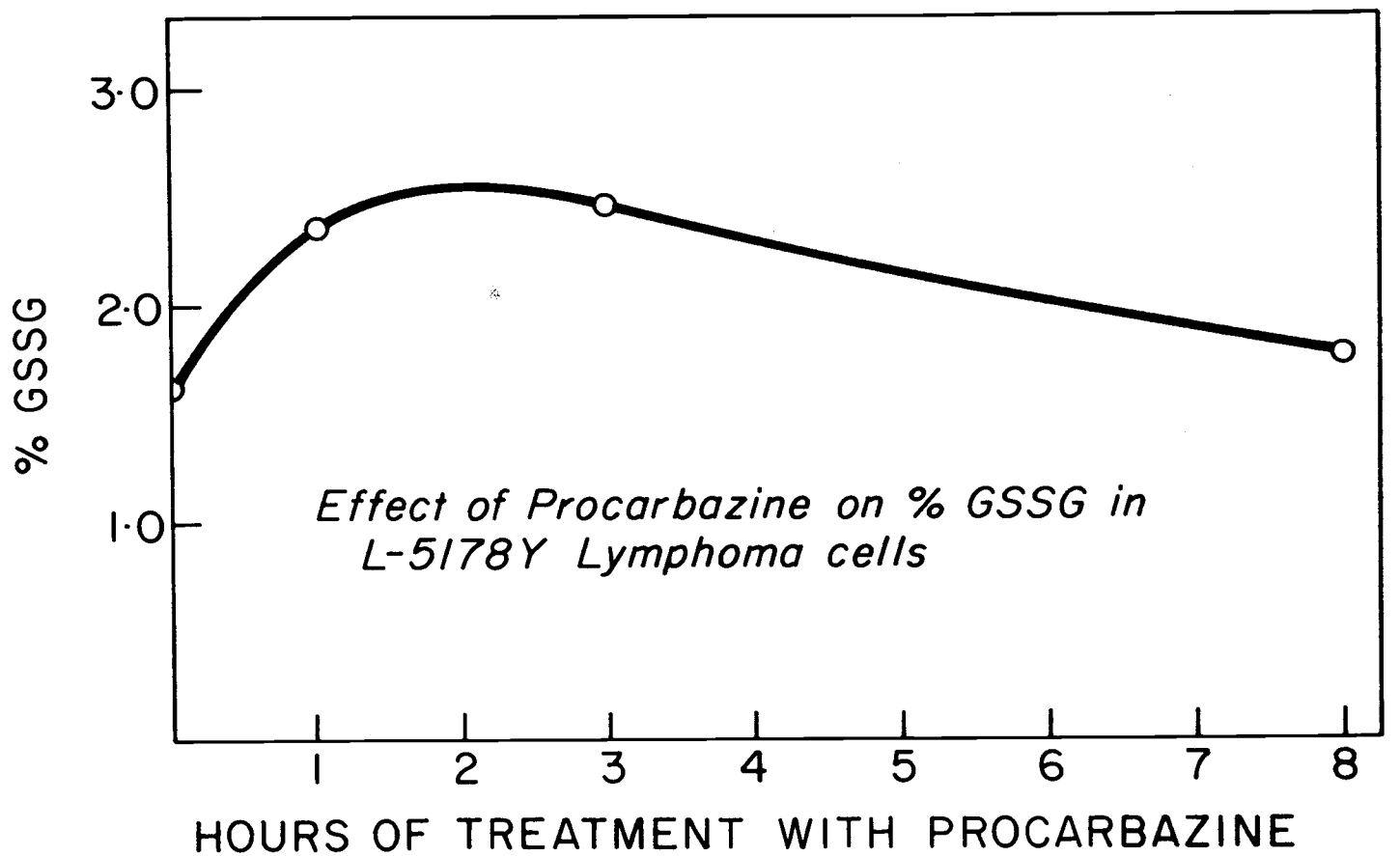


Figure 17. Effect of procarbazine on % GSSG in L-5178Y cells. Tumor bearing mice were treated with 300 mg/kg procarbazine and cells were harvested 1, 3, and 8 hours after procarbazine administration. Percent GSSG was determined by the reductase assay. Each data point represents an average of 10-80 mice.

Table 7. Total glutathione levels and % GSSG after in vivo treatment with procarbazine and/or diethylmaleate.

Number of Mice	Treatment	Hrs of Treatment	% GSSG [a]	GSH + GSSG (% of control)
84	Control	-	1.6 ± 0.09	100%
36	Procarbazine	1	2.4 ± 0.32	101%
27	Procarbazine	3	2.5 ± 0.41	91%
12	Diethylmaleate	3	2.1 ± 0.13	37%
12	Procarbazine plus Diethylmaleate	1 3	3.4 ± 0.35	32%
6	Procarbazine plus Diethylmaleate	3 3	2.6 ± 0.06	41%

L-5178Y lymphoma cells were harvested from DBA/2J mice after treatment with procarbazine and/or diethylmaleate on the 6th day after implantation. Cells were prepared as described on page 35 and GSSG and GSH were determined by the reductase method.

[a] Percent oxidized glutathione ± standard error of mean.

redox state was investigated, but the significance of the results is unclear, because diethylmaleate itself appeared to cause a slight oxidation of GSH (Table 7).

It is interesting to note that in three separate experiments carried out within a period of two weeks (Table 8), the effect of procarbazine on %GSSG was remarkably consistent. Much of the variability reflected in the overall average values could be due to the spacing of experiments over a period of a year, and possible divergence of the cell line during that time.

Evidence linking the redox state to hormonal influences from host mice was presented in the introduction. A diurnal variation in rat liver GSH levels has been reported (11). In the present study, a diurnal variation in the lymphoma cell redox state was noted (Figure 18). Values obtained at 10 A.M. were more than five standard deviations greater than values obtained at 5 P.M.

Another interesting observation regarding the redox state of glutathione is that washing with buffered saline (Table 9) resulted in selective loss of GSH at low pH values, before cell lysis was indicated by loss of protein, this resulted in a concentration of GSSG, increasing the apparent %GSSG. The effect is apparent at pH 5 or lower.

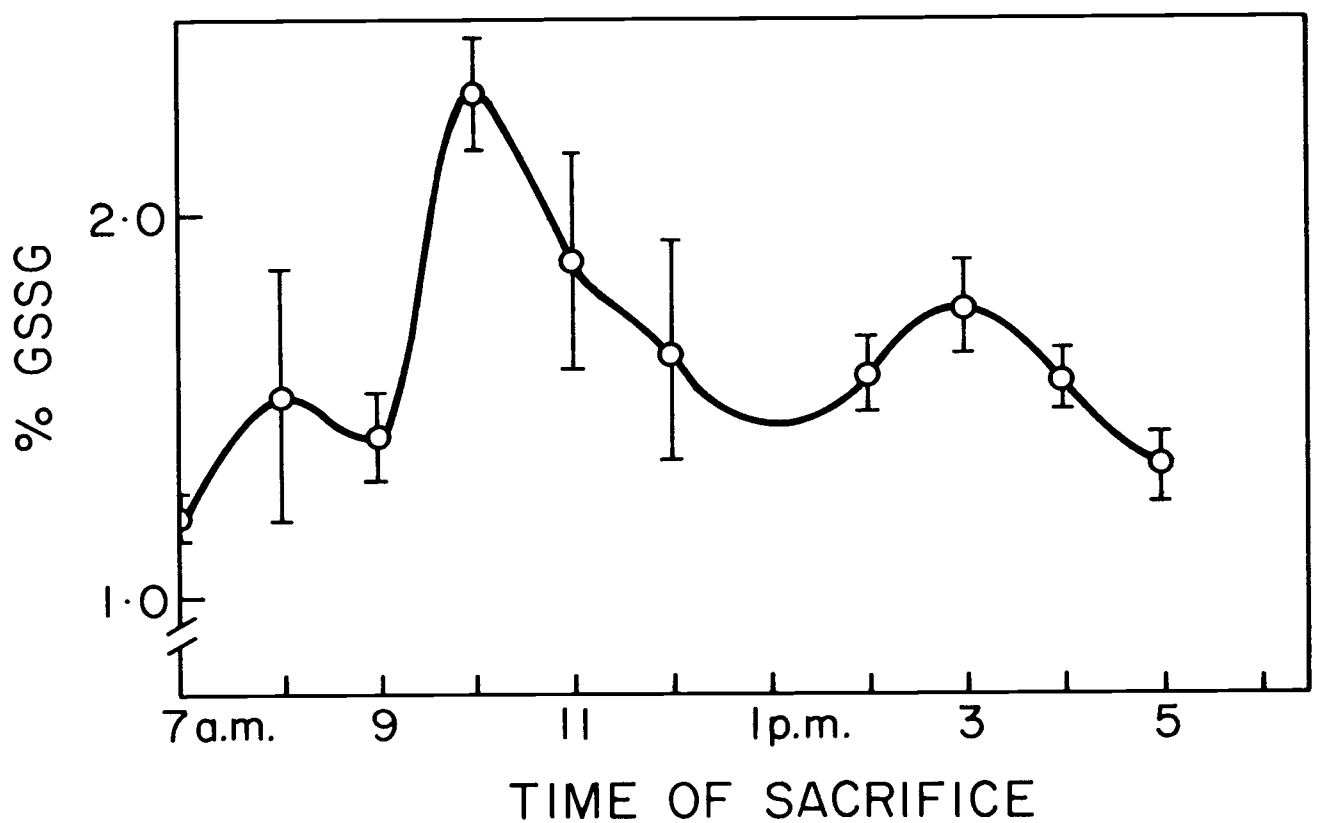


Figure 18. Percent GSSG values for untreated L-5178Y lymphoma cells as a function of time of sacrifice. Each point represents 6-10 mice. Standard deviations are indicated by error bars. Cells were washed as described on page 35 and GSSG and GSH were assayed by the reductase method.

Table 8. Effect of procarbazine on % GSSG in L-5178Y lymphoma cells.

Treatment	Date of Experiment		
	9/5/72	9/11/72	9/19/72
		% GSSG	
Control	2.2	2.0	1.9
Procarbazine (1 hr.)	3.9	3.9	3.8
Procarbazine (3 hr.)	4.9	4.5	1.9
Procarbazine (8 hr.)	2.0	1.3	2.1

Time of treatment with procarbazine indicates time between administration to tumor bearing mice and sacrifice. GSH and GSSG were determined by an enzymatic method.

Table 9. Effect of pH of wash solution on GSH levels and lymphoma cell integrity.

pH of Buffered KCl Wash Solution	% GSSG	GSH in Cells (μmoles/ml PCV)	GSH in Sup 3 (μmoles/ml PCV)	Protein in Sup (mg/ml)
KCl (no buffer)	3.1	2.3	0.02	4.1
7.4	3.0	2.2	0.02	3.3
5.4	2.6	2.2	0.04	3.3
4.7	9.0	0.5	0.29	3.8
3.0	-	0.0	0.37	5.5
Distilled H_2O	21.0	0.08	0.16	13.7

Inhibition of Protein Synthesis

Since protein synthesis has been found to be controlled by the redox state, and can be inhibited by GSSG/GSH ratios as low as 0.05, it is interesting to examine the relationship between the effects of procarbazine on this system and the effects on the redox state.

Initial studies were carried out following the method of Sartorelli and Tsunamura (184). Tumor bearing mice were given 0.2 ml of a solution of 5 μcuries/5 mg leucine-1-^{14}C in 10 ml saline, and one hour was allowed for incorporation. Diethylmaleate was used to decrease GSH levels in vivo in an attempt to potentiate the effects of procarbazine on the redox state, and in turn on protein synthesis, in order to determine the relationship between these parameters in this system.

The results of these studies are summarized in Table 10. Treatment with procarbazine one hour prior to sacrifice did not inhibit leucine incorporation, but a combination of procarbazine and diethylmaleate resulted in a slight inhibition (93% of control). Diethylmaleate did not appear to be inhibitory. Treatment with procarbazine three hours prior to sacrifice resulted in 30% inhibition (70% of control) when procarbazine was administered alone, and 45% inhibition when it was given in combination with diethylmaleate.

Table 10. ^{14}C-leucine incorporation in L-5178Y lymphoma cells after *in vivo* treatment with procarbazine and/or diethylmaleate. Leucine level was 4 mg/Kg body wt.

Number of Mice	Treatment	Hrs of Treatment	Leucine Incorporation (% of Control)
40	Control	-	100
10	Procarbazine	1	111
14	Procarbazine	3	70
10	Diethylmaleate	3	108
14	Procarbazine plus Diethylmaleate	1 3	93
16	Procarbazine plus Diethylmaleate	3 3	55

Leucine was administered one hour prior to sacrifice
Procarbazine was administered one and three hours prior to sacrifice

A diurnal variation in leucine incorporation was noted. Values from samples taken at the same time of day agreed reasonably well even when the samples were taken on different days, but agreement from morning to afternoon, or even over a period of a few hours, was very poor. The observed pattern was roughly complementary to the redox potential pattern observed earlier, but not enough data are available to be certain. A test of the dietary influence on incorporation did not reveal any significant effect with as much as 24 hours fasting.

Because of the observed time of day variation, samples from treated animals were taken as closely as possible to the time of sacrifice of controls. In order to compare values from different sets of data, all values reported are adjusted in such a way that the average value for controls in each set is brought to par with the overall average for controls. The average value was 142,000 $\pm$ 29,500 dpm/ml PCV under the conditions described above. Incorporation is not linear with time, however under these conditions. The kinetics of leucine incorporation are illustrated in Figure 19 (a & b). Cells from control mice ceased to incorporate leucine 20 minutes after its administration (Figure 19a), whereas cells from procarbazine treated mice continued to incorporate leucine throughout the entire 60 minute period. When only 15 minutes are allowed for incorporation both procarbazine (one hour treatment) and diethylmaleate are seen to cause inhibition.

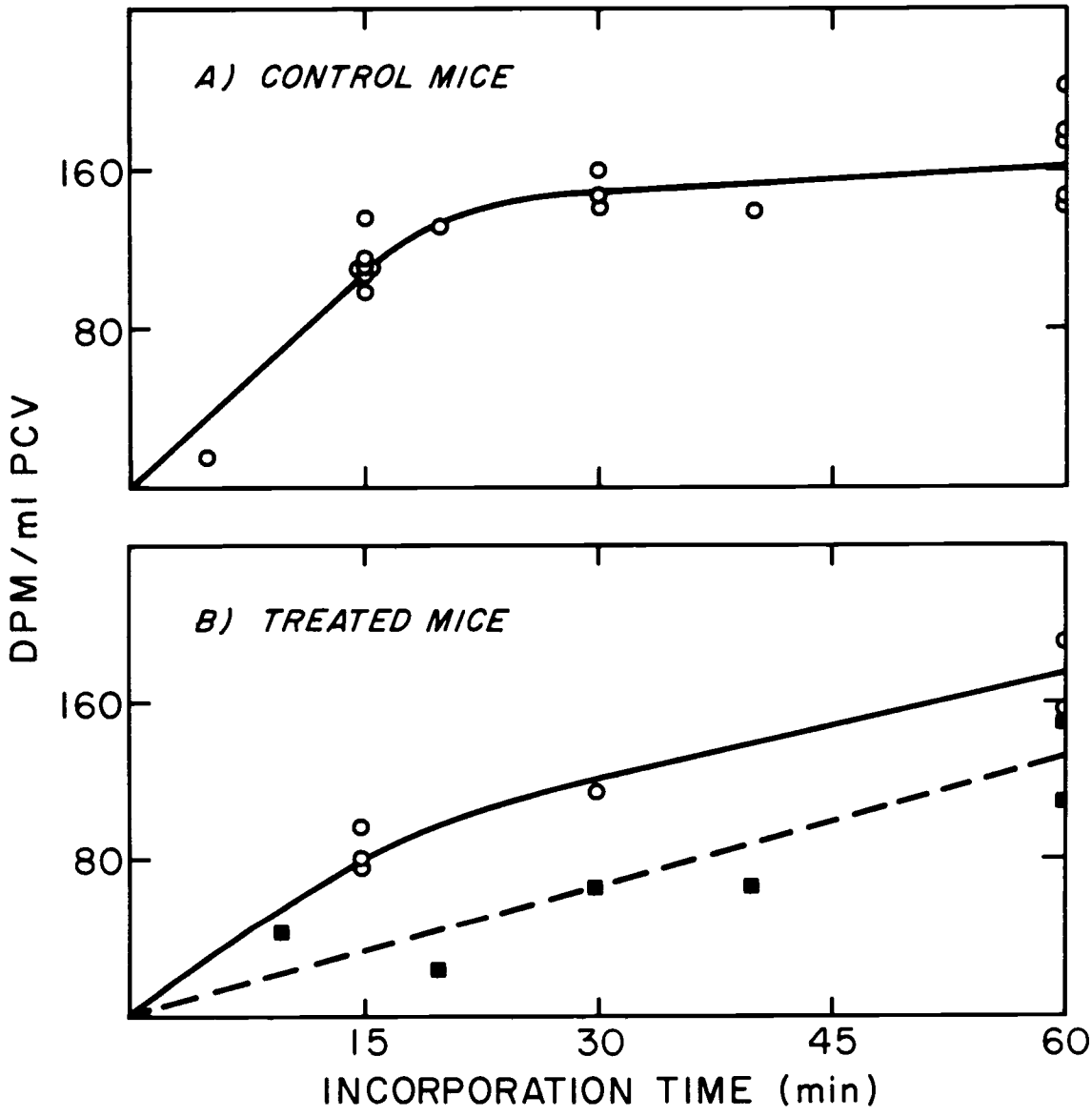


Figure 19a and b. Kinetics of leucine incorporation in L-5178Y cells (4mg/kg leu) Leucine was administered 15, 30, 45 and 60 min. before sacrifice. Procarbazine, 1 hr. prior to sacrifice. Diethylmaleate, 3 hr. prior to sacrifice.

High Leucine Levels

The effect of administration of increasingly large amounts of leucine on incorporation in control mice is seen in Figure 20. Incorporation is apparently linear for the first 15 minutes even at 4 mg/kg leucine, but a dose of 80 mg/kg is required to extend linearity over the entire 60 minute period.

The kinetics of incorporation at 80 mg/kg leucine are seen in Figure 21 (a & b). Linearity is apparent over the first 15 minutes in both control and treated mice, although at this level of leucine there appears to be some binding in addition to incorporation. Values of as much as 10,000 dpm/ml PCV were achieved by incubating washed cells with leucine at 37^{o} for a few minutes, and this apparent incorporation was not correlated with time of incubation. Further evidence of binding was obtained by administering leucine 15 seconds prior to sacrifice. In control mice no incorporation was seen, but in procarbazine treated mice 20,000 dpm/ml PCV appeared in the TCA insoluble fraction. It would seem that the slope of a line running through several points reflecting different times of incorporation would be an accurate picture of the actual rate of protein synthesis, especially if the intracellular specific activity of leucine is monitored. Table 11 shows the specific activity of leucine in several of the samples represented in Figures 21 (a & b). It is apparent that in all cases

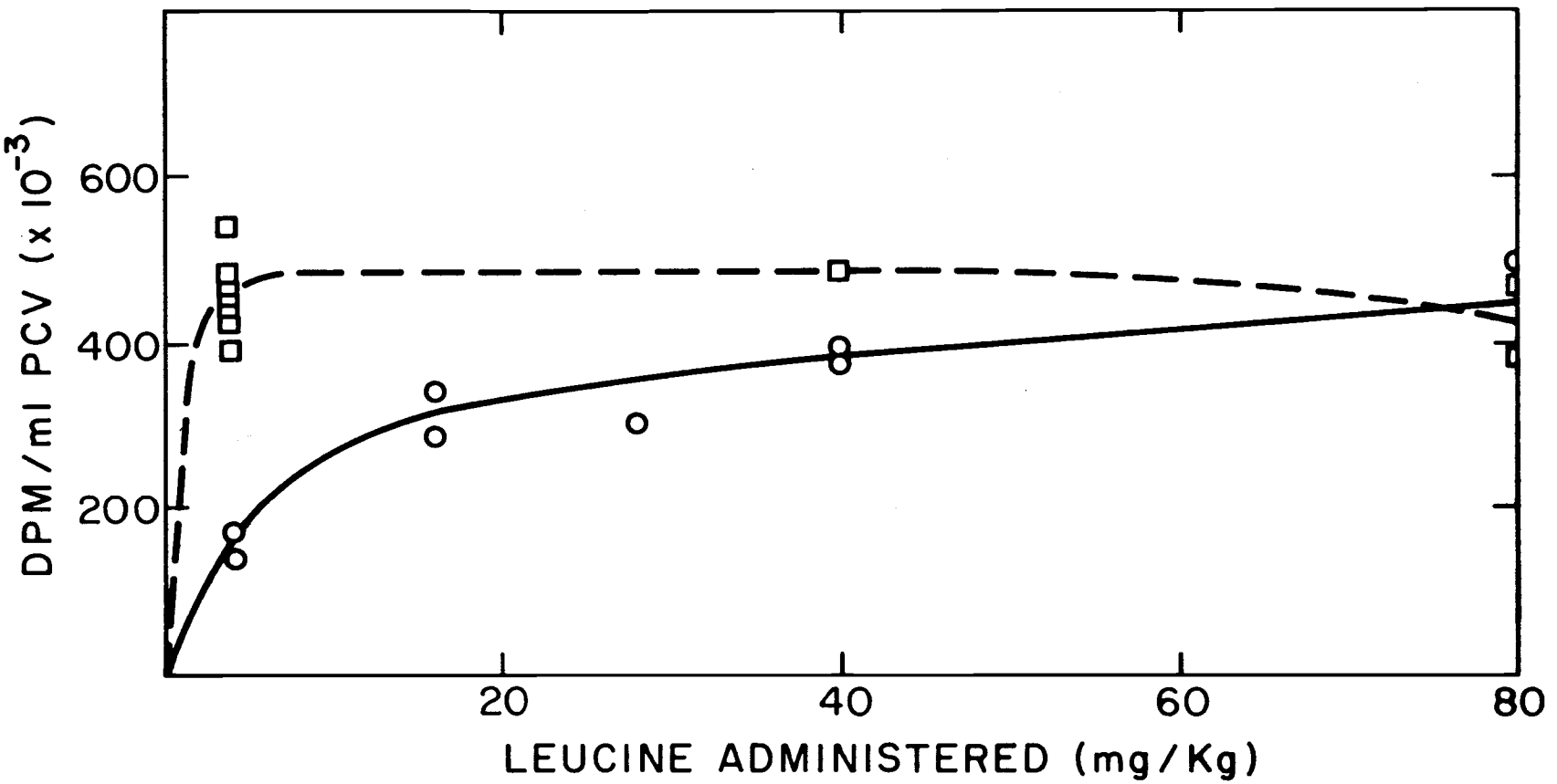


Figure 20. Relationship between amount of leucine administered and incorporation. DBA/2J mice carrying L-5178Y ascites tumors were given leucine-1-^{14}C on the 6th day after implantation, and sacrificed after 15 or 60 min. Cells were washed, and precipitated with TCA. The precipitate was combusted after exhaustive washing and the $^{14}CO_2$ was counted.
*Values are multiplied by a factor of 4 for comparison with 60 min. incorporation.

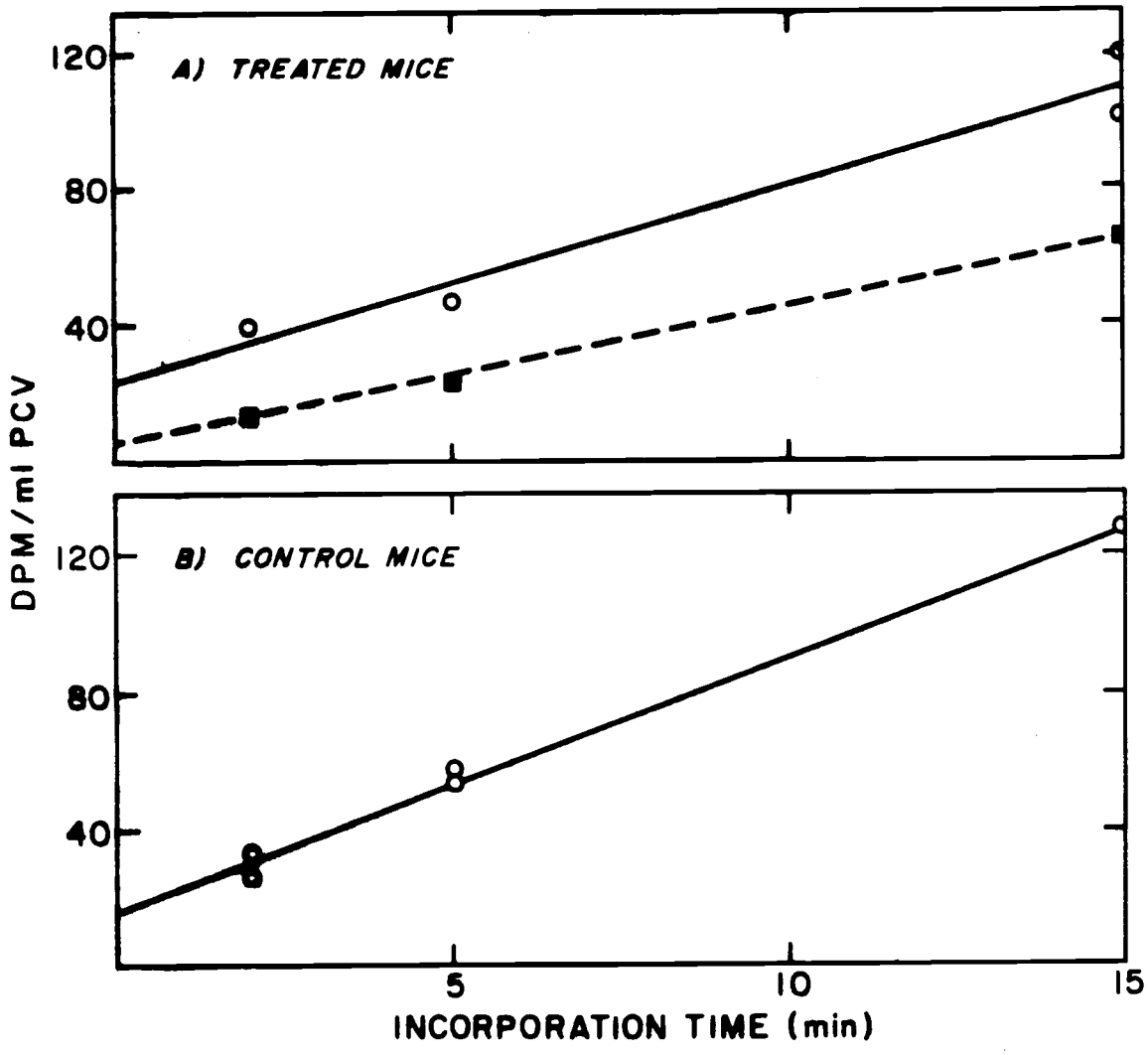


Figure 21a and b. Kinetics of leucine incorporation in L-5178Y cells (40 mg/kg leu). Leucine was administered 15, 30, 45 and 60 min. before sacrifice. Procarbazine was administered 50 min. prior to leucine. Diethylmaleate, 2 hrs. 50 min. prior to leucine.

Table 11. Intracellular specific activity of leucine in L-5178Y lymphoma cells treated in vivo.

Treatment	Time of leucine* incorporation (min)		
	2	5	15
Control	.107		.055
Procarbazine (1 hr)	.097	.097	.104
Diethylmaleate (3 hr)	.109		.106

* or valine

Specific activities are expressed in mmoles/μcurie
TCA extracts of lymphoma cells were reacted with ^{3}H-FDNB, and the DNP-amino acids formed were analyzed as described on page 51.

leucine enters the cells within 2 minutes and its specific activity remains constant over the first 15 minutes. The values for inhibition of protein synthesis based on Figure 21 (a & b) are 22% (78% of control) for 1 hr procarbazine and 45% (55% of control) for diethylmaleate.

Table 12 shows the effect of procarbazine and/or diethylmaleate on leucine incorporation when 80 mg/kg leucine are administered and 15 or 60 minutes are allowed for incorporation (not corrected for binding). One hour treatment with procarbazine causes 31 and 33% inhibition, respectively, when observed at 15 and 60 minutes, reflecting linearity over a 60 minute period of incorporation, as observed in controls. Following diethylmaleate treatment, however, incorporation does not continue at a constant rate after the first 15 minutes. When 15 minutes are allowed for incorporation, inhibition is 33%, but when 60 minutes are allowed for incorporation inhibition becomes an apparent 67%. The same effect is seen when diethylmaleate is administered in combination with procarbazine, with 45% inhibition at 15 minutes and an apparent 71% inhibition at 60 minutes.

It is interesting to note that one hour treatment with procarbazine reproducibly results in inhibition, regardless of the level of leucine used, provided incorporation kinetics are linear. At 4 mg/kg leucine, inhibition is 28% when observed at 15 minutes. This compares closely with the values of 22% and 31% noted earlier. Inhibition

Table 12. ^{14}C-Leucine incorporation in L-5178Y lymphoma cells after *in vivo* treatment with procarbazine and/or diethylmaleate. Leucine level was 80 mg/Kg body wt.

Treatment	Leu Incorporation (% of Control) 15 Min	60 Min	Incorporation Ratio (60 Min/15 Min
Control	100	100	4.7
Procarbazine (1 hr)	69	67	4.6
Diethylmaleate (3 hr)	67	34	2.4
Procarbazine (1 hr) + Diethylmaleate (3 hr)	55	29	2.4

Tumor-bearing mice were treated with procarbazine 50 min. prior to leucine administration and with diethylmaleate 2 hr. 50 min. prior to leucine. Leucine was given either 15 or 60 min. prior to sacrifice.

of protein synthesis had never been recognized to be an early event in procarbazine cytotoxicity. It now becomes more plausible to consider glutathione oxidation as a mechanism of inhibition of protein synthesis by procarbazine, considering the time-course of glutathione oxidation which is reported in this thesis.

Effects of Diamide on L-5178Y Cells In Vitro

Challenge - Recovery Studies

One question that arises in considering the significance of a certain magnitude of effect on the redox state is what magnitude of challenge is required to achieve it. The reductive capacity of these cells is quite high. In Table 13 it can be seen that even at 0°C L-5178Y cells are capable of regenerating reduced glutathione at an appreciable rate, following oxidative challenge with diamide. When enough diamide was added to oxidize cellular glutathione completely, recovery to 18% was seen in 50 minutes at 0°C. After partial oxidation (to 69% GSSG) the cells recovered to 13% GSSG in 50 minutes and to 7% GSSG in another 50 minutes.

Figure 22 shows the GSH regeneration rate after diamide challenge in whole ascites fluid at 37°C. Recovery from complete oxidation was rapid if no excess diamide was used. When twice as much diamide than required for complete oxidation of GSH was added, GSH

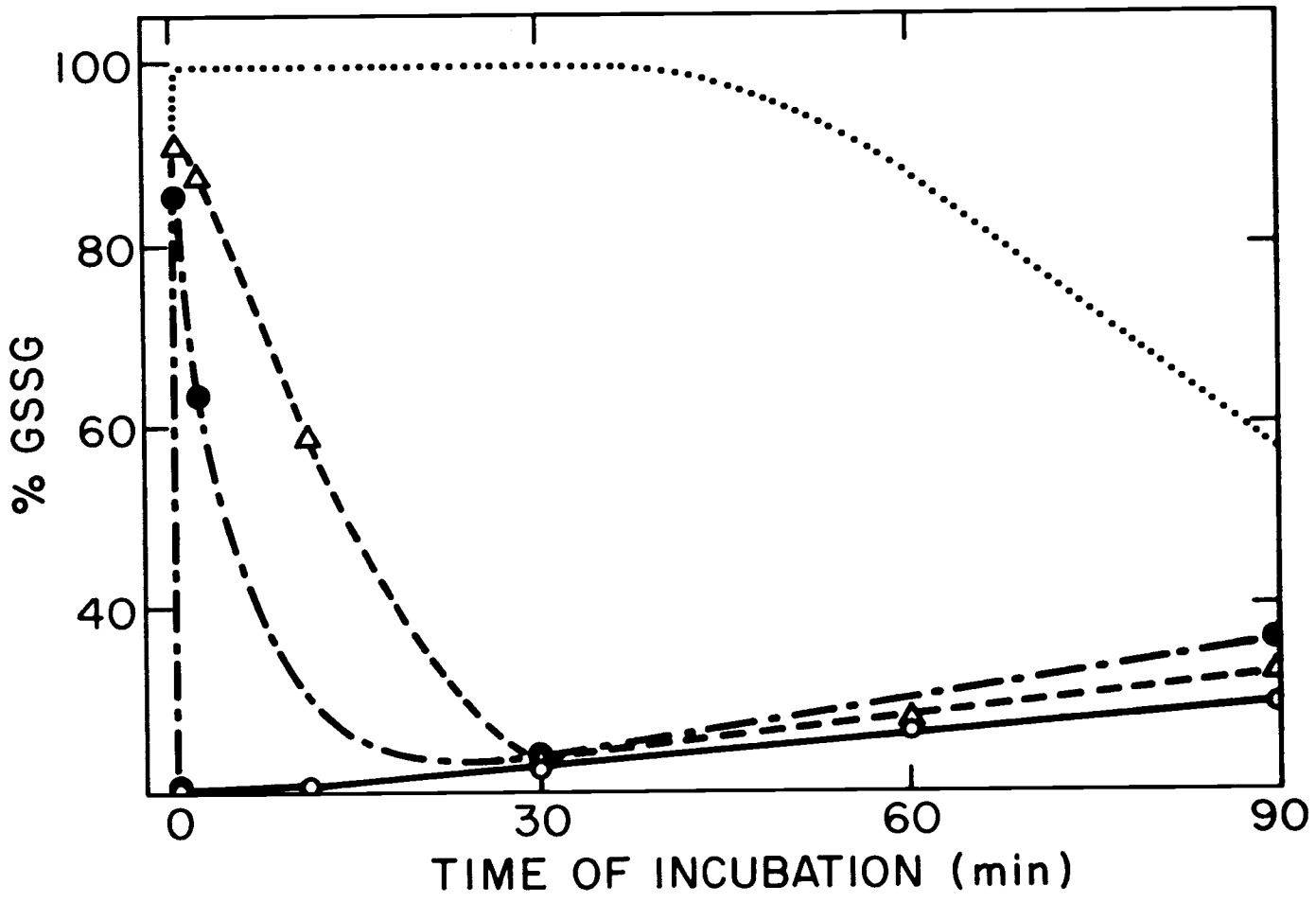


Figure 22. Regeneration of GSH after treatment with diamide, in whole ascites fluid. L-5178Y cells were harvested from tumor-bearing mice, treated with diamide at 0°C, and incubated at 37°C. No nutrients were added. Diamide levels (expressed as diamide/GSH molar ratios): 1:1 (●–·–·–●) 2:1 (Δ - - - - Δ) 4:1 (. . . . . .) Control (o — o)
Note that one mole of diamide oxidizes two moles of GSH.

Table 13. Recovery of %GSSG in L-5178Y cells following challenge with diamide.

Treatment	Incubation Time (Min.)	%GSSG
High diamide level	0	100
High diamide level	50	18
Low diamide level	0	69
Low diamide level	50	13
Low diamide level	100	7

Cells were harvested from mice bearing 6 day tumors, and mixed with diamide, while keeping at $0^{o}C$. Cells were run through the cell preparation procedure of page 35 (about 50 min.), and one sample was allowed to remain on ice for another 50 minutes.

regeneration was complete in 30 minutes. A 4X molar excess resulted in incomplete recovery, after as much as 90 minutes incubation. All samples were subject to air oxidation, as indicated by an increasing baseline. Addition of glucose-6-phosphate curbed the increase in GSSG, but would not reverse the trend, indicating that most of the GSSG may have been extracellular.

Incubation of unwashed lymphoma cells in Fischer's Medium at 37^{o} did not lead to an increasing baseline, and also resulted in complete recovery from diamide challenge in less than 10 minutes (Table 14). MMH and procarbazine had no effect on %GSSG in this system after as much as 120 minutes incubation.

L-5178Y cells grown in spinner flasks (page 36) were concentrated to contain 1 ml PCV in 100 ml growth medium, and diamide (2 mg/ml in Fishcer's medium) was dripped into the stirring suspension at a rate of 20 ml/hr., for one hr. Although this proved to be a serious metabolic challenge, on the basis of lactate production, no change in the glutathione redox state was observed. This corresponds to a turnover of the entire cellular content of GSH about every minute. These studies make it clear that in the presence of an adequate nutrient supply, which is the physiological condition, the challenge required to achieve even the slightest change in the redox state is quite large. In order to ameliorate the metabolic stress, and in order to administer a sufficient amount of diamide over a prolonged period, hollow

Table 14. Effects of single doses of diamide, procarbazine and monomethylhydrazine on L-5178Y cells incubated in Fischer's medium in vitro at 37°C.

Treatment	%GSSG Time of Incubation (min.) 0	10	30	60	120
MMH	5.8	3.7	3.4	7.6	5.7
Procarbazine	6.6	3.7	2.8	7.2	4.9
Diamide	77.0	2.6	3.5	7.7	6.2
Control	7.6	2.4	2.6	6.5	9.1

Cells were harvested from tumor bearing mice, centrifuged once, and resuspended in Fischer's medium. Reagents were added in 0.1 ml Fischer's medium at the following concentrations: Diamide, 5 mg/ml; MMH, 35 mg/ml, procarbazine, 35 mg/ml. Each sample contained 6 ml of cell suspension, for a packed cell volume of 0.9 ml. Cells were washed for TCA precipitation.

fiber perfusion was utilized.

Hollow Fiber Perfusion

L-5178Y cells were harvested from tumor-bearing mice, washed according to the procedure on page 35, resuspended in Fischer's medium, and placed in Biofiber^{R} minibeakers as described on page 38. Each minibeaker contained approximately 0.3 ml packed cells, in a volume of about 7 ml. Perfusion media, with or without diamide, was pumped through the fibers at a rate of 90 ml/hr. Diamide was administered over a period of 3 hr., and then the cells were returned to control medium perfusion for another hour before taking final samples.

Figure 23 shows the effect of this treatment of the gluthathione redox potential at the time of diamide administration. The response was somewhat erratic, but values close to those observed after procarbazine treatment *in vivo* were achieved. The ability to cope with the challenge was lost at diamide concentrations much higher than 20 mg/ml/ml PCV, and cellular GSH became completely oxidized. Once this happened, the cells never recovered, and viability appeared to be decreased.

It is interesting to note (Figure 24) that the redox state remains altered even after the 1 hr. recovery period. The cells appeared to acquire a new redox state, which was reasonably well correlated with the extent of oxidative challenge experienced. These altered cells

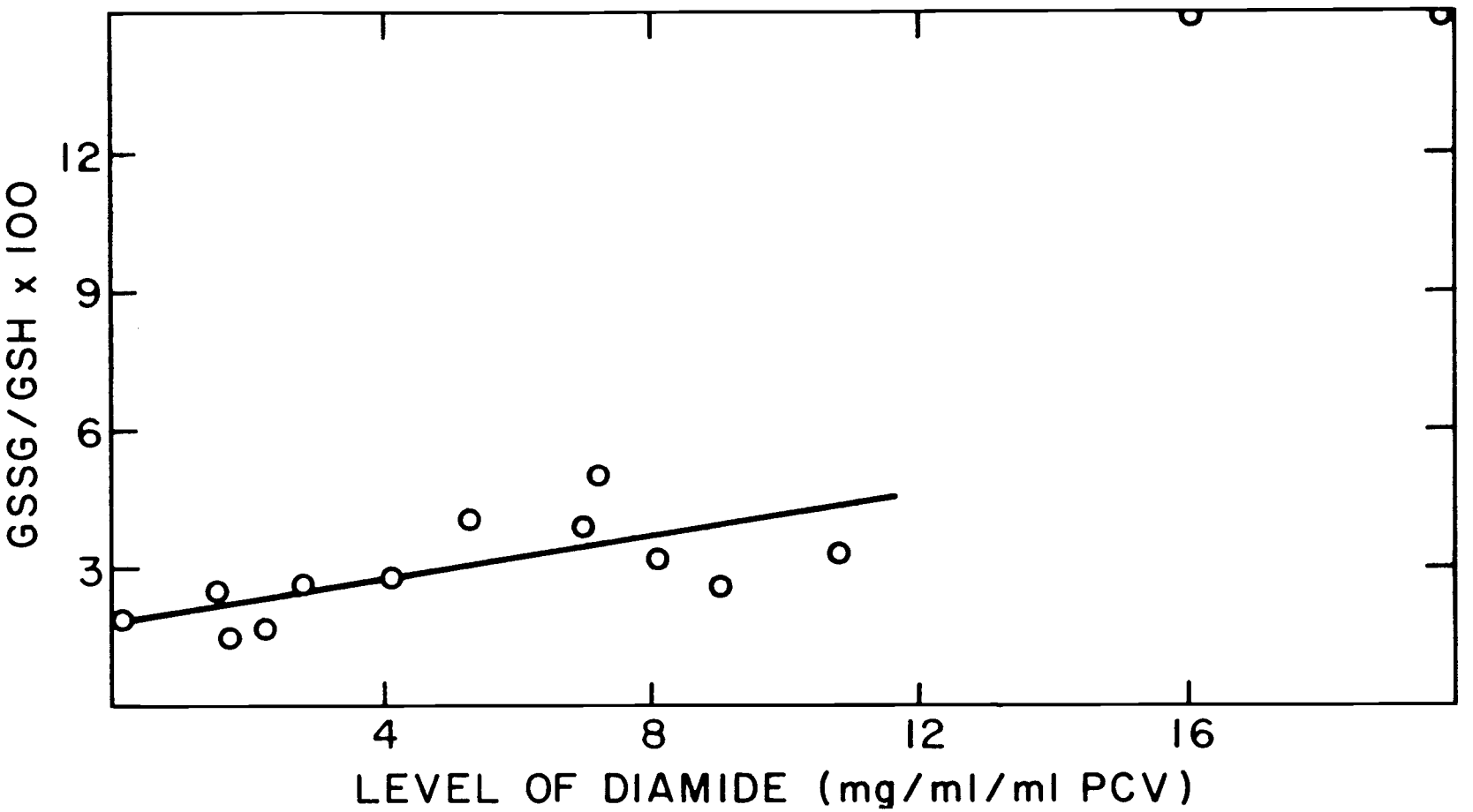


Figure 23. GSSG/GSH ratio in L-5178Y lymphoma cells during treatment with diamide in vitro. Cells harvested from tumor-bearing mice were treated with diamide by hollow fiber perfusion. Diamide levels indicated are levels in perfusion medium, based on total packed cell volume in the minibeakers. Glutathione was determined after 60 or 120 minutes of diamide treatment.

were used to measure the rate of protein synthesis and also were transplanted back into mice, and their ability to produce tumors was observed.

Protein Synthesis

Leucine incorporation was measured during and after treatment with diamide in hollow fiber devices by incubating treated cells containing ^{14}C-leucine, in Fischer's Medium for 20 minutes at 37°C. As can be seen in Table 15 a substantial decrease in leucine incorporation was seen, and the degree of inhibition was correlated with the level of diamide used. A comparison with Figure 23 suggests that diamide treatment to result in 2.5% GSSG could correspond to as much as 70% inhibition of protein synthesis.

Transplantability and Viability

Following the recovery period, diamide treated cells were transplanted to the intraperitoneal cavity of BDF_1 mice, and their ability to produce tumors was observed. An increase in survival time in relation to mice bearing tumors from untreated or control cells was noted, and the extension in lifetime was correlated with the final %GSSG values in these cells (Figure 25). Variability was great, as expected in such a system, but the trend was apparent. A substantial increase in survival time, from 10 days to 20 was seen at %GSSG values greater than 4%.

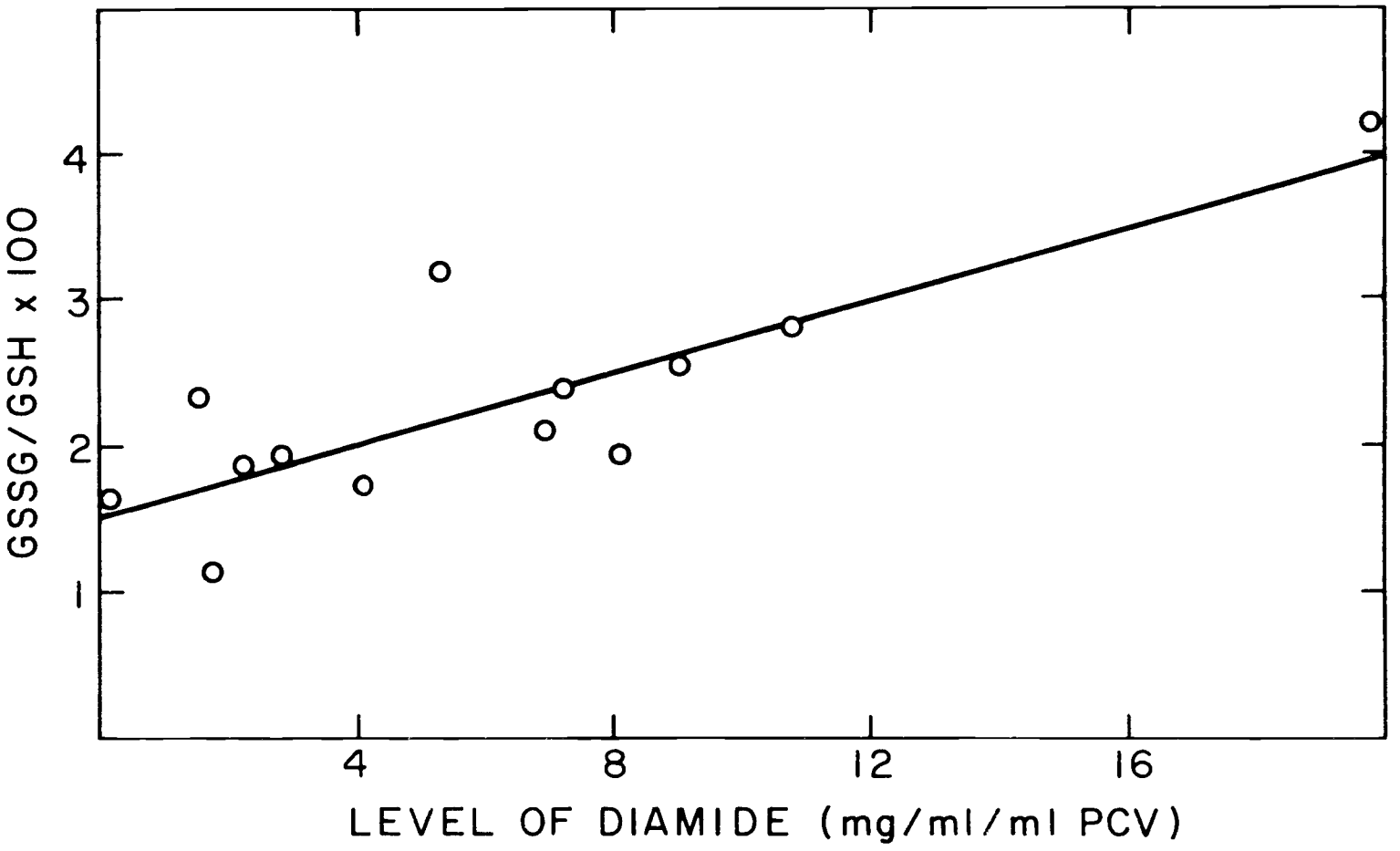


Figure 24. GSSG/GSH ratio in L-5178Y lymphoma cells after treatment with diamide in vitro. Cells harvested from tumor-bearing mice were treated for 3 hr. with diamide by hollow fiber perfusion. Diamide levels indicated are levels in perfusion medium, based on total packed cell volume in the minibeakers. Glutathione was determined following a 1 hr. recovery period.

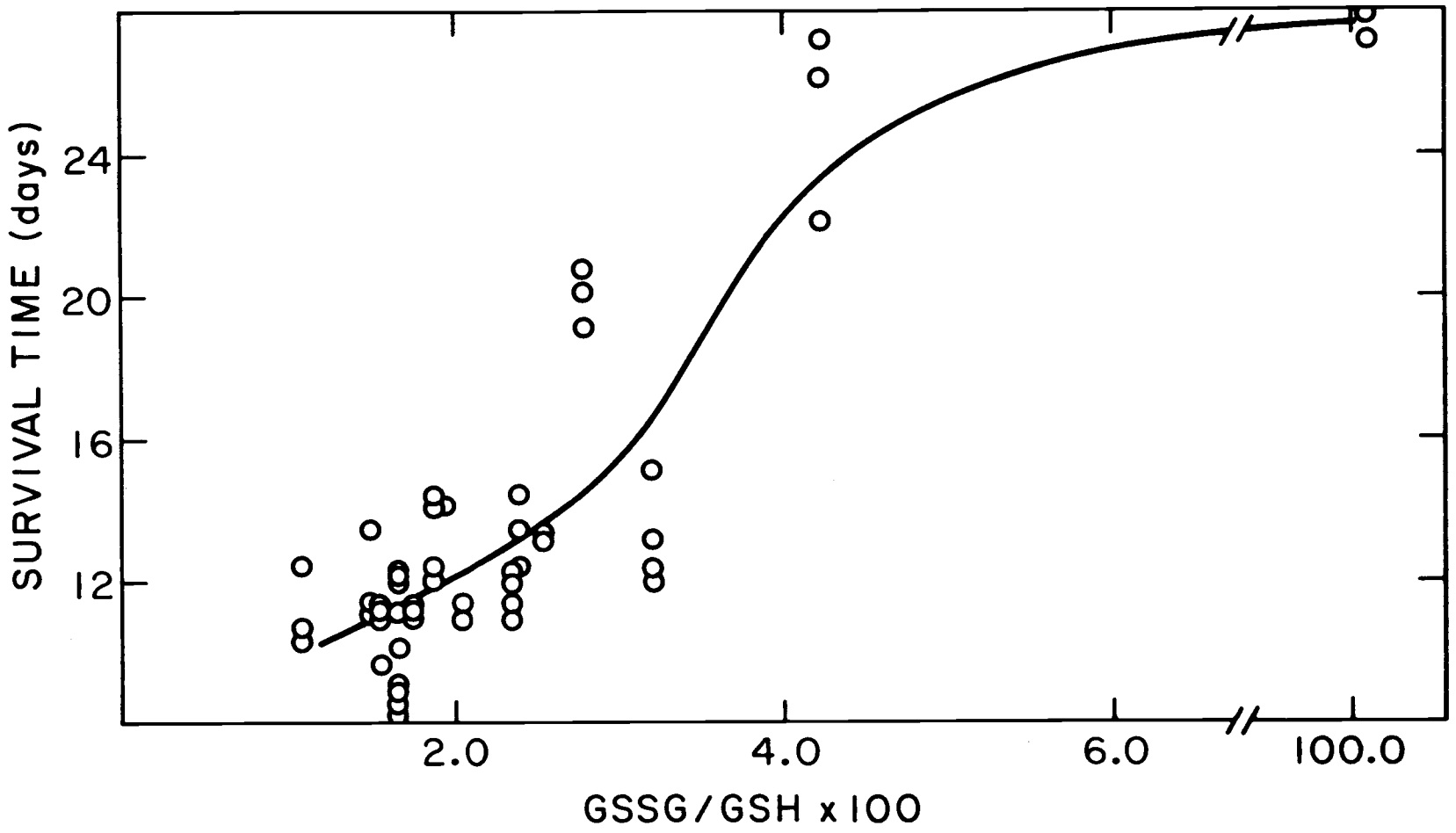


Figure 25. Survival time of mice bearing L-5178Y tumors from diamide treated cells, as a function of %GSSG at the time of transplantation. Cells were treated by perfusion with various levels of diamide in hollow fiber minibeakers, and reinjected into BDF_1 mice. Each data point represents one mouse.

Table 15. Leucine incorporation in L-5178Y lymphoma cells treated *in vitro* with diamide.

Treatment	Leucine Incorporation (dpm/ml PCV) x 10^{-3}	
	120	180 + 60
Untreated (8/18)	1960	
Untreated (9/26)	1390	
Control (8/18)	1625	1420
Control (9/26)	1580	1275
2.1 mg/ml/ml PCV (8/18)	730	1330
5.2 mg/ml/ml PCV (8/18)	650	495
7.1 mg/ml/ml PCV (9/26)	570	900
8.9 mg/ml/ml PCV (9/26)	360	420
10.7 mg/ml/ml PCV (9/26)		160

Cells were treated by perfusion with various levels of diamide in hollow fiber minibeakers. Aliquots were taken at 120 min. and at 240 min., 60 min. after discontinuing diamide treatment. 20 min. were allowed for incorporation, at 37°C, in Fischer's medium.

Table 16. L-5178Y cell viability and metabolic activity following diamide treatment in vitro.

Diamide Treatment (mg/ml/ml PCV)	Viable Cell Count (% viable)	Lactate Production 60[a]	120	180	180+60	Oxygen Uptake[b] 120	180+60
Untreated cells	99					515	
Control (no diamide)	93	.34	.25	.23	.28	512	455
1.4 - 4.0	93	.30	.28	.29	.20	590	750
5.2 - 10.7	78	.39	.13	.33	.32	330	210
14.2 - 21.4	5	.28	.13	.19	.15		
40.0	0	.13	.12	.09	.08		

a. Time of diamide treatment (min.), or for controls, time of incubation in hollow fiber devices. Untreated cells were not incubated. 180 +60 indicates 60 min. of perfusion with control medium were allowed for recovery from effects of diamide
Lactate production is expressed in μmoles/min., as measured in perfusate.

b. Oxygen uptake is expressed in μmoles 0_2/min./ml PCV
All values, except those for 40 mg/ml/ml PCV diamide, are averages over 5-6 experiments.

The question that arises at this point is whether transplantability was impaired because of a change in the neoplastic characteristics of the cells, or whether they were simply killed by the challenge, leaving a smaller number of viable cells to produce a tumor. Either way, the observation is significant, because the basic question is whether glutathione oxidation, to the extent observed with procarbazine, can result in tumor inhibition.

Table 16 lists a number of metabolic parameters as indicators of cell viability following treatment with diamide. Vital staining with trypan blue suggests more than 70% of the cells survive the treatment, except in the extreme cases where glutathione is completely oxidized. Oxygen uptake and lactate production show metabolic activity which is at least 50% of that of controls in most cases (Table 16).

Mixed Disulfides in Fetal Calf Serum

Mixed Disulfide Analysis

Fetal bovine serum was found to be a convenient source of protein-glutathione mixed disulfide for calibration and evaluation of analytical techniques. Mixed disulfide levels in commercially prepared sera (Flow Laboratories, Inc., Rockville, Md.) averaged 5 μg GSH as GSSP per ml, while free glutathione levels were of the order of 0.2 μg/ml. Values varied from pool to pool (Table 17), but for a given

Table 17. Protein-glutathione mixed disulfides in various sera.

Serum	GSH as GSSP (μg/ml)
Fetal Bovine Serum	6.5
Fetal Bovine Serum	3.4
Fetal Bovine Serum	4.8
Fetal Bovine Serum	<0.1
Fetal Bovine Serum (3 mo.)	3.3
Fetal Bovine Serum (5 mo.)	6.3
Fetal Bovine Serum (Near Term)	<0.1
Calf Serum (2 mo.)	<0.1
Adult Bovine Serum	<0.1
Adult Bovine Serum	<0.1
Fetal Horse Serum	<0.1
Adult Horse Serum (Gelding)	<0.1
Adult Horse Serum (Gelding)	<0.1
Adult Horse Serum (Not Gelding)	<0.1
Swine Serum	<0.1

Each Value Represents a Different Pool or Source
GSSP was reduced with NBS and GSH assayed by reductase method.

pool, they remained constant over a period of at least one year of storage at -85°C or at least two weeks at 0°C, allowing reference to a common standard over a prolonged period of time.

Analysis of Various Sera

It is of interest to note that of all sera tested (Table 17), only fetal calf serum contained appreciable amounts of protein-glutathione mixed disulfide. Commercially prepared sera was obtained from Flow Laboratories, Inc. Other samples were obtained from Dr. Katherine Sanford (NCI, NIH, Bethesda, MD.) and Dr. Donald E. Mattson (Oregon State University). Additional horse serum samples were obtained from Grand Island Biological Co., Berkeley, California.

Three month and five month fetal calf sera contained large amounts of the mixed disulfide (3.3 - 6.3 μg/ml), whereas near term fetal calf serum mixed disulfide levels were below the limits of detection (less than 0.1 μg/ml). One particular pool of fetal calf serum obtained from Flow Laboratories did not contain detectable amounts of mixed disulfide. It could be that the period of gestation of the fetal calves used would account for this observation. Free glutathione was separated from fetal calf serum and other sera by sephadex G-25 filtration and Amicon^{R} PM 10 ultrafiltration. No serum tested contained significant amounts. Fetal calf serum was found to contain no more than 0.2 μg/ml.

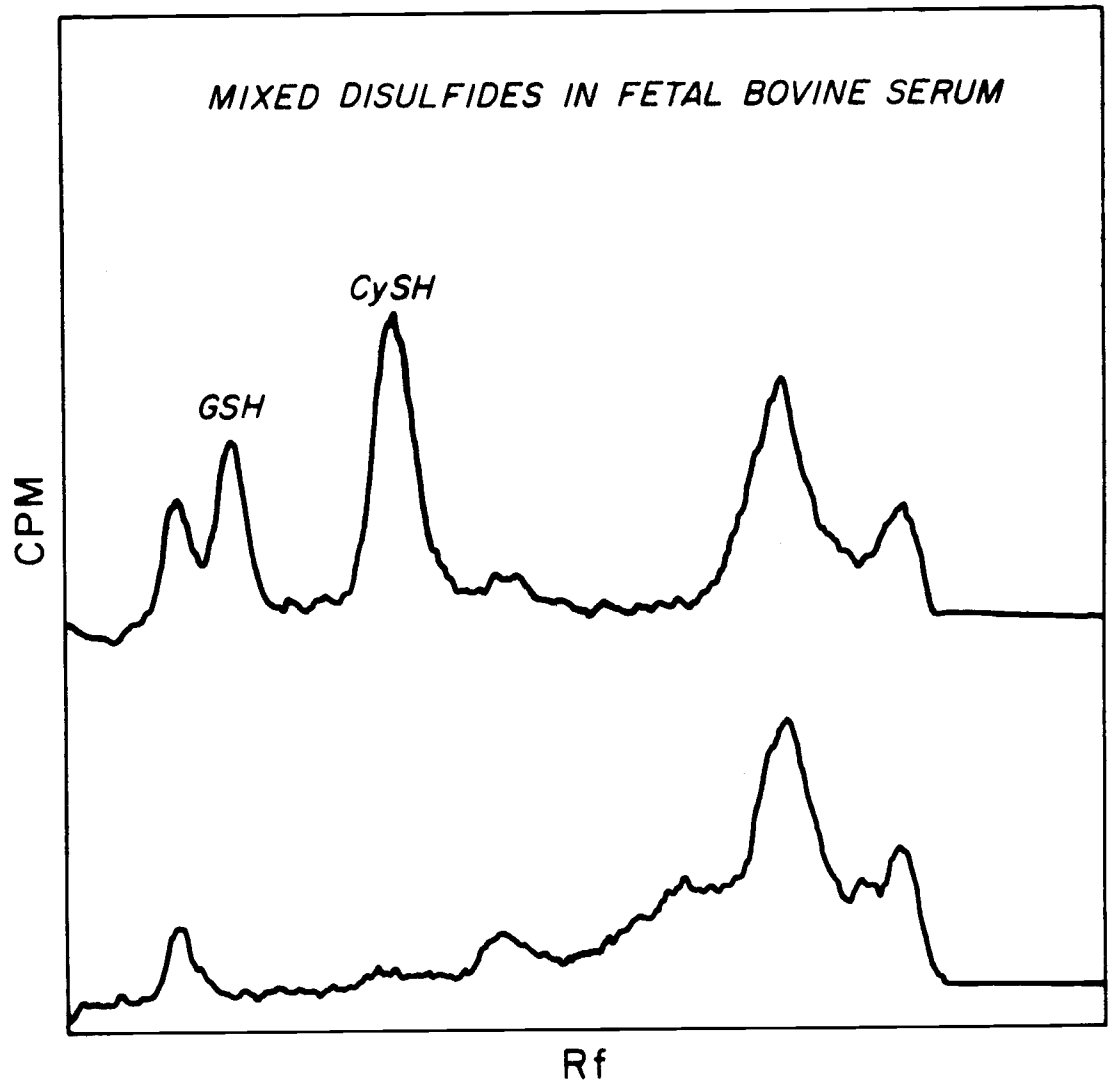


Figure 26. Mixed disulfides in fetal calf serum. Fetal calf serum was reduced with dithiothreitol and reacted with ^{14}C-NEM in the presence of arsenite. The adducts formed were separated by chromatography, and the radiochromatogram is shown above. A reagent blank radiochromatogram is shown below for comparison. The presence of GSH and Cysteine are apparent.

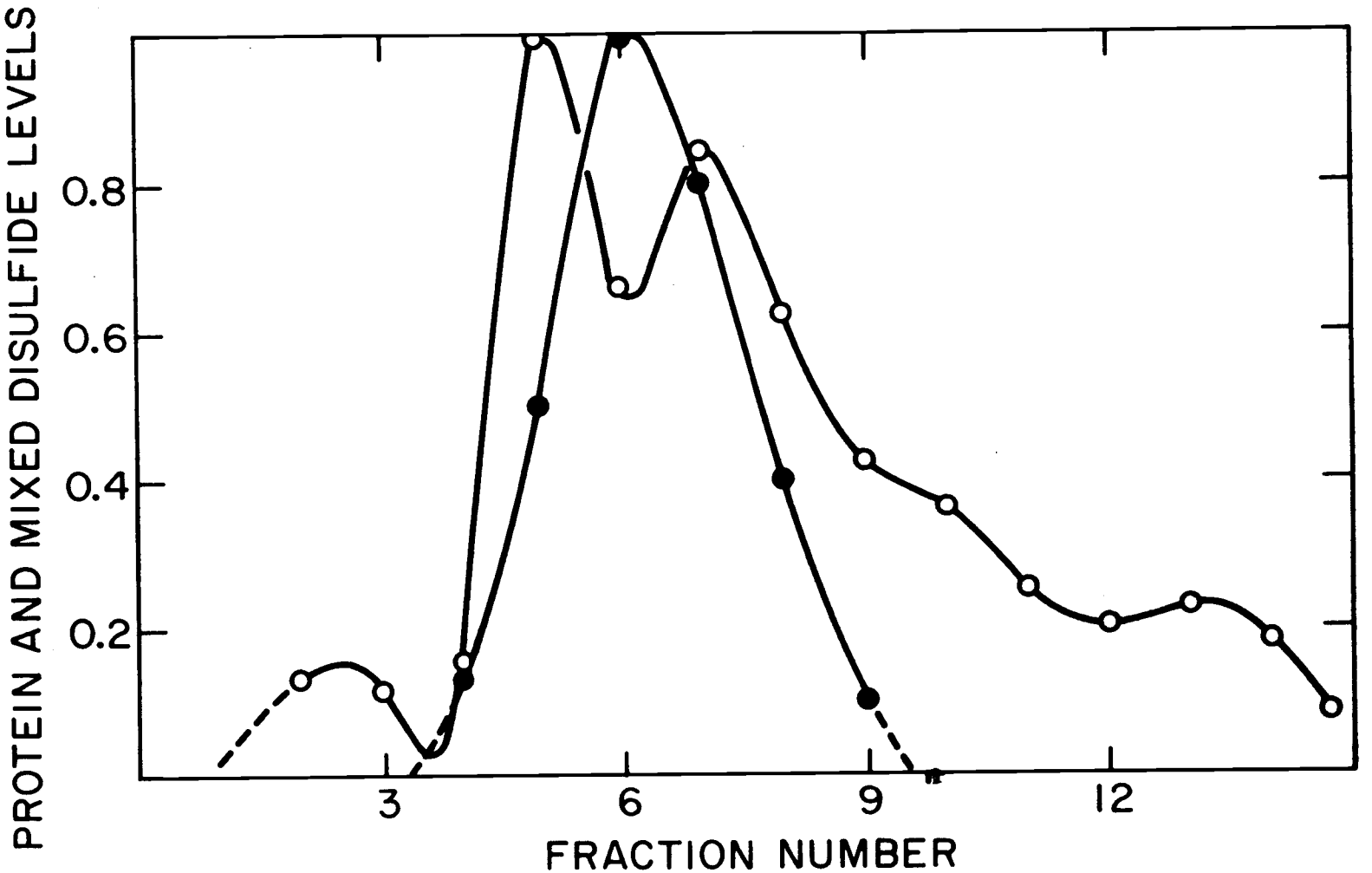


Figure 27. Sephadex G-200 filtration of fetal calf serum. Protein (o—o) and mixed disulfide levels (● — ●) were measured in the eluate. Protein is given in mg/ml. Mixed disulfide values are expressed in relative units, with the value 1.0 assigned to the highest level observed.

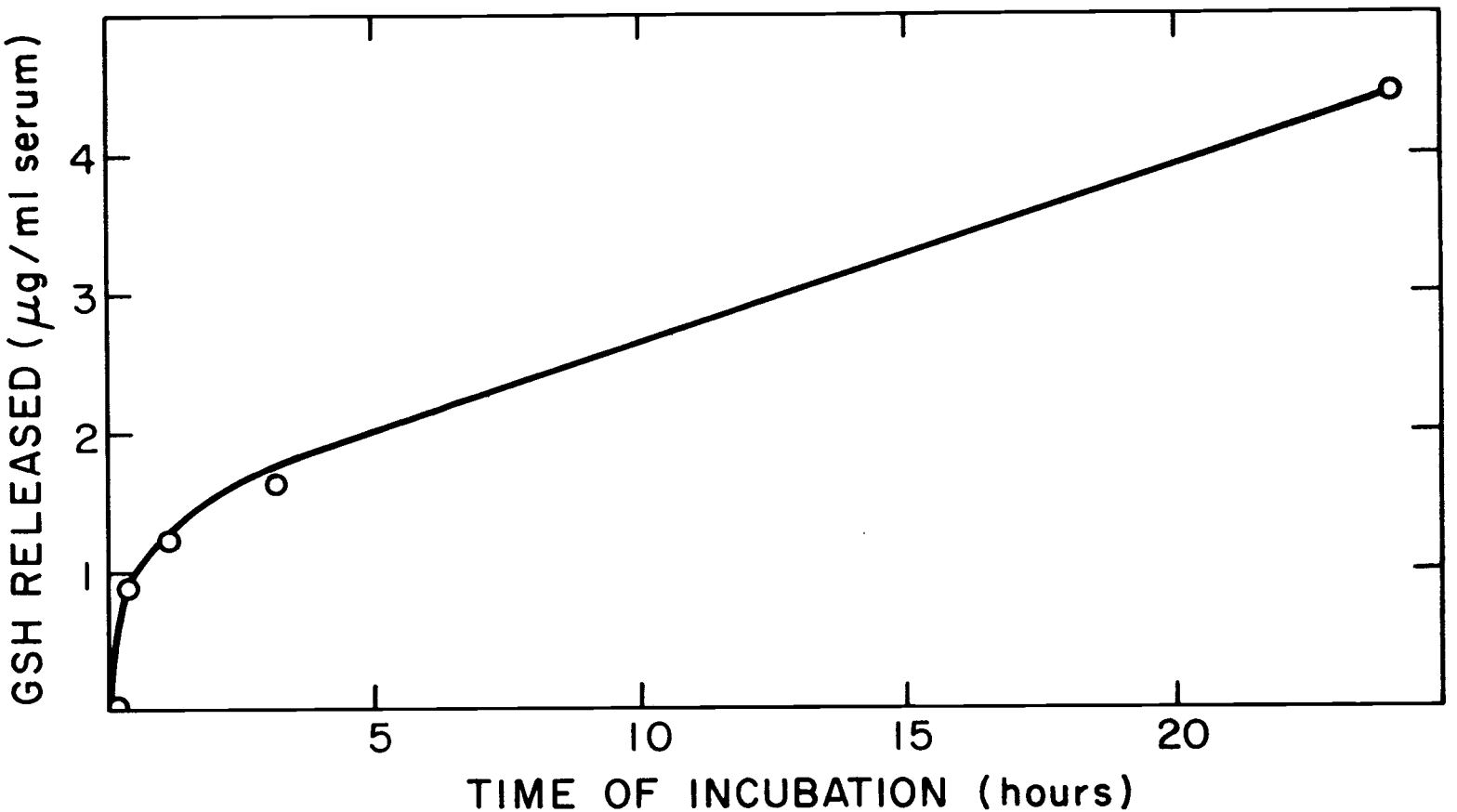


Figure 28. Mixed disulfide formation by incubation of fetal calf serum with GSSG. Fetal calf serum was incubated with 1 mg/ml GSSG in phosphate buffer, pH 7.5, at 37°C. After various periods of incubation the mixtures were filtered through a short Sephadex G-25 column, and then dialyzed against phosphate buffer, at pH 7.5. Mixed disulfides were determined by the NBS^- procedure (page 71).

Figure 26 shows the relative amounts of cysteine and glutathione (about 4x more cysteine than glutathione) found upon reduction of fetal calf serum mixed disulfides, as detected with ^{14}C-N-ethylmaleimide (page 62). Sephadex G-200 fractionation of fetal calf serum (Figure 27) showed mixed disulfide activity to be confined to one molecular weight region. Samples of partially purified fetuin supplied by Dr. Katherine Sanford (NCI, NIH, Bethesda, MD.) contained appreciable levels of mixed disulfide, but so did fetal calf serum fractions which were free of fetuin.

Mixed Disulfide Synthesis

Incubation of fetal calf serum with 1 mg/ml GSSG at 50°C for 24 hr., followed by filtration through Sephadex G-25 to remove GSSG resulted in a serum containing about seven times more mixed disulfide than normal. This method could be useful in studying both the chemistry of mixed disulfides and their biological properties. The kinetics of mixed disulfide formation at 37°C are seen in Figure 28.

DISCUSSION

Glutathione Oxidation by Diamide

Partial oxidation of the cellular glutathione pool in L-5178Y lymphoma cells by hollow fiber perfusion with diamide resulted in inhibition of protein synthesis (Table 15) and prolongation of survival (Figure 25) of DBA/2J mice implanted with treated cells, without severely affecting cell viability.

The level of diamide required to achieve a minimal effect on the GSSG/GSH ratio in this system was sufficient to cause a turnover of the entire cellular content of glutathione every minute. This is consistent with the observation of Biaglow and Nygaard (17) regarding the non-mitochondrial reducing capacity of Ehrlich ascites cells. In their experiments, washed cells, resuspended in phosphate buffered saline, were incubated with enough diamide to oxidize five times the cellular content of GSH, but no effect could be seen on the redox state of glutathione after just two minutes incubation. A 10X molar excess of diamide to GSH (note that one mole of diamide oxidizes two moles of GSH) was required to achieve 50% oxidation of GSH, when observed after two minutes incubation, and full recovery was again seen in just eight minutes.

The high reductive capacity of these cells may account for the observation of Harris, et al. (81) that a 2:1 molar excess (diamide to GSH) was required for complete oxidation of GSH when washed Ehrlich ascites cells were incubated for 3-5 minutes at room temperature

following addition of diamide at 0°C. The observed stoichiometry was attributed to non-specific reactions of diamide, since sonicated cells, or cells starved by one hour incubation in glucose-free buffered saline, still required the same excess of diamide for complete oxidation of glutathione. It is not clear that this conclusion is valid.

The reductive capacity is probably limited by the activity of glucose-6-phosphate (G-6-P) dehydrogenase, since most of the reducing equivalents utilized in the reduction of GSSG by glutathione reductase are derived from NADPH supplied by the hexose monophosphate shunt. Veech, et al. (220) find the ratio of G-6-P dehydrogenase activity to glutathione reductase activity in rat liver to be approximately 1:5. However, GSSG can activate G-6-P dehydrogenase (46), and the reducing capacity may be increased during prolonged oxidative challenge. Under normal conditions, the redox state of the $NADPH/NADP^+$ couple is several orders of magnitude more reducing than that of the glutathione couple (220), insuring a quick response to oxidative challenge. However, since the cellular content of NADPH (220) is only 1/100 of the cellular content of glutathione, the capacity to respond to an extended oxidative challenge is dependent upon the steady state flux capability, which is a function of nutrient availability as well as enzymatic activity.

The teleological importance of maintaining a proper glutathione redox potential is further suggested by the existence of a mechanism for the unidirectional active transport of GSSG toward the outside of the cell (202). Rat liver perfusion with a bicarbonate-buffered saline

solution of t-butyl hydroperoxide, which is claimed to be a specific reagent for enzymic glutathione oxidation, via glutathione peroxidase, resulted in oxidation of glutathione at a steady-state rate of 1.5 μmoles per gram wet weight per minute (assuming a 1:1 stoichiometry), which is comparable to that observed in the hollow fiber experiments reported here. This treatment (190) resulted in the loss of 75% of the cellular GSH content to the perfusate over a course of 20 minutes. Discontinuation of the treatment resulted in discontinuation of GSSG efflux. It is interesting to note that during the hollow fiber perfusion experiments with diamide no decrease in total glutathione levels was seen except in the extreme case where GSH was 100% oxidized. It may be that a rather high GSSG level is required to activate this transport system in L-5178Y cells. Or it may be that the phenomenon relates to the involvement of glutathione peroxidase in the mechanism of oxidation.

The inhibition of protein synthesis by hollow fiber perfusion with diamide at a %GSSG value as low as 2.5% is in agreement with the observation of Kosower, et al. (120) that as little as 5% GSSG can cause 80% inhibition of protein synthesis in rabbit reticulocyte lysate, at a total glutathione level in the physiological range. Evidence of redox control of protein synthesis has been outlined in the introduction. Several mechanisms appear to be involved. High GSSG/GSH ratios cause a reversible inhibition of translation (238). Low GSSG/GSH

ratios do not affect translation but inhibit initiation, and can cause a time-dependent dissagregation of polysomes to monosomes which is not readily reversible (121). This could explain the failure of protein synthesis to recover after removing the hollow fiber perfusion challenge with diamide and after partial recovery of the redox state. Since thiols are involved in the binding of ribosomal subunits to each other (148) and to the membrane (145) it is conceivable that mixed disulfide formation with ribosomal protein thiols could interfere with this binding. Mixed disulfide formation was observed to be correlated with loss of t-RNA binding capacity in GSSG treated ribosomes (62). It would be interesting to measure mixed disulfide levels as a function of %GSSG, and because of the extensive work that has been done with ribosomal proteins it may be feasible to isolate a particular protein for characterization of its redox state in relation to the redox state of glutathione.

Diamide has been found to inhibit amino acid accumulation in rat kidney cortex slices (84). Tissues were preincubated with enough diamide to oxidize completely intracellular glutathione and then were allowed a 15 min. recovery period at 25°C before addition of ^{14}C-amino acids in Krebs-Ringer bicarbonate buffer, at 37°C. The ratio of intracellular to extracellular amino acid concentrations was determined at various time intervals. A 50% decrease in the distribution ratios was seen following diamide treatment. Since

γ-amino-isobutyric acid transport was inhibited also, and it is not a substrate for γ-glutamyl transpeptidase, it was concluded that the effect was not due to a decrease in the activity of the γ-glutamyl cycle proposed by Orlowski and Meister (157) for amino acid transport. They attributed the effect to indirect oxidation of membrane protein thiols. All indications are that protein thiol oxidation is a longer-lasting effect than glutathione oxidation, which is in line with the proposed mechanism. However, in the hollow fiber perfusion experiment, there was no evidence of deficiency in amino acid transport, since treated cells accumulated ^{14}C-leucine with the same efficiency as control cells, and the intracellular specific activity of leucine was the same in both cases, when observed after 20 min. incubation at 37°C. It may be that a high GSSG/GSH ratio is required for this effect, or that kidney cells possess a different transport mechanism than L-5178Y cells.

Oxidation of glutathione presents a considerable metabolic challenge, and the question of cell viability following treatment with diamide must be dealt with. Many investigators have been satisfied with the ability to regenerate GSH, as an indicator of cell viability. Harris, et al. (81) have found, however, that complete oxidation of glutathione with diamide in the absence of glucose for a period in excess of ten minutes can lead to a loss of the ability of Ehrlich ascites cells to exclude vital dye, and to morphological changes

characterized by blebbing. Decreased plating efficiency was also seen. The blebbing observed may be a reflection of membrane fragility, which, as noted by Kosower, et al. (118), occurs when erythrocyte glutathione is completely oxidized with methyl phenyldiazene carboxylate. In the latter case, the mechanism appeared to involve free radical attack on the membrane, in the absence of the protective effect of the glutathione peroxidase system or the free radical scavenging ability of GSH. Nevertheless, free radicals arise continuously as a consequence of normal cell activity (29), and it may not be necessary to have an external challenge.

Some toxicity at higher diamide levels was noted in the hollow fiber perfusion experiments. When glutathione was completely oxidized for the entire period of incubation, a decrease in packed cell volume and cell count was seen, and vital staining indicated almost total loss of viability, although tumors were produced when the cells were transplanted into mice. Some decrease in oxygen consumption was noted at the higher diamide levels, even without complete oxidation of GSH, although this could be due to the uncoupling of oxidative phosphorylation by thiol oxidation reported by Siliprandi, et al. (191). They found that rat liver mitochondria treated with diamide exhibited a decreased respiratory control index, accompanied with increased ATPase activity and a failure to accumulate Ca^{++}. All these effects were reversed with dithioerythreitol, demonstrating the mechanism

involved a redox change, and no permanent damage. Similar observations were reported by Kaback, et al. (101) in relation to the mechanism of action of the uncoupling agent, carbonyl m-chlorophenylhydrazone.

Cell viability was not significantly affected by partial oxidation of the cellular glutathione pool in L-5178Y cells by hollow fiber perfusion with diamide (Table 16). Values for trypan blue exclusion, lactate production and oxygen uptake were at least 50% of control values. This is in agreement with the observation of Harris, et al. that partial or transient complete oxidation, which resulted in decreased rates of protein and nucleic acid synthesis, did not affect viability to a significant extent (81). They did observe, however, that growth curves for treated cells were not as steep as those of controls, indicating that although the cells remain viable, they reproduce more slowly. This is in agreement with the transplantability data reported in this thesis.

The validity of the trypan blue exclusion test as an indicator of cell viability has been tested by several authors (213). Tennant (213) compared vital staining values with in vitro and in vivo cultivability, with various cell types. The values agreed within 15% of each other for suspension cultures. Correlation was not as good when cells had to be mechanically dispersed from solid tissues or monolayer cultures. The method should be applicable to the present situation

according to these criteria. It should also be pointed out that as in the case of X-ray irradiated cells, trypan blue staining allows the determination of viability even when the cells are incapable of division, and would not be scored as viable by the plating efficiency method. The one hour recovery period prior to transplantation and viability determination allows the manifestation of latent toxicity, and cell survival after that period of time is a good indication of long-term viability.

The ability of L 5178Y lymphoma cells to produce large amounts of lactate, even under aerobic conditions, makes it possible to monitor lactate in the hollow fiber perfusate during diamide treatment. Lactate production from glucose is 1.5 μmoles/mg protein/hr under aerobic conditions, compared with 2.5 μmoles/mg protein/hr under anaerobic conditions, and accounts for as much as 90% of the pyruvate that is produced (69). Although the rate of lactate production at any given time is of limited diagnostic value, the ability of the cells to maintain a constant rate over a prolonged period of incubation is evidence that their viability is not impaired during this period. In combination with oxygen uptake measurements and vital staining, this evidence provides a fairly accurate picture of the metabolic state of the cells during and following diamide treatment.

Apffel and Walker (8) have found that Ehrlich ascites tumor cells treated with low levels of the thiol blocking reagents iodoacetate and

N-ethylmaleimide, lost transplantability, while retaining viability, as evidenced by vital stain exclusion and oxygen uptake. Furthermore, they observed that thiols could protect against the effect, and thiols could reverse the loss of transplantability. These observations closely parallel the results presented in this thesis. However, the mechanism of inhibition is unclear. The high reducing capacity of these cells would preclude a direct oxidative challenge by depletion of reduced glutathione, and would suggest an efficient redox buffering system should still be possible at GSH levels well below normal. Alkylation of essential protein thiols would offer a mechanism which could account for the efficacy of low levels of thiol reagents in achieving a long term effect. However, the reversal of the effects with reduced thiols suggests a redox mechanism. Apffel and Walker observed decreased activity of glutathione reductase and other disulfide reductases following treatment with NEM and iodoacetate. This could be a direct effect of alkylation and could also have a secondary effect on the redox state.

Effects of Procarbazine on L-5178Y Cells

The results presented in this thesis demonstrate that procarbazine treatment in vivo leads to oxidation of intracellular glutathione in L-5178Y lymphoma cells (Table 7), carried as ascites tumors in DBA/2J mice. It was further demonstrated by the hollow fiber

perfusion experiments with diamide (Table 15) that the extent of glutathione oxidation that is seen with procarbazine treatment is sufficient to account for the magnitude of inhibition of protein synthesis observed, as reported by Sartorelli and Tsunamura (184) and Gutterman, et al. (73). Although effects on the redox state were only measured during the first few hours treatment with procarbazine, the extended inhibition of protein synthesis that is observed could be accounted for as a long term consequence of GSH oxidation, since recovery of protein synthesis does not immediately follow recovery of the redox state after prolonged oxidative challenge, as discussed earlier.

Inhibition of nucleic acid synthesis by glutathione oxidation has been observed (239), and mitotic arrest can be caused by the GSH oxidizing reagent, diamide (81). So glutathione oxidation could account for the cytotoxic manifestations of procarbazine treatment. However, the mechanism of action of procarbazine could involve a number of cytochemical lesions, and it would be difficult to single out any one of them as the primary and essential mediator of carcinostatic activity.

Methane formation in vivo (168) following treatment with procarbazine is evidence of the formation of methyl radicals in its metabolism. The selectivity of methyl radicals (163) suggests they would attack predominantly thiol groups, and because of the high

cellular concentration of glutathione, it would be a primary target. Other radicals which may be formed during procarbazine metabolism include the methyldiazene radical, which Huang and Kosower (90) have demonstrated to result from reaction of methyl diazene with molecular oxygen, with the concommittant generation of a superoxide anion radical:

$$CH_3N{=}N\text{-}H + O_2 \rightleftharpoons CH_3N{=}N\cdot + HOO\cdot$$

$$HOO\cdot \rightleftharpoons H^+ + O_2^-\cdot$$

Nicolau and Dertinger (145) have examined the mechanism of reaction of thiols and disulfides with various organic free radicals. Protection of cellular components can be achieved either by direct reaction between GSH and the free radical or by repair of the cellular component attacked by a free radical:

$$RSH + R'\cdot \rightleftharpoons RS\cdot + R'H$$

$$RS\cdot + RS\cdot \rightleftharpoons RSSR$$

This mechanism could lead to mixed disulfide formation at a rate not much lower than that of GSSG production, since PSH and GSH are present in the cell at the same order or magnitude of concentration. However, the reductive capacity with respect to mixed disulfides has not been examined, although the enzymic thiol-disulfide interchange between GSH and PSSG has been described (215). It is possible that there is a lag in reduction of mixed disulfides even when

the glutathione redox state is maintained at control levels. During prolonged challenge, this could lead to an accumulation of mixed disulfides and secondary effects, such as polysome disaggregation, which might only be slowly reversible.

Inhibition of protein synthesis by procarbazine, reported in this thesis, occurs after as little as one hour treatment in vivo. Sartorelli and Tsunamura (184) did not see inhibition until three hours after administration of procarbazine. The present report shows that under the conditions used by Sartorelli and Tsunamura, the kinetics of leucine incorporation are not linear, and are affected by procarbazine treatment. Inhibition was apparent when higher leucine levels or shorter incorporation periods were used. The validity of the data reported here is established by determination of intracellular specific activity of leucine, and by kinetic studies. The observation that inhibition of protein synthesis occurs as early as one hour after administration of procarbazine is consistent with the kinetics of GSH oxidation and the observed inhibition of protein synthesis by diamide.

Inhibition of the rate of ^{14}C-leucine incorporation into protein by diethylmaleate (Figure 12) was also seen when high leucine levels were utilized, although low leucine level incorporation studies gave no indication of inhibition. Diethylmaleate had appeared to be a promising reagent for decreasing total glutathione levels in vivo over a prolonged period of time (31). The enzymatic conjugation of

diethylmaleate with glutathione (21) presented the possibility of specificity, and the transport and reaction rate observed allowed a limited continuous challenge. However, the present results raise serious questions about the usefulness of the reagent, since it does result in cytotoxic manifestations which are not easily related to a simple decrease in total glutathione levels. A slight increase in the GSSG/GSH ratio was also observed. The most feasible mechanism for inhibition of protein synthesis would be direct alkylation of protein thiols, and this possibility should be examined before the reagent can be considered specific for glutathione.

Determination of Leucine Specific Activity

The need for determination of the specific activity of radioactive precursors in incorporation studies designed to measure the rate of macromolecular synthesis is underscored by the results presented in this thesis. A method is also provided, which allows the simple and accurate determination of the specific activity of leucine or valine, and possibly other amino acids, with as high a sensitivity as desired.

High performance liquid chromatography allows the rapid separation of MeDNP-amino acids, compared to conventional silica column chromatography (106) or TLC (170) of the DNP-amino acids, and affords a better resolution of the leucine and isoleucine derivatives,

which is also a problem in the liquid chromatographic separation of the PTH-amino acids (67). The reaction with radioactive FDNB allows the determination to be made from the isolated derivative with no need for quantitative recovery, as would be required with the free amino acids.

The kinetics of amino acid uptake by L-5178Y lymphoma cells grown as ascites cultures in DBA/2J mice are such that the intracellular specific activity rises quickly (within 2 min.) to the extracellular values, and then slowly declines over the period of incorporation. Under these conditions, it is the intracellular specific activity that would best reflect the specific activity of the pool utilized for protein synthesis, since the extracellular fluid would be slow to reflect changes resulting from synthesis and proteolysis that would quickly be equilibrated at the sites of protein synthesis.

The source of amino acids utilized for protein synthesis has been the subject of considerable controversy. In systems which are slow to equilibrate it appears that the extracellular specific activity may resemble more closely that of the amino acids actually incorporated (218). The only way to be certain, if there is a significant difference between the extracellular and intracellular specific activities, is to measure the specific activities of the t-RNA bound amino acids or nascent peptides (92).

Tissue Culture in Hollow Fiber Devices

The system described in this thesis is unique in that it allows the growth of cells at a high density, while keeping them in suspension, insuring an even distribution of substances in the perfusion medium to all cells, and allowing repeated sampling of the cell suspension during perfusion. The potential of hollow fiber devices for cell culture has been explored (187), but the trend has been toward establishing cultures with tissue-like density, where cells actually become attached to the fibers, and these act as capillaries would in vivo. Certainly there is a need for such a system as well.

Cell viability did not seem to be affected by incubation in the Biofiber^{R} 50 minbeakers for as long as five hours. Some damage might result from continued stirring with a magnetic bar, but a shaking mechanism should work just as well. The resistance of the apparatus to 70% ethanol allows a convenient method of sterilization, and it should be possible to maintain sterility during prolonged incubation. The system is particularly appropriate for studying the effects of drugs which react too quickly to allow permeation through layers of cells, and may prove useful in the detection of metabolites.

Glutathione Oxidation and Neoplasia

The role of thiols in cell division and neoplasia has long been theorized. Known carcinogens have been observed to affect both thiol levels (77) and enzymes involved in glutathione metabolism (55, 160). Specific thiol reagents have been found to have antitumor activity and have been used in the clinical treatment of cancer (112).

It is interesting to note that hydrogen peroxide, which has been used as a reagent for the intracellular oxidation of glutathione, has been found to have antitumor activity (123), and iodine, which could also act by oxidizing glutathione, is also effective in tumor inhibition (221). A two-fold increase in the activity of glutathione reductase was seen with diethylnitrosamine treatment of rat liver (160). Glutathione peroxidase activity was decreased by the treatment, resulting in a situation favoring glutathione reduction. This and other evidence in the literature led Pinto and Bartley (160) to suggest:

> The consequence of this (treatment) might be a change of thiol disulfide ratio in the cells to a highly reduced state which might favour the increase in cell division. It appears that a rise in the concentration of any acid-soluble thiol is a pre-requisite for mitosis and therefore oxidoreduction of glutathione and/or degradation of such a thiol may be in part responsible for proliferation.

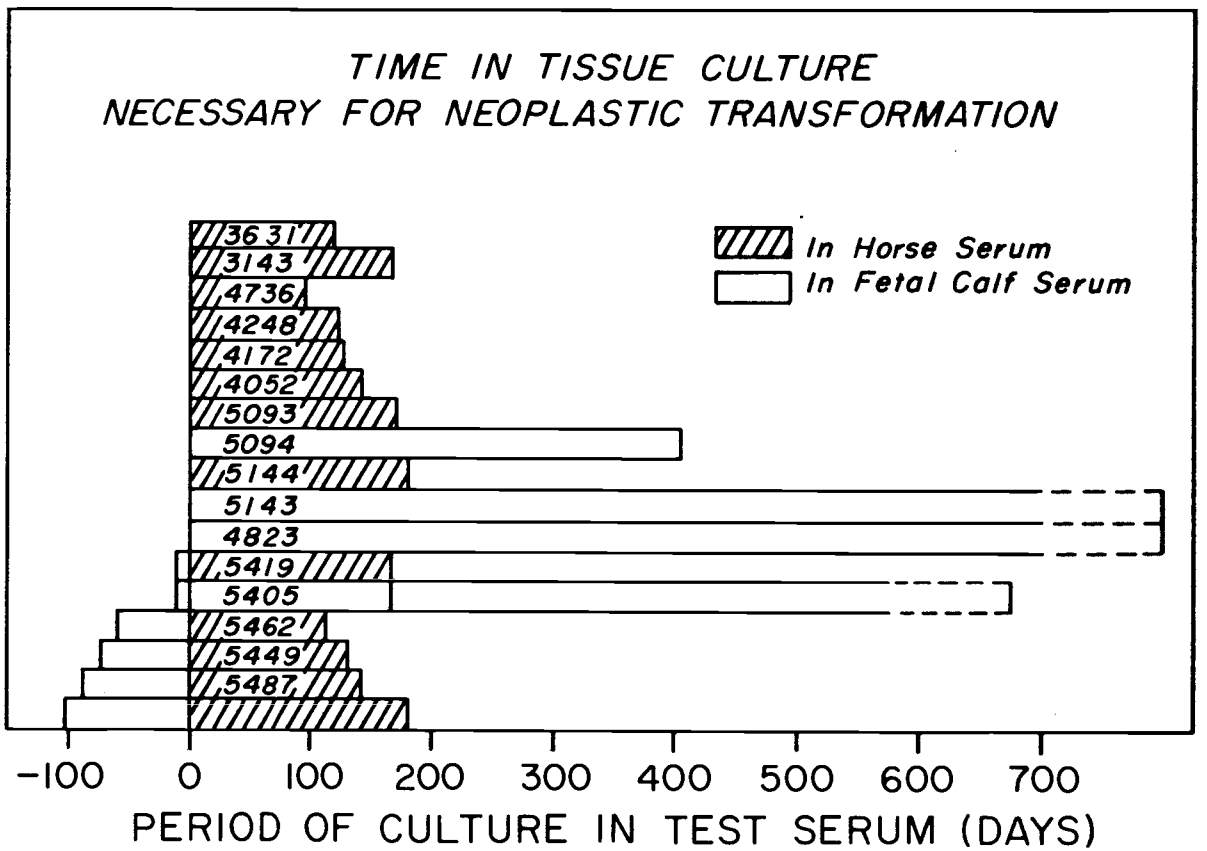
Much remains to be understood about the neoplastic state. A number of mechanisms, ranging from electronic phenomena (209) and free radical involvement (159) to membrane changes (38, 44) and

virus infection (212) have been implicated. It would be premature to conclude that any one mechanism could be responsible. But in the light of the present investigations and the mounting evidence in the literature, the involvement of thiols and disulfides, and particularly the glutathione redox potential, is well deserving of further investigation.

Mixed Disulfides in Fetal Calf Serum

Fetal calf serum has been reported to inhibit "spontaneous" neoplastic transformation *in vitro*. No other serum has been found to have this effect. The present study indicates that fetal calf serum is also unique in containing high levels of protein-glutathione mixed disulfides (Table 17).

Evans and Andresen (51) found that cell lines initiated from minced mouse embryos or mouse kidneys, and grown on NCTC 135 chemically defined medium supplemented with 10% gelding horse serum, invariably became neoplastic after 100-200 days in tissue culture (Figure 29). Cell lines grown on the same medium, supplemented instead with 10% fetal calf serum, did not become neoplastic within that length of time, and most of these cell lines remained untransformed at the time of publication, after as much as 777 days in culture. This difference is particularly striking when you consider three paired sets (cell lines 5143 & 5144; 5093 & 5094 and 5419 &



Evans and Andresen, 1966 & 1969; Parshad and Sanford, 1968

Figure 29. Time in tissue culture necessary for neoplastic transformation. Data were taken from references 7, 51 and 158. Mouse embryo or mouse kidney cells were grown on media supplemented with either horse serum or fetal bovine serum. Neoplastic transformation was detected by intraocular transplantation. Bars to the left indicate the period of time cells were grown on FBS-supplemented medium before transfer to the test serum. Dashed lines indicate cell lines not transformed at the time of publication.

5405 in Figure 29), in which cell lines initiated in chemically defined media were split into horse serum and fetal calf serum supplemented media (6, 51, 136, 158).

Calf serum, adult bovine serum, fetal horse serum and other sera, do not inhibit this "spontaneous" neoplastic transformation (47, 149). Neoplastic transformation has also been observed in chemically defined medium (6). This and other evidence has lead to the conclusion that there is an agent in the fetal calf serum that is protective, rather than there being cytotoxic agents in other media, and the protective effect of fetal calf serum appears to involve its macromolecular fraction (183).

The presence of protein-glutathione mixed disulfides in fetal calf serum may be a handle to further exploration of its unique protective qualities. It would be interesting, for instance, to examine the effect of reduced fetal calf serum, or fetal calf serum from a lot which is for some reason deficient in mixed disulfide, on "spontaneous" neoplastic transformation. Or, on the other hand, the effect of horse serum which had been treated with GSSG to contain high levels of mixed disulfide.

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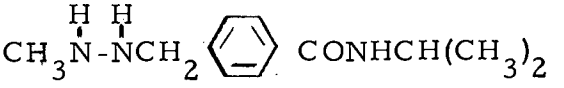
APPENDICES

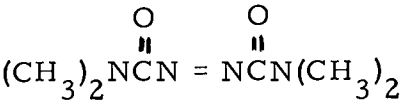
APPENDIX I

STRUCTURES OF COMPOUNDS OF INTEREST

PROCARBAZINE:



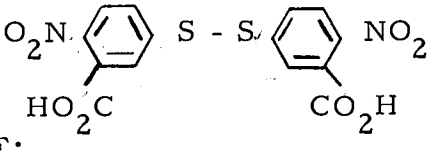
DIAMIDE:



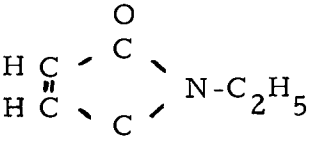
GLUTATHIONE:

γ-glutamyl-cysteinyl-glycine

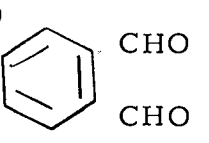
DTNB:



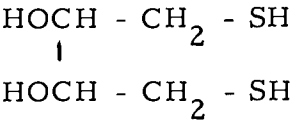
N-ETHYLMALEIMIDE:



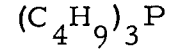
O-PHTHALALDEHYDE:



DITHIOTHREITOL:



TRIBUTYLPHOSPHINE:



APPENDIX II

PROGRAM FOR CALCULATION OF LEUCINE SPECIFIC ACTIVITY

MeDNP-leucine samples, obtained as described in the methods section, were counted in the $^{3}H/^{14}C$ mode, and the information relayed to an HP 9821A calculator. The program allows data to be entered by punch tape, or manually, through the calculator keyboard. Data can be analyzed on the basis of external or internal standard, or, having established the proper constants, on the basis of total DNP content, as indicated by the ^{3}H and ^{14}C counts and the specific activity of the FDNB used (Figure 30).

The following constants were calculated for the system utilizing 6 g/l PPO and 50 mg/l POPOP in toluene:

R19: Difference in efficiency in ^{3}H channel/DPM ^{3}H: 0.00000024

R20: Difference in efficiency in ^{14}C channel/DPM ^{14}C: 0.00000040

R21: Difference in efficiency in ^{3}H channel/DPM ^{14}C: 0.00000010

R22: Efficiency for ^{3}H in ^{3}H channel: 0.376

R23: Efficiency for ^{14}C in ^{14}C channel: 0.576

R24: Efficiency for ^{14}C in ^{3}H channel: 0.121

R30: Least squares fit constant A: 17

R31: Least squares fit constant B: -41

R32: Least squares fit constant C: 33

R33: Least squares fit constant D: - 8

A sample printout can be seen in Figure 31.

Figure 30. **Program for calculation of leucine specific activity from $^{3}H/^{14}C$ data using an HP 9821A calculator**

```
0:
0→A;FMT "ANALYSI
S OF CPM",/;WRT
2;ENT "DATE",R0;
FMT "DATE",2X,
FXD 9.4;WRT 2,R0
⊢
1:
ENT "BKG H-3",R1
;ENT "BKG C-14",
R2;FMT "BKG H-3"
,FXD 6.0,5X,"BKG
 C14",FXD 6.0⊢
2:
WRT 2,R1,R2;ENT
"UCURRIE/MM FDNB
",R27;FMT "SP AC
T FDNB",FXD 9.4;
WRT 2,R27⊢
3:
ENT "LAST VIAL",
R3;DSP "ENTER IF
 STATMNT";DSP ;
DSP ;DSP ;DSP ;
DSP ;DSP ;DSP ;
DSP ;DSP ;DSP ⊢
4:
FMT 1,/,FXD 3.0,
2;FMT 2,FXD 7.0,
2;FMT 3,FXD 7.4,
[illegible]
5:
ENT "4",R4;ENT "
5",R5;ENT "6",R6
;ENT "7",R7;ENT
[illegible],R8;ENT "9",R
[illegible] →11, →20"
[illegible] ⊢
[illegible]
FMT [illegible] NO.",2X,"
[illegible] 2X,"C C14
[illegible] "E H-3",
[illegible] 2X,[illegible]
[illegible]⊢
[illegible]
[illegible] FMT 2X [illegible]
[illegible] UC/M [illegible]
[illegible] UC",2[illegible]
[illegible] WRT 2[illegible]
```

```
8:
RTN 1,R10,R11,R1
[illegible],R13,R14,R15,R1
[illegible],R17,R18;IF R11
>[illegible];SFG 4⊢
9:
RDB 1→Z;RDB 1→Z;
RDB 1→Z;RDB 1→Z;
RDB 1→Z;RDB 1→Z;
RDB 1→Z⊢
10:
RDB 1→Z;RDB 1→Z;
RDB 1→Z;IF Z≠141
;FMT /,"ERROR",Z
;WRT 2;WRT 2.3,R
10,R12,R14,Z⊢
11:
IF R11=R4;FMT 2/
,"EXP 3.0",/;
WRT 2;GTO 6⊢
12:
IF R11=R5;FMT 2/
,[illegible] 2 L",/;WRT 2
;GTO 6⊢
[illegible]
[illegible]11=R6;FMT 2/
,[illegible] 2 L",/;WRT 2
;GTO 6⊢
14:
IF R11=R7;FMT 2/
,"[illegible].025 MG VALIN
E [illegible]10 MIN COUNTS
",/;WRT 2;GTO 6⊢
[illegible]
[illegible]R11=R8;FMT 2/
[illegible];WRT 2;
GTO 6⊢
16:
IF R11=R9;FMT [illegible]
[illegible]/;WRT 2;
GTO 6⊢
[illegible]
[illegible] R11[illegible]
[illegible] R18;[illegible]
[illegible] R19-R[illegible]
[illegible] FLG 1=1[illegible]
[illegible]
[illegible]
[illegible] 2=1;GTO 3
```

```
19:
IF FLG 2=1;GTO 4
6⊢
20:
PRT "SET FLGS","
1=[illegible]DMP AA]","2=I
NT STD","3=EXT S
TD","5=NO TAPE";
STP ⊢
21:
IF FLG 1=0;GTO 2
5⊢
22:
ENT "DIFF E H/DP
M",R19;ENT "DIFF
 E C/DPM",R20;
ENT "IN H CH",R2
1⊢
23:
ENT "E H-3",R22;
ENT "E C14",R23;
ENT "IN H CH",R2
4⊢
24:
FMT FXD 9.8;WRT
2,R19,R20,R21,R2
2,R23,R24⊢
25:
IF FLG 2=1;ENT "
DPM STD H-3",R25
;ENT "DPM STD C1
4",R26⊢
26:
IF FLG 2=1;FMT "
[illegible] STD";WRT 2;
WRT 2.2,R25,R26,
[illegible];IF FLG 3=0;
[illegible] 51⊢
[illegible]
[illegible] EFF H EFF C
[illegible];ENT "EFF C
[illegible] EFF C",R29
[illegible]",R30⊢
[illegible]
[illegible]",R31;ENT
[illegible]R32;ENT [illegible]",
[illegible]FMT [illegible] STD
[illegible] 2⊢
[illegible]
[illegible],R28,R29,
[illegible],R32,R33;
[illegible] 51⊢
```

```
30:
R22→R34;R17/R34→
R35⊢
31:
R35*R20→R36;R23-
R36→R37;R35*R21→
R38;R24-R38→R39⊢
32:
R18/R37→R40;R40*
R39→R41;R17-R41→
R42;R42/R34→R43⊢
33:
R43*R19→R44;R22-
R44→R45⊢
34:
1+A→A;IF A>15;
FMT 5X,"I CAN'T
DO IT",Z;WRT 2;0
→A;GTO 51⊢
35:
R34-R45→R46;IF R
46>.0001;R45→R34
;R43→R35;GTO 31⊢
36:
IF R46<-.0001;R4
5→R34;R43→R35;
GTO 31⊢
37:
WRT 2.3,R45,R37;
WRT 2.2,R43,R40;
R43/2220/R27→R41
;R40/2220→R42⊢
38:
R42/R41→R43;1/R4
3→R44;WRT 2.2,R4
3;WRT 2.3,R44;
IF FLG 2=0;GTO 4
6⊢
39:
FMT 4,"SPK ",Z;
WRT 2;ENT "SPK H
-3",R34;ENT "SPK
 C14,H3",R35⊢
40:
ENT "SPK C14,C14
",R36;WRT 2.2,R3
4,R36⊢
41:
R35-R34→R35;R36-
R18→R36;R34-R18→
R34⊢
42:
R34/R25→R34;R35/
R26→R35;R36/R26→
R36;R18/R36→R37;
R37*R35→R38⊢
43:
R17-R38→R39;R39/
R34→R39;WRT 2.3,
R34,R36;WRT 2.2,
R39,R37⊢
44:
R39/2220/R27→R40
;R37/2220→R41;R4
1/R40→R42;1/R42→
R43⊢
45:
WRT 2.2,R42;WRT
2.3,R43,R40;IF
FLG 2=0;GTO 51⊢
46:
IF FLG 4=1;PRT "
LAST VIAL";STP ⊢
47:
FMT 2,"AES",Z;
WRT 2;WRT 2.2,A,
0;R20→R10;3;R31→
R10;2,R32*R10→R3
3→R34⊢
48:
R18/R34→R35;R35(
R29*R34)→R36;(R1
7-R36)/(R28*R34)
→R37⊢
49:
R34/2220/R27→R38
;R37/2220→R39;R3
9/R38→R40;WRT 2.
3,R28*R34,R34⊢
50:
1/R40→R41;WRT 2.
2,R37,R35,R40;
WRT 2.3,R41,R10;
IF FLG 4=1;PRT "
LAST VIAL";STP ⊢
51:
IF FLG 5=1;GTO 5
3⊢
52:
GTO 8⊢
53:
ENT "NO.",R11;
ENT "C H-3",R17;
ENT "C C14",R18;
ENT "AES",R10;
IF R11=R3;SFG 4⊢
54:
GTO 11⊢
55:
END ⊢
123092
R33
```

Figure 31. Sample printout of specific activity data. Left to right: counts, efficiency, DPM, specific activity. See Figure 30 for program.

C 2 V, + 0.025 LEU

NO.	C H-3	C C14	E H-3	E C14	D H-3	D C14	UC/MM	MM/UC
228	8260	775	.3713	.5674	21747	1334	43	.0232
AES	0	0	.3722	.5694	21697	1330	43	.0232
229	3304	287	.3744	.5726	8606	470	38	.0261
AES	0	0	.3795	.5807	8490	463	38	.0261