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Jonas Antanas Gylys
*Loyola University Chicago*

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# THE EFFECT OF SOME AROMATIC SULFUR COMPOUNDS ON THE GROWTH OF THE WALKER 256 TUMOR

by

Jonas Antanas Gylys

A Dissertation Submitted to the Faculty of the Graduate School
of Loyola University in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy

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1957

## LIFE

Jonas Antanas Gylys was born in Kaunas, Lithuania, on June 27, 1928.

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Biochemorphological Effects of N-Allyl Substitution on Barbiturate Compounds, Trans. Illinois Academy of Sciences, Vol. 48: 97-107, 1956.

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# CHAPTER I

## INTRODUCTION AND STATEMENT OF PROBLEM

Two new compounds with structures somewhat analogous to the well known bacteriostatic sulfonamide compounds have been prepared (see Figure 1, Table I). The first compound - TSN (para-thiolbenzenesulfonamide) is characterized by the substitution of the sulfhydryl group for the para amino radical on the benzene ring of the p-aminobenzenesulfonamide molecule. The second compound - TSN-TSN (4:4'-sulfonamidediphenyldisulfide) has two TSN molecules linked by the disulfide bond in the para position to the sulfonamide radicals. It appeared of interest to determine whether or not these compounds might exert cancerostatic effects on the experimental animal tumor - Walker 256 carcinosarcoma for reasons outlined in the following paragraphs.

Reimann and Hall (1936) demonstrated that topical application of para-thiocresol would reduce the incidence and size of skin cancer induced by 1:2:5:6-dibenzanthracene. Structurally para-thiocresol and TSN have a thiophenolic nucleus in common. The difference between the two structures lies in the nature of the para substituents of the two compounds. It was felt that the exchange of a para sulfonamide substituent for the para methyl group present in thiocresol might yield a compound (TSN) with less systemic toxicity than thiocresol. If this resulted, some insight into the possible systemic effects of thiophenolic compounds on neoplastic growth might be obtained following parental administration. There is evidence which points

out that the substitution of the para-sulfonamide radical on aniline reduces the toxicity of the parent aniline molecule (Northey, 1948). Secondly, Boyland (1938) has indicated that sulfonilamide might possess some cancerostatic properties. Therefore, it was felt that the use of a parasulfonamide substituent on the thiophenolic nucleus might afford a compound relatively devoid of toxicity but with possible cancerostatic effect.

There is some evidence that aliphatic thioacids (thiomalate, thioglycolate) might stimulate the growth of the transplanted tumors in rats (Brunschwig et. al., 1946). Therefore, it seemed of interest to determine the analogous properties of a compound (TSN) which possesses the aromatic SH group.

Additional work by Boyland (1938) on aromatic sulfur compounds has revealed that the following compounds: 4:4'-dinitrodiphenylsulfoxide, 4:4'-diaminodiphenylsulfide, Na-sulfanylsulfanilate, 4:4'-dinitrodiphenylsulfone, and 4:4'-diaminodiphenylsulfoxide had cancerostatic effect on the spontaneous mouse carcinoma while the last three compounds were effective additionally against the Crocker sarcoma 180. The later work by Boyland (1946) indicated that 4:4'-diaminodiphenylether, benzidine (pp'-bianiline), 4:4'-diaminodiphenylmethane, 4:4'-diaminodiphenylsulfone, 2:4-dinitro-2'-aminodiphenylsulfide, and 2:2'-diaminodiphenyldisulfide exhibited cancerostatic properties against spontaneous mouse carcinoma but were without any significant effect on the transplanted tumor. While these studies suggested that the presence of the aromatic sulfur linkage between two phenyl radicals was not critical they nonetheless indicated that some activity might be present in compounds with

such sulfur groups. On a basis of these considerations it appeared of interest to test the cancerostatic properties of TSN-TSN which is characterized by the disulfide linkage between two phenyl radicals in the para position to the sulfonamide groups. It appeared probable that TSN-TSN might possess some cancerostatic properties on a basis of the substituted phenyl radicals. Secondly, it appeared of interest to ascertain whether or not the substitution of phenyl rings by the sulfonamide group (as opposed to $NO_2$ or $NH_2$) would have any effect on the chemotherapeutic properties of this type of compound.

In case the TSN compounds proved to posses cancerostatic properties some work will be done in elucidating their biochemical effects. This phase of the work will be based on the following assumptions: a) the action of TSN compounds might be due to an effect on some enzymatic reaction, b) since these compounds possess sulfur atom(s) as a structural component, their effect might be linked with the sulfur metabolism and possibly with that of cysteine or cystine, c) there is some evidence that some aliphatic thiolacids might stimulate the growth of the transplanted tumors (Brunschwig et. al., 1946) while analogous compounds would tend to inhibit the enzymes which catalyze the $H_2S$ release from cysteine (Lawrence and Smythe, 1943). Therefore, it appeared of some value to obtain information concerning the effects of TSN compounds on the analogous enzyme systems.

## CHAPTER II

## LITERATURE REVIEW

A. Chemotheraphy of Cancer with Compounds Related to TSN and TSN-TSN.

In 1936 Reimann and Hall studied the effect of topically applied parathiocresol on production and growth of tumors caused by painting the skin of albino mice with the carcinogenic hydrocarbon 1:2:5:6-dibenzanthracene. They found that the incidence of the tumors (both carcinomata and papillomata) caused by the application of the carcinogen was reduced by this use of para-thiocresol. The effect was most pronounced when the para-thiocresol was applied prior to the application of the carcinogen, the incidence and the size of both types of tumors being greatly reduced under this procedure. The limiting effect of the aromatic sulfhydryl agent when it was applied on alternate days with the carcinogen (with the latter applied first) was not too marked in terms of over-all tumor incidence, but the growth of the tumors which resulted seemed to be inhibited by the regimen. It is interesting to point out that the para-thiocresol treated skin showed more epithelial cells than the normal skin, but the various layers of epidermis were very clearly defined, and the basement membrane was sharply observable. No inflammatory reaction was observed in either the epidermis itself or in the underlying tissues. The 1:2:5:6-dibenzanthracene treated skin was thickened and showed relatively well defined layers, but nonetheless a distinct difference from normal skin was detected in the cellular organization. The cells of the basal

layer tended to be oriented less regularly, more variation in the shape and sise of the individual cells was observed and the basement membrane was poorly defined. These findings were explained by Reimann and Hall (1936) in the following manner: a stimulation of the rate of the cell proliferation such as that caused by para-thiocresol does not lead to neoplasia, while on the other hand the carcinogenesis, as caused by the carcinogen, induced damage to the cellular potencies of differentiation and organization. It is difficult to predict how a compound showing the para-thiocresol properties would act in cases of transplanted tumors. But if one would make an analogy with the findings in the chemically induced tumors one might say that such a compound helps the malignant cells to obtain a more advanced differentiation and more regular organization.

The author's search of the literature has failed to reveal any experimental data indicating that systemic administration of an aromatic mercaptan is capable of producing such an effect on a neoplasm. In the case of systemic administration of several aliphatic sulfhydryl compounds the results reported by Brunschwig et. al., (1946) would seem to indicate that these compounds not only exert no cancerostatic effect, but rather they stimulate growth of transplanted tumors in rats. A simultaneous administration of iodoacetate and maleate (mercaptide forming agents) would reverse the tumor growth stimulating effect of the thioacids. These findings indicate that the transplanted tumors appear to require SH groups for their development and in case where the aliphatic SH groups were provided an augmentation of tumor growth was produced. However, these findings do not preclude the

possibility that the aromatic SH groups may have an effect entirely different from that observed with aliphatic SH groups.

Boyland (1938) screened a number of aromatic compounds containing sulfur as a structural component against spontaneous tumors and transplanted Crocker sarcoma 180 in mice, of strong and dilute Brown strains. Their chemotherapeutic agents were administered orally at a daily dose which was approximately one fourth of the toxic dose. Four mice with spontaneous carcinoma and ten mice (5 treated, 5 controls) with transplanted sarcoma were used for each compound. The tumors were measured by caliper three times a week and the size of tumor was expressed as a sum of the length and breath.

The following compounds with one phenyl radical showed an inhibition (regression) of the spontaneous tumor. They are listed in the order of apparent efficiency : 1) para-aminobenzenesulfonamide, 2) Na-para-aminobenzenesulfonate, 3) Na-para-hydrazinobenzenesulfonate, 4) para-heptylaminobenzenesulfonate, 5) Na-ortho-aminobenzenesulfonate, 6) Na-meta-aminobenzensulfonate (no effect). Of these compounds only numbers 1 and 5 had any effect on the Crocker sarcoma 180.

The following aromatic sulfur compounds with two phenyl radicals showed cancerostatic effect on spontaneous mouse tumor (listed in the order of efficiency): 1) 4:4'-diaminodiphenylsulfoxide, 2) Na-sulfanilylsulfanilate, 3) 4:4'-diaminodiphenylsulfone, 4) 4:4'-dinitrodiphenylsulfoxide, 5) 4:4'-dinitrodiphenylsulfone, 6) 4:4'-diaminodiphenylsulfide, 7) 4:4'-dinitrodiphenylsulfide (not effective). Compounds 1,2,5, had some effect against transplanted sarcoma.

Some general conclusions which could be drawn from this work are as follows: a) the diphenyl compounds were more potent on a weight basis, b) the spontaneous carcinoma was more susceptible to chemotherapy, while only a few compounds showed any effect on transplanted sarcoma, c) the therapy did not produce any long lasting effect, because tumors grew after the therapy was stopped, d) the sulfoxides were more effective than either sulfides or sulfones, e) the compounds with amino substitution on phenyl radical proved to be more effective than those having nitro groups.

In general the work described by Boyland (1938) appears to be a report of a screening investigation and, therefore, the data should be regarded only as indicative of preliminary results. No statistical analysis, no quantitative data on transplanted tumors were given. The dosage schedules varied from one animal group to another.

Later work by Boyland (1946) was extended to include the diphenyl compounds where the binding of two phenyl radicals was accomplished by linkages different from S atoms. The spontaneous mammary cancer of mice and transplanted sarcoma MCDBI of dilute Brown mice were employed. This experimental series was performed under more uniform conditions than the previous work by Boyland (1938). The dosage schedules were uniform for all agents employed (12 doses of one-fourth of the $LD_{50}$ in 14 days). Statistical analysis was employed for the results obtained with spontaneous tumors. The results on spontaneous carcinoma were expressed as the ratio of the growth during the therapy period to that of the control period.

A complete inhibition of spontaneous carcinoma was obtained with 4:4'-diaminodiphenylsulfoxide (2 mg. per mouse x 12), 4:4'-diaminodiphenylether (5 mg. x 12), and methylene blue (8 mg. x 12) although none of these compounds had any effect on the transplanted sarcoma. With several other compounds the observed inhibition was statistically significant on the basis of the "t" test: 1) 2-amino,2'-nitrodiphenylsulfide (5 mg. x 12), 2) benzidine (3 mg. x 12), 3) 4:4'-diacetamidodiphenylether (10 mg. x 12), 4) 2:4'-diaminodiphenylether (5 mg. x 12), 5) 4:4'-diaminodiphenylmethane (3 mg. x 12), 6) 4:4'-diaminodiphenylsulfone (2 mg. x 12), 7) 2:4'-dinitro-2'-aminodiphenylsulfide (5 mg. x 12), 8) 2:2'-diaminodiphenyldisulfide (2 mg. x 12).

It is interesting to note that compound (8) shows structural similarity to our TSN-TSN, on the basis of the disulfide linkage between the two phenyl radicals.

The conclusions which could be drawn from these results by Boyland (1948) are as follows: a) the diaminodiphenyl compounds are more effective than the sulphonamide compounds, b) the lengthening of the chain between the phenyl radical does not have any significant effect on chemotherapeutic action, c) the position of the substitutions on the phenyl radicals may have an effect on the activity. For example, it was demonstrated that 4:4'-substitution is more effective than that on 2:4'-position as was the case with diaminodiphenylethers, or the 4:4'-substitution was more effective than that of 2:2'-as was the case with diaminostilbenes.

B. <u>The Effect of Protein and Certain Amino Acid Deficiencies on Tumor Growth.</u>

The scope of this review is limited primarily to the effects caused

by the deficiencies of certain amino acids and proteins on tumor development. No attempt is made to discuss the role of certain vitamins or fat substances and, therefore, the reader is referred to the major reference sources for reviews on these subjects (Tannenbaum and Silverstone, 1953; Greenstein, 1954).

It has been established that the deficiency of certain amino acids may influence the development of the spontaneous mammary carcinoma in mice. The animals on low cystine diet failed completely to develop any tumors. The histological study of the mammary tissue revealed a regression of the glandular tissue. The experimental animals showed lack of estrus cycle (White and Andervont, 1942-43). The implantation of diethlstilbestrol pellets raised the incidence of tumor appearance from zero per cent to 44.7 per cent (White and White, 1943-44). Mice on a low lysine diet showed a 25 per cent incidence of spontaneous mammary tumor in comparison to 74 per cent incidence in control animals, while the mice on low cystine diet did not show any single case of tumor development. The regularity of the estrus cycle was disturbed in the deficient animals. Therefore, it might be concluded that the hormonal influence might be one of the contributing factors (White and White, 1944-45). Virgin mice on caloric restricted diets showed little evidence of the mammary tissue growth, while breeding females on the same diet exhibited atrophic changes of glandular tissue (White, 1944-46a). A fifty per cent reduction of caloric intake without alteration in the amounts of dietary essentials reduced the incidence of mammary gland tumor from 100 to 12.5 and 18.2 per cent in C3H mice, virgin and breeding females, respectively (White et. al., 1944-45).

The incidence of leukemia induced by methylcholanthrene painting was markedly reduced (from 90 to 10 per cent) in mice on low cystine diet (White et. al., 1942-43). The diet low in lysine did not have any effect. Therefore, it would appear that cystine played some role in the tumor formation, perhaps similar but not identical to an amino acid essential for normal growth. The inclusion of gelatin or methionine in the cystine low diet increased the rate of leukemia from 10 to 32 per cent (White et. al., 1943-44). In three groups of animals where the dietary cystine, lysine and tryptophane were restricted, only the cystine low group showed a reduction in leukemia occurrence. This experiment was designed in such a way that the caloric intake per unit of body weight was identical in all groups (White et. al., 1946-47). A reduction of the caloric intake decreased the rate of leukemia from 96.2 to 35 per cent and increased the duration of the latent period three fold (White et. al., 1944-45). In the spontaneous mouse leukemia, a similar picture (reduction from 65 to 10 per cent) was observed, while the life expectancy was markedly prolonged and the malignant transformation of the lymphoid cells was delayed (Saxton et. al., 1944).

The growth of UCLA fibrosarcoma and Jensen sarcoma in rats of the Long-Evans strain was retarded by administration of ethionine. The tumors showed a tendency for both regressive and necrotic changes. These findings suggested that methionine might play an essential role in the growth of these transplanted tumors. The observed retarding effects might be brought about by the action of the competitive inhibition of the ethionine which is a metabolic antagonist to methionine (Levy et. al., 1953).

The restricted caloric intake or cystine low diet did not have any effect on the development of spontaneous pulmonary tumors (Larsen and Heston, 1945-46).

Tannenbaum showed that the crucial point of dietary restriction does not occur during the initiation phase (time of the carcinogen application) but in the developmental phase, when tumors are formed and make their appearance (Greenstein, 1954).

The influence of diet on a tumor (induced epithelioma and sarcoma, spontaneous mammary and lung tumors) already established is relatively small (Tannenbaum, 1940; Greenstein, 1954). The growth rate of mammary tumors (mouse) could be slightly inhibited in the early stages by lysine deficiency, but no effect was observed on the more advanced tumors (Kocher, 1944). White and Belkin (1944-45) showed that the tumor transplants (mouse adenosarcoma) in mice on a protein deficient diet grew at 74 per cent of the rate of control tumors. Later, White (1944-45b) reported that the tumor bearing animals on restricted protein diet showed a negative nitrogen balance, indicating that the normal tissues of host animals were probably broken down to supply the needs of the growing tumor. Additionally, it was shown that the catheptic activity of the organs of tumor bearing animals was markedly increased (Maver et. al., 1945-46, 1948-49). The growth of the Walker 256 tumor was markedly reduced in the protein depleted rats (Green et. al., 1950). If the protein depletion was reinforced by the feeding of the carcinogens during the growth period of the Walker 256 tumor, the effect of dietary restriction was more evident (Elson and Haddow, 1947; Elson and Warren, 1947; Green and Lushbaugh, 1949).

The mechanism of the inhibitory effect by carcinogens is not fully explained, but it might be associated with a production of deficiency of a specific sulfur containing amino acid (White and White, 1939; Stekol, 1943). Stekol (1947) showed that the inhibition of animal growth by benzyl chloride could be caused by the formation of benzylmercapturic acid. On the other hand Elson et. al., (1947) showed that the growth inhibition induced by carcinogens could not be reversed by the addition of cystine to the diet. Gutmann and Wood (1950a) used radioctive l-cystine for the determination of the mercapturic acid formation after the administration of bromobenzene (noncarcinogenic) and 3:4-benzpyrene (carcinogenic). It was found that only in the case of bromobenzene the mercapturic acid containing radioactive sulfur could be detected. Later work by the same authors (Gutmann and Wood, 1950b) with labeled methionine indicated an excretion of radioactive mercapturic acid only with bromobenzene and anthracene, but this was not the case with benzpyrene. The major source of the mercapturic acids appeared to be the tissue sulfur compounds.

C. Some Enzymatic Systems of Sulfur Metabolism.

Since an implication was made that TSN compounds might have some effect on the sulfur metabolism some general references on this topic will be mentioned here. Smythe (1945) and Fromageot (1947) present a detailed account on the metabolism of organic sulfur. A book by Umbreit (1952) contains an excellent presentation of the metabolic pathways of sulfur containing amino acids in chart form. Description of the enzymes involved in the sulfur metabolism is found in reference books by Sumner and Somers (1953) and Colowick and Kaplan (1955).

Since in our work some attempts will be made to study the enzyme systems releasing $H_2S$ from cysteine, applying the polarographic method of cadmium determination as an indirect measure of $H_2S$ production, a somewhat more detailed account of these enzymes will be given.

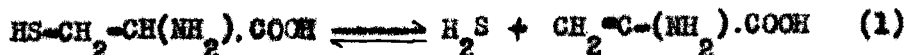
1. Cysteine Desulfhydrase (CD).

History. Tan, in 1933, demonstrated that the formation of $H_2S$ from cysteine or cystine by P. vulgaris was due to an enzyme which was obtained in a cell-free solution. Fromageot and Desnuelle (1939) found the same enzyme in E. coli. In higher animals, a similar enzyme was discovered by Fromageot, Wookey and Chaix in 1939 (Fromageot, 1951).

Reaction Mechanism. The overall enzymatic reaction for CD is as follows (Fromageot, 1951):

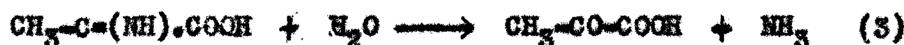
$$HS-CH_2-CH-(NH_2).COOH \longrightarrow H_2S + NH_3 + CH_3-CO-COOH$$

The intermediate steps are:



$$HS-CH_2-CH(NH_2).COOH \rightleftharpoons H_2S + CH_2=C-(NH_2).COOH \quad (1)$$

$$CH_2=C-(NH_2).COOH \rightleftharpoons CH_3-C=(NH).COOH \quad (2)$$



$$CH_3-C=(NH).COOH + H_2O \longrightarrow CH_3-CO-COOH + NH_3 \quad (3)$$

The specific action of the enzyme is represented by equation (1), while (2) and (3) correspond to spontaneous reactions. The intermediate reactions justify the name cysteine desulfhydrase. For the discussion of the mechanism involved in these reactions, the reader is referred to the work by Smythe (1942). Smythe and Halliday (1942) have shown that enzymatic conversions of radioactive sulfur ($Na_2S^{35}$) to cysteine sulfur can occur, a fact that would raise doubt about the irreversibility of the reaction.

Distribution. CD is found in a great number of bacteria. The following animals possess this enzyme: rat, guinea pig, rabbit, dog, hog, horse, ox, and man. The enzyme is essentially localized in the liver; small quantities are found in the pancreas and kidney, while very small amounts are found in the striated muscle, stomach wall and intestinal mucosa. The following organs or tissues do not contain the enzyme: spleen, testicles, brain, duodenum, and blood (Fromageot, 1951).

The one reference found by the writer concerning the occurrence of CD in neoplastic tissue reported its absence in stomach carcinoma (Fromageot et. al., 1940). A more recent work by Rutenburg et. al. (1950) suggested the presence of CD in some neoplastic tissues (Bagg lymphosarcoma and Walker 256 carcinoma). But some doubt about the specificity of the method for $H_2S$ determination used in this study could be raised, because they found that kidney was more active than liver, a finding which is just the opposite to the prior reports in the literature.

Assay Methods. There are some quantitative methods used in the determination of the enzymatic degradation of cysteine. They fall into three major classes: $H_2S$, $NH_4OH$, and pyruvate determinations (Smythe, 1955). Usually $H_2S$ is measured by absorbing it in solutions containing cadmium ions. The precipitated CdS is dissolved in acid and $S^{--}$ is oxidized to free sulfur by means of iodine solution. The amount of iodine used up in the oxidation of $S^{--}$ ion is measured. The lowest amount of S which could be determined is about 0.20 mg. Masters, 1938). The measurement of the light scattering (absorption) by colloidal PbS may be used too. The sensitivity of this method is the range of

3 to 100 micrograms of S was ± 2 micrograms of S (Delwiche, 1951). Other methods of $H_2S$ determination involve a reduction of various organic dyes by $H_2S$ e.g. formation of methylene blue or diformacan (Smythe, 1955; Rutenberg et. al., 1950). The ammonia production may be measured by Nessler's method the pyruvate can be determined by means of the hydrazone formation (Smythe, 1955).

Activators. There are a few indications that the proper functioning of the CD may be dependent upon vitamin-like compounds which may be co-enzymic factors.

There occurred a decrease of the CD concentration in the rat liver before any other signs of the vitamin $B_6$ avitaminosis could be observed (Sherman, 1954). Districh and Shapiro (1953) found that the CD was more sensitive to the vitamin $B_6$ deprivation than the transaminase and dopa decarboxylase systems, but the known pyridoxine antagonist, desoxypyridoxine, was without any effect on the CD, while it inhibited the other two enzymes. Similar results were obtained by Meister et. al., (1953) and they could reactivate the CD by the addition of the pyridoxal phosphate.

Delwiche (1951) found that the CD in E. coli could be activated by adenosine-5-phosphate, pyridoxal phosphate, biotin and alpha-ketoglutarate. Kallio (1951) showed that pyridoxal phosphate was involved in the CD system of Proteus morganii.

The information about the specificity, kinetics and effects of metallic ions on CD may be found in the following references: Fromageot et. al. (1940), Fromageot and Grand (1943-44), Fromageot (1951), Smythe (1942), Lawrence and Smythe (1943), and Binkley (1943).

2. Cystine Exodesulfhydrase (ECD)

The enzymatic effects of the cystine desulfhydrase are closely related to those of the exocystine desulfhydrase (ECD) in so far as these enzymes are concerned with the metabolism both of cystine and cysteine. The differences observed were only quantitative in nature, as revealed by the $NH_3$ production from the l-cystine and l-cysteine. The production of $NH_3$ from cysteine or its peptides was smaller than that observed with cystine or its peptides (Greenstein and Leuthardt, 1942-43, 1944-45b).

The ECD acts on the cystine molecule both in free form and when it is incorporated into the peptide chain. The cystine in peptide form has to be in the terminal position, and must possess either a free alpha amino group or alpha carboxyl radical (Greenstein and Leuthardt, 1944-45b). The capacity of certain normal tissues to split cystine peptides and to produce ammonia from cystine, goes parallel with the ability to degradate cysteine. The following tissues: liver, kidney and pancreas possessed the ability to perform these two enzymatic reactions, while spleen, brain and striated muscle did not show any such activity.

The absence of the ECD in various tumors in rats and mice was reported (Greenstein and Leuthardt, 1944-45a; Greenstein, 1954), although some hepatomas (primary and secondary) induced by chloroform possessed the ECD activity (Greenstein,1954).

CHAPTER III

MATERIALS AND METHODS

## A. Procedure of Transplantation and Growth Study of Walker 256 Tumor

All instruments and saline solution used in transplantation procedure were boiled for at least 15 minutes before their use and care was taken to minimize any later contamination.

The surface area of a tumor and surrounding tissue in a freshly killed tumor bearing rat was washed with 75 per cent EtOH and the tumor tissue carefully dissected out. One to three grams of tumor tissue were gently ground by pestle and mortar, or cut into small pieces with scissors in 6-10 ml. of saline. Incorporation of a small amount, 1000 Units/ml. of crystalline penicillin G (K salt) was undertaken in some experiments, but this proved not to be critical. Therefore, in a majority of experiments, plain saline was used. This cell suspension was filtered through gauze and 0.2 ml. of filtered cell suspension was injected subcutaneously into the inguinal region of the rat.

Sprague-Dawley rats of both sexes, weighing 150-220 grams, were used for transplantation purposes. They were fed Purina Chow diet *ad libitum*. Care was taken to have an equal distribution of animals with respect to sex and weight among control and treated animals. All drug solutions were administered intraperitoneally in a fashion indicated in the results chapter.

Growth of the tumor was followed by repeated measurements of three diameters of tumor, using a vernier caliper. The size of the tumor was

expressed as a product of three diameters in cubic centimeters (ccm.). Tumors were usually palpable 7-10 days after transplantation. The observed differences in the size of control and treated tumors were evaluated statistically; for the methods used see Hill (1952) and Appendix.

The tissues for the histological examination were taken from the freshly sacrificed animals and fixed in 10 per cent formalin for at least 24 hrs. before preparation of the paraffine blocks. The histophathological evaluation of tissues was made with the help and supervision of a qualified pathologist (Dr. P. D. Toto of the School of Dentistry, Loyola University).

B. Properties of TSN and TSN-TSN Compounds.

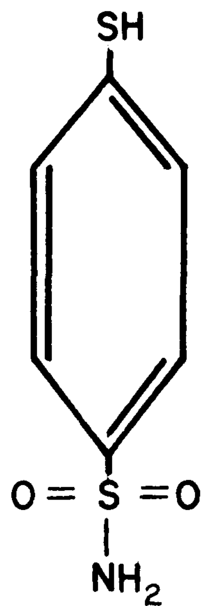
Para-thiolbenzenesulfonamide (TSN) and the disulfide of TSN were prepared by Dr. C. D. Proctor (Stritch School of Medicine). The elemental analysis was performed by Dr. L. I. Diuguid (St. Louis, Mo.). These compounds exhibited poor $H_2O$ solubility, but were soluble in slightly basic solutions. See Table I and Figure 1.

TABLE I

ELEMENTAL ANALYSIS OF TSN AND TSN-TSN

| Compound | Molecular Weight* Calcd. | Molecular Weight* Found | Observed Melting Point** °C | Formula | Nitrogen Per Cent Calcd. | Nitrogen Per Cent Found | Sulfur Per Cent Calcd. | Sulfur Per Cent Found |
|---|---|---|---|---|---|---|---|---|
| TSN | 189 | 187<br>188 | 139-140 | $C_6H_7O_2NS_2$ | 7.40 | 7.48 | 33.86 | 33.91 |
| TSN-TSN | 376 | 381<br>379 | 243-245 | $C_{12}H_{12}O_4N_2S_4$ | 7.44 | 7.44<br>7.57 | 34.04 | 34.02<br>34.13 |

* Boiling point elevation in acetone.
** Fisher-Johns apparatus used.



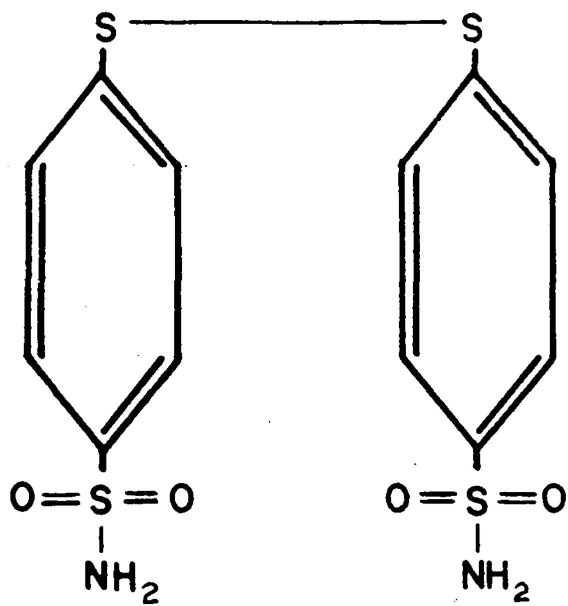




FIGURE 1. STRUCTURAL FORMULAE OF TSN COMPOUNDS

C. Polarographic Procedure for Cysteine Desulfhydrase Determination.

Apparatus. The Sargent model XXI polarograph was used to record the polarograms. Voltage span used lay between -0.30 and -1.30 volts, while applied voltage constituted 25.0 to 55.0 per cent of this span. The dropping mercury electrode (t=2.88 - drop time, sec., m = 2.9 mg./sec.- mercury flow per sec.) was the cathode, while one ml. of mercury on the bottom of a test tube (14 mm. x 125 mm.) used as the polarograph cell served as a stable anode. The amplitude of the galvanometric deflections caused by the mercury drop was reduced by dampening. The drop distance in the test solution and the height of the mercury column were kept constant. The galvanometric deflections (diffusion currents) due to reduction of the $Cd^{++}$ ions (half wave potential = -0.68 volts) were recorded using a sensitivity of 0.060 or 0.030 mu a/mm. A deflection of 10.0 mm. represented a current intensity of 0.6 mu a or 0.3 mu a. The method used for determining wave heights was the procedure described by Willard et. al. (1951). The polarographic waves exhibited well defined form and were easily analyzed by conventional methods (Figure 2). Oxygen free nitrogen was used for deoxygenation of the test solution effected by bubbling nitrogen through the test solution for at least five minutes prior to the polarographic determinations.

The 125 ml. Erlenmeyer flasks were used for carrying out the enzymatic reaction and in the procedure used for calibration of the polarographic wave. They were closed by a rubber stopper which contained two glass tube openings. One tube (inside diam. 3.5 mm.) extended near the bottom of the flask and served as an inlet channel for the $N_2$ which served as the gas phase for the

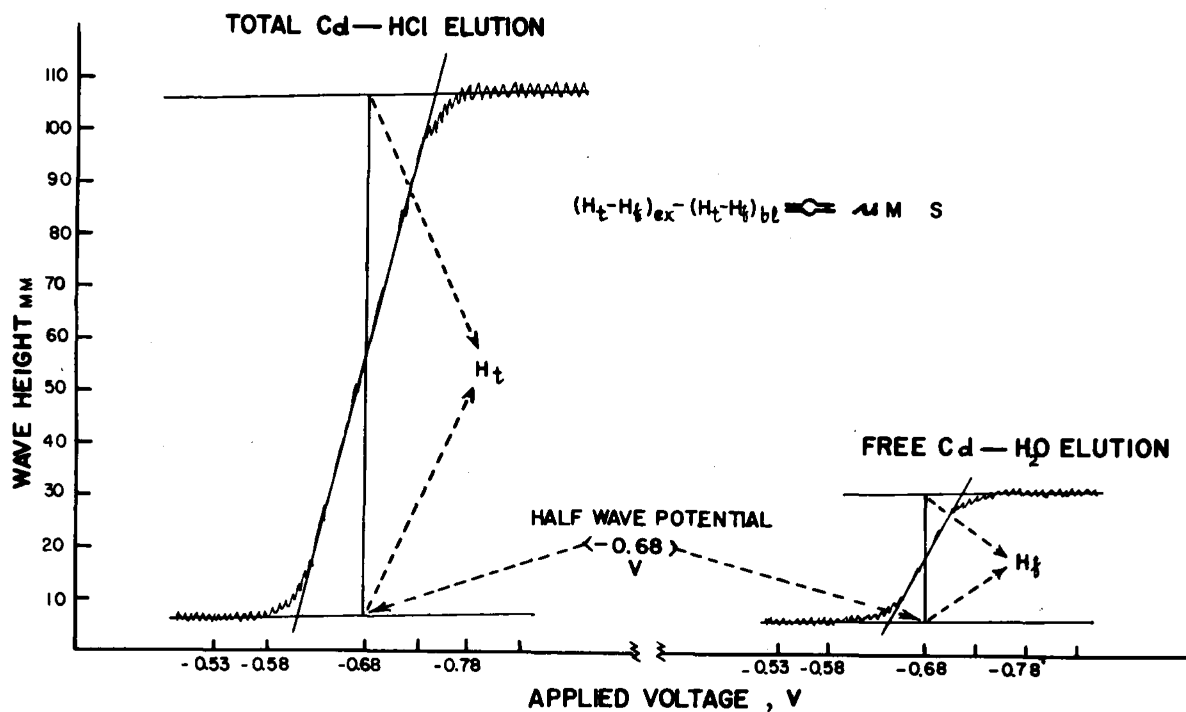



FIGURE 2. QUANTITATIVE METHOD FOR $H_2S$ DETERMINATION

enzymatic reaction mixture. The second tube (inside diam. 1 mm.) served as an outlet tube for $N_2$ and $O_2$ during deoxygenation procedure and, secondly, it was used for delivery of one ml. of mixture containing 10 per cent trichloroacetic acid (TCA) and 4.5 N HCl used for deproteinization and for quantitative release of $H_2S$ from the solution. These tubes were closed right after deoxygenation and opened before removal of the filter paper cups containing Cd acetate and Cd sulfide.

The filter paper cups (Bodansky and Levine, 1940) were prepared in an identical manner from the same filter paper (Whatman #1, diam. 12.5 cm.) in order to obtain cups of uniform size and mass, because these factors might affect the polarographic waves due to Cd reduction (Figure 3). It was demonstrated, employing equivalent amounts of Cd, that the presence of filter paper tends to lower the height of the polarographic wave to a measurable degree. Although this presents some disadvantage to use filter paper cups, one advantage afforded by the relatively large surface area for spreading of the Cd solution (thus effecting an efficient trapping of the $H_2S$ gas) overweighs the disadvantage.

<u>Calibration of Polarographic Wave</u>. The principal chemical reaction involved in the indirect polarographic determination of the $H_2S$ is as follows:

$$(CH_3COO)_2Cd + H_2S \longrightarrow \downarrow CdS + 2CH_3COOH$$

The cadmium ions were present in an excess in respect to the $H_2S$ produced. The free cadmium was determined and subtracted from the total cadmium present. The difference between these two values represented the amount of the $H_2S$ released (Figure 2).

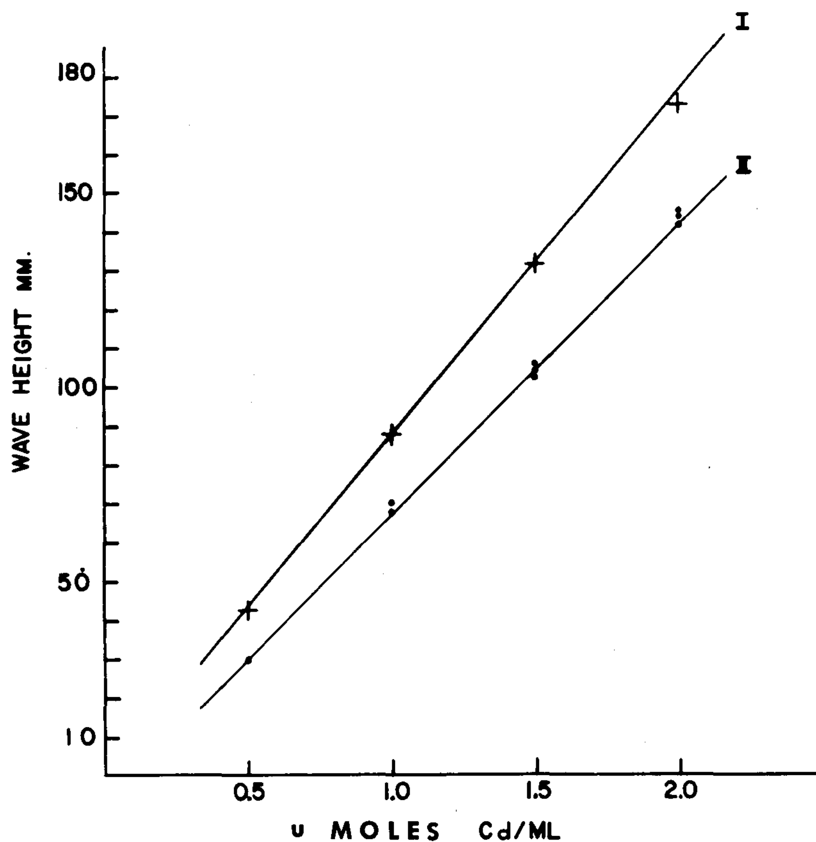



FIGURE 3. EFFECT OF FILTER PAPER ON THE Cd WAVE HEIGHT

CURRENT SENSITIVITY 0.060 mu a/mm

I. 10.0 ml. $H_2O$
II. 10.0 ml. $H_2O$ elution from filter paper

$Na_2S \cdot 9H_2O$ crystals (molecular weight - 240.19) were dried in a desiccator, weighed and dissolved in 0.5 M Tris 7-9 buffer (Tris (hydroxymethyl) aminoethane) of pH 7.4 to yield a 0.015 M solution (approximately). Varying volumes of $Na_2S$ solution (0.10 - 1.00ml.) were added to the 125 ml. Erlenmeyer flasks which contained 4.0 ml. of physiological saline solution. Final volume was adjusted to 5.0 ml. by appropriate volumes of Tris buffer solution. Filter paper cups containing 0.20 ml. of 0.10 M cadmium acetate solution (Cd. $(C_2H_3O_3)_2 \cdot 2H_2O$; mol. wt. -266.53) were inserted into the flasks, which were then tightly stoppered. One ml. of 10 per cent trichloroacetic acid and 4.5 M HCl mixture was added to the solution to obtain a quantitative release of $H_2S$. Precaution was taken to avoid any loss of $H_2S$ gas in the course of the manipulations. After 30 minutes, the paper cups containing CdS and excess of ionic Cd were transferred to 10.0 ml. cooled freshly boiled distilled water in 125 ml. Erlenmeyer flasks. Free Cd ions were removed by repeated washings of the filter paper with a pipette, leaving the CdS on the filter paper unaffected. (An addition of 1.0 ml Tris buffer of pH 7.4 to the eluate medium did not have any polarographic measurable effect on the amount of free Cd eluted). This test was undertaken in order to determine whether or not distilled water (which exhibited a slightly acidic reaction) could dissolve the CdS. The solubility characteristics of CdS are as follows: solubility product $-3.6 \times 10^{-23}$ (18° C); solubility in gm. per 100 ml. water - $9 \times 10^{-7}$ gm. (18° C). Two (2.0) ml. of water eluate containing free ionic Cd were mixed with 2.0 ml. of 1.0M HCl and analyzed polarographically. The height of the wave caused by free Cd was designated as $h_f$ (Figure 2). To the remaining 8.0 ml in the Erlenmeyer

flasks were added 8.0 ml. of 1.0 M HCl and allowed to stand covered overnight to effect a complete solution of CdS. Four (4.0) ml. of this solution were analyzed polarographically, giving a wave height designated as $h_t$ which represented the total Cd present. The difference in wave heights in mm. between $h_t$ and $h_f$ was corrected by subtraction of the analogous value obtained with Cd cups not exposed to $Na_2S$ solution (Figure 2). These relationships may be summarized by the following formula:

$(h_t - h_f)_{ex} - (h_t - h_f)_{bl}$ = corrected difference of the wave height in mm.

The corrected wave differences and the corresponding $H_2S$ amounts were plotted on the ordinate and abscissa, respectively (Figures 4 and 5), yielding a satisfactory linear relationship. The difference between $h_t - h_f$ observed with Cd filter paper cups not exposed to $H_2S$ might be explained by the Cd absorption characteristics of the filter paper (Figure 3).

Since $Na_2S$ crystals varied to a considerable degree in $H_2O$ of crystallization and showed a tendency to decompose, it was, therefore, necessary to ascertain the actual molarity of the $Na_2S$ standard solutions. The standardization was carried out by determining the difference in wave heights (due to free Cd in the $H_2O$ elution) of a Cd filter paper cup (I) not exposed to $Na_2S$ and a cup (II) which was exposed to 1.0 ml. of $Na_2S$ solution. Both cups contained 20 micromoles of Cd ion. Wave height in mm. which corresponded to one micromole of ionic Cd in $H_2O$ eluate was found by dividing the wave height of cup I by twenty (number of micromoles of Cd present). The observed differences of the wave height of cup I and cup II was divided by the value in mm. which corresponded to one micromole of Cd, thus finding the number of micromoles of

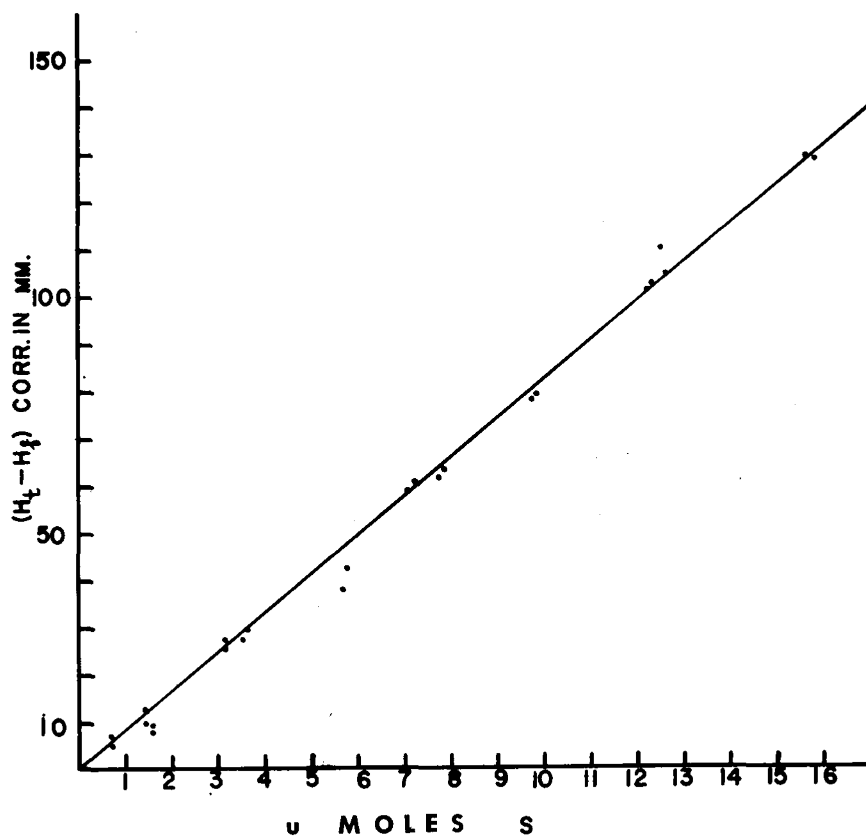



FIGURE 4. CALIBRATION FOR $H_2S$ DETERMINATION I
(Current Sensitivity 0.060 mu a/mm.)

Cd used by binding the S which was released. Since the reaction between $Cd^{++}$ and $S^{=}$ occurs on equimolar basis, the number of Cd micromoles "used up" would represent the number of micromoles of S present in 1.0 ml. of $Na_2S$ used. In such a way one finds the actual molarity of the $Na_2S$ solution used. The number of micromoles of $Na_2S$ in smaller volumes delivered was found by multiplying the number of micromoles of $S^{=}$ in one ml. by the fraction of one ml. under consideration. This standardization procedure was repeated every day with each new $Na_2S$ solution used in determination of the linear relationship between the corrected difference of wave height $(h_t - h_f)_{ex}$ in mm. and the amount of $Na_2S$ added (Figure 4).

The same procedure was employed with lower concentration of Cd acetate solution (0.20 ml. of 0.06 M CdAc) and correspondingly smaller amounts of $Na_2S$ using greater polarographic sensitivity - 0.030 mu a/mm. for the recording (Figure 5). The standardization line obtained was used primarily for determination of the tumor enzymatic activity which was considerably lower than that of the liver. Increasing the sensitivity of the determination by these changes afforded an advantage in the measurement of enzyme activity from sources of low activity so that greater magnification of slight relative differences was yielded.

Presuming that a corrected difference $(h_t - h_f)$ of five millimeters might lie within the range of error involved in the process of extrapolation of the wave heights, one may safely assume that a minimal detectable amount of $H_2S$ evolved with the lower sensitivity - 0.060 mu a/mm. was 0.7 micromoles of S, while that with higher sensitivity - 0.030 mu a/mm. was 0.3 micromoles of S.

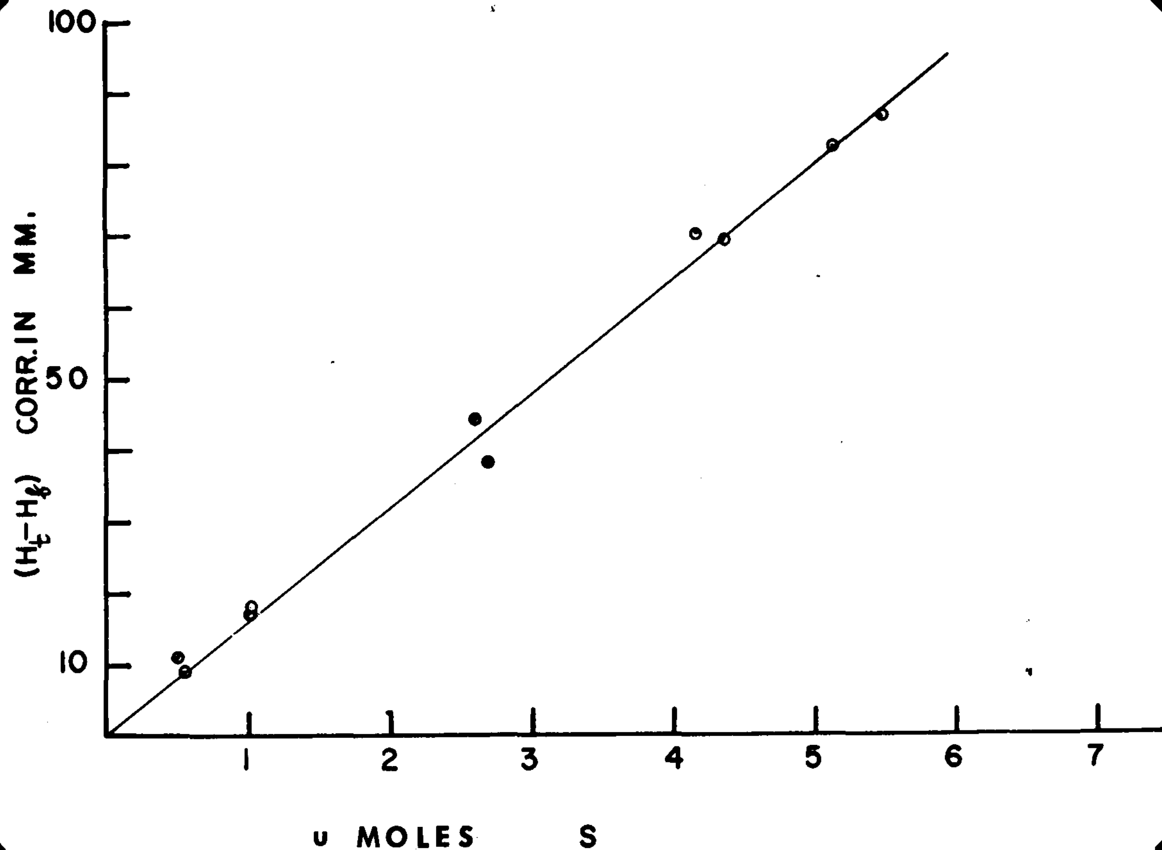



FIGURE 5. CALIBRATION FOR H2S DETERMINATION II

(Current Sensitivity 0.030 mu a/mm.)

Determination of the Enzymatic Release of $H_2S$.

Liver or tumor tissue (or any desired organ) was carefully excised from the animal sacrificed by a blow on the head and homogenized with a pestle and sand (sand, purified, Baker Chemical Company) in a precooled mortar in cold 0.9 per cent NaCl solution. The volume of saline was varied from one to three times the weight of tissue (see individual tables). The ground tissue was centrifuged for 10 minutes at 600 x g and the supernatant fluid obtained was kept in the refrigerator before the preparation of the reaction mixture. Three (3.0) mls. of the supernatant were used as an enzyme source for the $H_2S$ production from cysteine.

L-cysteine solution was prepared by dissolving its hydrochloride salt (mol. wt. - 157.62) in physiological salt solution and adjusting the pH approximately to 7.0 with NaOH using indicator paper (hydrion B), just before using. 1.0 ml. of a neutralized l-cysteine HCl solution of concentrations indicated under the individual tables was used as a substrate. The test compounds (activators and inhibitors) were dissolved in $H_2O$ and NaOH solution was added to effect dissolving and neutralization of the solutions. (TSN was dissolved in an excess of NaOH and neutralized back with HCl). One (1.0) ml. of a 0.1 M phosphate or 0.5 M Tris 7-9 buffer solution of a pH 7.4 was used in the reaction mixture. But in some experiments no buffer solution was used, since it was shown that there was no significant difference in the $H_2S$ production over a pH range of 7.0 - 8.0. Secondly, parallel experiments with and without buffer solution did not show any observable differences in $H_2S$ production.

The enzymatic reaction was carried out in a 125 ml. Erlenmeyer flask

(described previously) to which were added homogenate, buffer, cysteine test compound solutions (for reaction mixtures see individual tables). The control flasks received identical volumes of saline in place of the test solutions. For the incubation times see individual tables. The incubation temperature was 37° C. The enzymatic reaction was stopped by addition to one ml. of a mixture containing 10 per cent trichloroacetic acid in 4.5 M HCl acid, whereupon the flasks were allowed to stand for 30 minutes. This procedure helped to release any bound $H_2S$ from the reaction mixture.

The polarographic analysis of Cd cups exposed to enzymatic $H_2S$ release was performed in an identical manner as was discussed in the calibration procedure where the $H_2S$ was released by acidifying the solution containing the $Na_2S$. The standardization line of the appropriate current sensitivity was used to convert the corrected differences in wave height $(h_t - h_f)_{ex}$ to the micromoles of S produced.

A higher polarographic sensitivity (0.030 mu a/mm.) was used in the majority of tumor experiments. Although it was necessary to employ smaller amounts of Cd (0.20 ml. of 0.050 M Cd solution) in such cases, the height of the Cd diffusion current was not changed significantly, while the relative differences due to variation of the enzyme activity could be magnified. In other words, the observed quantitative differences in enzyme activity could be increased or a weak enzymatic tumor activity might be better detected.

The difference of wave height $(h_t - h_f)$ due to enzyme activity was corrected by subtracting the value of $(h_t - h_f)$ obtained with Cd cups exposed only to a reagent blank (no enzyme present). The control (homogenate, no

cysteine) determination for endogenous $H_2S$ release by liver and tumor tissue did not demonstrate any measurable $H_2S$ production. Therefore, only reagent blanks were used in the majority of experiments to make the correction of the observed wave height differences before the conversion into the micromoles of S produced.

# CHAPTER IV

## RESULTS

### A. Chemotherapy of Walker 256 Tumor

Preliminary Experiment with TSN-TSN. The therapy in this experimental series was started on 8th day after tumor implantation (Table II). Experimental animals were divided into three groups: 1) Control animals receiving 1cc of 1.0% tragacanth) suspension intraperitoneally; 2) TSN-TSN animals receiving four doses of 500 mg/kg of TSN-TSN (10% suspension in 1.0% tragacanth) on four consecutive days followed by rest interval of one day and administration of 250

TABLE II

THERAPY OF WALKER 256 TUMOR BY TSN-TSN AND TSN-TSN PLUS VIT. $B_{12}$

| Group | Days after Implantation | | |
|---|---|---|---|
| | 8 | 12 | 14 |
| Control | palpable<br>n** = 9 | 5.5*<br>n=8 | 15.3*<br>n=7 |
| TSN-TSN | palpable<br>n=9 | 2.2<br>n=6 | 6.8<br>n=4 |
| TSN-TSN $B_{12}$ | palpable<br>n=8 | 2.2<br>n=4 | 5.6<br>n=4 |

* Product of three tumor diameters in cubic centimeters

** n refers to the number of animals with tumors in each case

mg/kg of TSN-TSN; 3) A TSN-TSN and $B_{12}$ group which received 30 micrograms of $B_{12}$ with each injection of TSN-TSN. The experimental results summarized in Table II show that there was a marked decrease in tumor size of the animals under therapy in comparison to that of the control animals. But at the same time the mortality rate in treated animals was considerably high (Control group - one death out of nine. TSN-TSN group - five deaths out of nine; TSN-TSN and $B_{12}$ group - four deaths out of eight animals). There was no significant weight loss observed in the treated groups while control animals showed a slight weight gain (from 188 gm to 195 gm) at the end of the experiment. This small weight gain might be explained by the presence of the larger volume of tumor in untreated animals. The results obtained in this experimental series indicated a need for experiments employing smaller doses before any definite conclusions about this particular compound could be reached.

Large Scale Experiment with TSN-TSN. The therapy in this experimental series was started one day after implantation of tumor. The experimental animals were divided into two groups: 1) Control animals receiving 1.0% tragacanth suspension intraperitoneally; 2) TSN-TSN animals receiving seven doses of 250 mg/kg of TSN-TSN (5% suspension in 1.0% tragacanth) intraperitoneally every second day. The volume of the drug preparations injected was 1.0 cc per 200 gm of body weight. The results were summarized in Table III and Figure 6. On the 12th day after implantation (after five doses of TSN-TSN) the first measurements of tumor size were made and there were definite differences observed in the size of tumors of both groups. The treated animals showed a marked reduction in tumor size which was statistically significant. The measurements on

two later periods indicated that there was a continuous tumor growth in treated animals but the size of these tumors was considerably smaller than that observed in control animals. The observed differences were statistically significant.

Since there was a marked reduction in the tumor volume of treated animals a question may arise as to whether or not this form of the therapy had any effect upon the establishment of successful tumor growth. It was found

TABLE III

THERAPY OF WALKER 256 TUMOR BY TSN-TSN

| Days after Implantation | Tumor size ccm. (Product of 3 diams.) Control, m±SE** | Treated, m±SE** | $SE_{D}$** | t** p |
|---|---|---|---|---|
| 12 | 6.3±1.3 n*=10 | 2.0±0.3 n=18 | 1.3 | 3.3 < 0.01 |
| 15 | 12.3±1.8 n=11 | 5.1±0.9 n=19 | 2.1 | 3.4 < 0.01 |
| 17 | 16.8±2.6 n=11 | 7.8±1.3 n=15 | 2.8 | 3.2 < 0.01 |

* n refers to the number of animals with tumors in each case
** See Appendix for the formulae used in statistical analysis

that the number of successful transplantations was greater in the treated group than that of the control animals (four animals out of sixteen controls did not "take" vs. one animal out of 24 treated). Therefore, it is probably safe to conclude that this type of therapy did not have any effect upon the establishment of tumor growth.

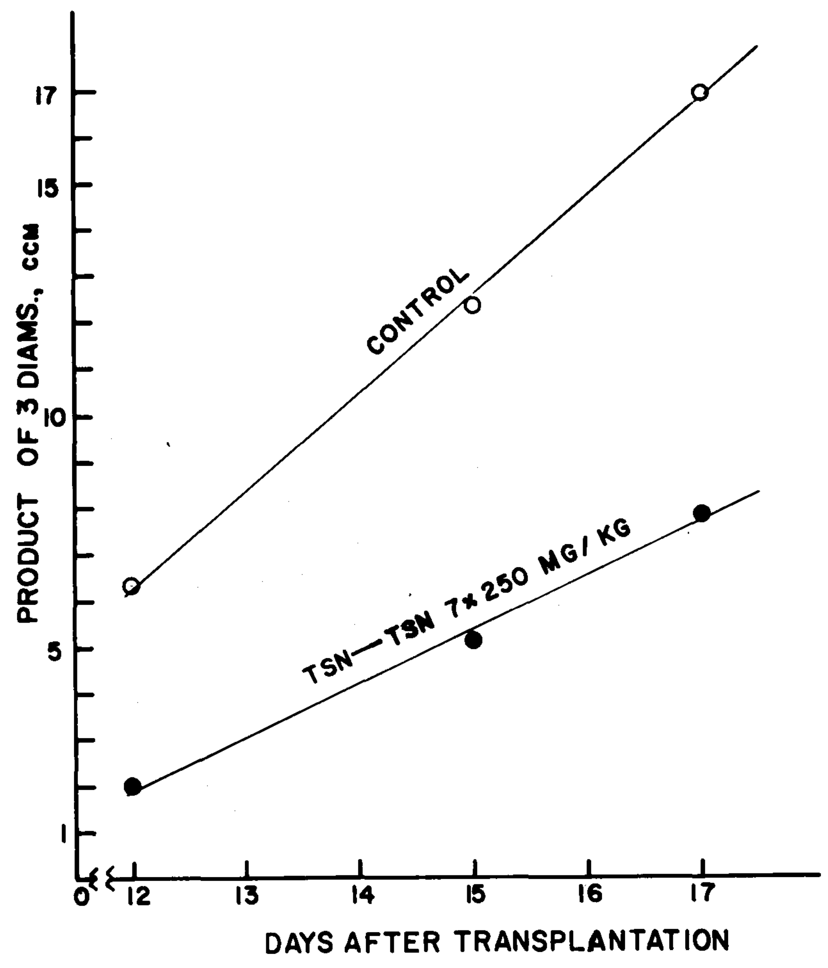



FIGURE 6. THERAPY OF WALKER 256 TUMOR BY TSN-TSN

(See Table III)

A comparison of the death rates between the control and treated groups indicated a considerably higher mortality in the treated group (six treated animals died out of 24 vs. two control deaths out of 16 animals). These deaths included animals which did not show any tumor growth but were treated according to the group to which they belonged.

A most common toxic phenomenon grossly observable in treated animals was the accumulation of edematous fluid in the peritoneal cavity. Upon opening of the peritoneal cavity there was observed a deposition of drug on the viscera and especially on the surface of liver.

No metastatic tumor growth was observed in control animals while in two treated rats metastatic tumors were found. In one animal there was a large tumor mass with characteristic histological structure located below the stomach. The other metastatic tumor, which was observable only microscopically, was located on the liver surface and exhibited an encapsulated growth which did not invade the host liver tissue. The last finding is worth mention because the metastatic growth in liver tissue has not been reported to the writer's knowledge in the case of Walker 256 tumor.

The accumulation of edematous fluid in the peritoneal cavity suggested that there might be kidney damage induced by this form of therapy. But the kidney sections stained by hematoxylin eosin technique did not reveal any extensive damage which could be compatible with edema formation. The kidney slides from 12 treated animals which were sacrificed after the cessation of the therapy did not show any essential structure difference as compared with control animals. The most commonly observed pathological phenomenon was cloudy swelling

of the proximal convoluted tubuli which occurred both in control and treated groups. The other structures (glomeruli, renal pelvis) did not reveal any deviation from the normal picture. In some treated animals there were observed focal accumulations of inflammatory cells in the interstitial tissues between tubuli although no severe inflammation could be detected.

The liver sections (hematoxylin eosin stain) of treated animals (8) showed a relatively constant picture. The liver capsule demonstrated a proliferation of collagenous fibers and presence of polymorphonucleocytes suggesting presence of foreign body reaction due to the deposition of injected drug on the liver surface. This inflammatory reaction showed tendency to spread along the septa (hepatic triads) and sometimes reached the hepatic cords causing a mild hepatitis on an inflammatory basis. Occasionally, small areas of focal fibroblastic tissue were observed. The hepatic cords appeared to be swollen and the lumen of sinusoid was reduced. The liver slides of control animals (3) did not show any foreign body reaction but the hepatic cords exhibited some cloudy swelling. In one control liver the cloudy swelling was more severe than that observed in treated animals. Despite that fact the tumor of the same animal showed a large volume. Therefore, it would appear that the presence of the cloudy swelling both in control and treated animals probably does not have any significant effect on tumor growth.

The histological analysis of tumor tissue of control and treated animals did not reveal any significant differences. A more detailed description will be given later together with experiments where TSN was employed as the chemotherapeutic agent.

Despite the fact that there occurred a marked reduction of tumor size in treated animals and there was no extensive damage to liver and kidney tissues the role of TSN-TSN as a chemotherapeutic agent cannot be evaluated in a simple manner because of the following complicating factors: 1) general toxicity as shown by increased mortality of treated animals, 2) absence of an effect upon the establishment of tumor growth, 3) presence of metastatic growth in treated animals, 4) absence of any clearly defined histological effect on tumor tissue.

TABLE IV

THERAPY OF WALKER 256 TUMOR BY TSN AND TSN PLUS VIT. $B_{12}$

| Days After Implantation | Tumor Size ccm (Product of 3 diams.) | | | $SE_D$** | | t** p | |
|---|---|---|---|---|---|---|---|
| | Control, m±SE** | TSN, m±SE** | TSN $B_{12}$, m±SE** | TSN | TSN $B_{12}$ | TSN | TSN $B_{12}$ |
| 8 | 4.61±.45<br>n*=13 | 3.31±.21<br>n=20 | 3.36±.34<br>n=20 | .57 | .55 | 2.5<br>0.01 | 2.3<br>$<0.05$ |
| 11 | 12.1±1.1<br>n=13 | 4.97±.48<br>n=19 | 5.47±.59<br>n=19 | 1.2 | 1.2 | 6.0<br>$<0.01$ | 5.0<br>$<0.01$ |
| 14 | 19.4±3.1<br>n=11 | 8.30±.90<br>n=16 | 11.2±1.4<br>n=16 | 3.3 | 3.4 | 3.3<br>$<0.01$ | 2.4<br>$<0.05$ |
| 16 | - | 10.3±1.4<br>n=14 | 14.6±2.6<br>n=14 | 3.4 | 4.1 | 2.7***<br>$<0.05$ | 1.1***<br>$>0.10$ |

* n refers to the number of animals with tumors in each case
** See Appendix for the formulae used in statistical analysis
*** Between 14th day of control animals and 16th day of treated animals

Chemotherapy by TSN and TSN plus Vit. $B_{12}$. Table IV and Figure 7 summarize results obtained with TSN and a combination of TSN plus Vit. $B_{12}$ as chemotherapeutic agents.

The experimental animals were divided into three groups: 1) Control animals receiving saline intraperitoneally; 2) TSN animals which received eight doses of 200 mg/kg of TSN as the sodium salt intraperitoneally for eight consecutive days (drug was partially in crystalline suspension form); 3) TSN and $B_{12}$ animals received in addition to TSN as described above a dose of 5 micrograms of vit. $B_{12}$ simultaneously. The volume of the preparations to be injected was 1.0 ml per 200 gm of body weight. The animals were distributed among the separate groups taking care to get an equal distribution in respect to the animal weight and size of tumors which were at the palpable stage of the growth. The therapy was begun on the 7th day after tumor implantation. Only three measurements of tumor size in the control group were taken because tumors of untreated animals showed extensive necrotic processes at later stages of tumor growth. The same necrotic processes occurred in treated animals but at later stages of tumor growth than in control animals. The appearance of necrosis may be explained by the fast growth of tumor tissue thus outgrowing its nutritional supply since these necrotic changes occurred both in control and treated animals. In no case was the appearance of necrosis attributed to the chemotherapeutic effect.

The effect of TSN on tumor growth was clearly indicated statistically by comparison of mean values of tumor size. Even the difference between mean

values of the control tumor size on 14th day and that of TSN treated animals on 16th day was statistically significant. It would appear that the cancerostatic effect by TSN was more pronounced in the early stages of therapy as is evident from the slope of the growth line (see Figure 7). A similar phenomenon was observed in the TSN with $B_{12}$ group, where the increased rate of tumor growth in later stages was more clearly observable after an initial inhibitory effect. The differences between the mean value between control and TSN plus $B_{12}$ group were significant on the 11th and 14th days after implantation while there was no real difference between the mean control value for the 14th day after transplantation and that for the 16th day of the treated group. These findings would suggest that the initial cancerostatic effect caused by TSN plus $B_{12}$ therapy was less sustained than with TSN alone.

From these experimental findings one may conclude that the cancerostatic effect by both forms of the chemotherapy (TSN alone and in combination with $B_{12}$) was more pronounced at the initial stages of tumor growth. Later treated tumors showed an increased growth rate which approached that of the control animals. The attempts to follow the tumor growth after the cessation of the therapy were unsuccessful because the tumors tended to show extensive necrotic changes. But it did not appear likely that this form of the therapy might have any permanent cancerostatic effect, because tumors continued to grow even during the period of drug administration.

The comparison of the death rates in these separate groups revealed that the treated groups showed a significant mortality while there were no

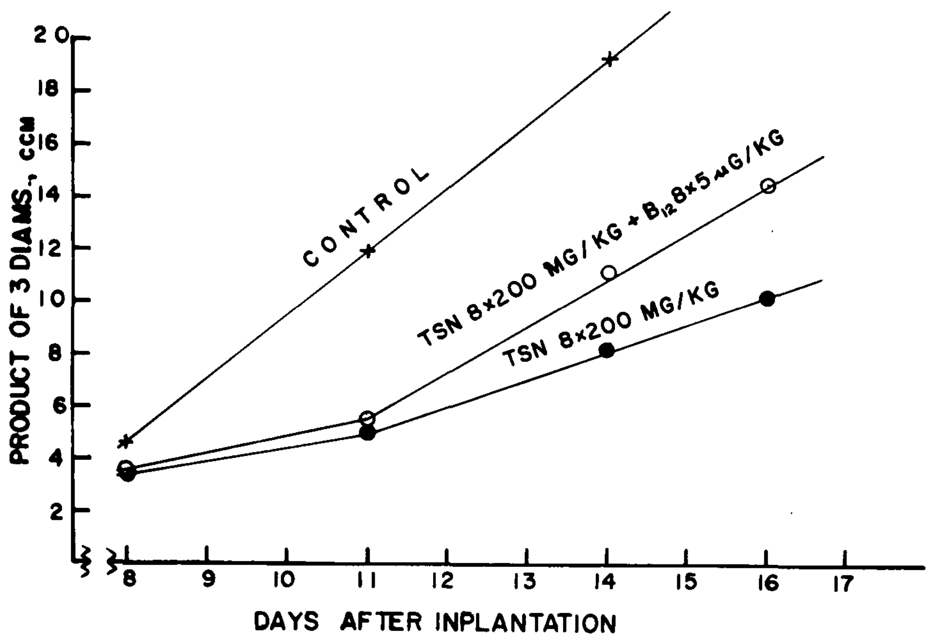



FIGURE 7. THERAPY OF WALKER 256 TUMOR BY TSN AND TSN PLUS VITAMIN $B_{12}$

(See Table IV)

deaths in the control groups during the observation time (16 days after tumor implantation). There were five deaths out of 20 TSN treated animals and two deaths out of 20 TSN with $B_{12}$ treated animals. The changes in weight during therapy in comparison to the weight before the start of therapy were not significant in all animal groups. The control animals showed some gain in weight (from 170 gm to 186 gm) while in TSN and TSN plus $B_{12}$ groups the weights stayed approximately constant (174-176 gm and 172-178 gm respectively). These differences in weight gain between control and treated groups might be partially explained by the larger weight of the tumor mass of the control animals.

The examination of kidney sections (hematoxylin eosin stain) of treated animals revealed damage as the result of this therapy. The kidney of an animal which showed an accumulation of edematous fluid in the peritoneal cavity exhibited a severe hydropic degeneration of renal tubuli and an interstitial edema causing enlargement of the organs. Although the glomeruli were intact there was some albumin-like exudate inside the Bowman's capsule and the endothelial cells showed some swelling and granulation. In the majority of cases the renal interstitial edema, tubular swelling and hydropic degeneration were of lesser degree. The glomeruli were not severely affected and only in some cases there were traces of albuminous exudate in capsule and some evidence of swelling and granulation of endothelial cells. In no cases were there observed any traces of blood pigment either in the tubuli or in Bowman's capsule. It appeared that in the majority of cases the observed changes in renal tissue were reversible in nature.

Liver sections of treated animals (7) showed a variable degree of

damage. The sinusoidal space was collapsed due to the cloudy swelling of hepatic cords. The liver cells showed albuminous degeneration, some evidence of plasma vacuolization, and loss of cell wall demarcation. The changes were more pronounced in the vicinity of central veins (evidenced by weaker staining intensity of cells at this site). The general microscopic appearance gave the impression that a majority of these changes could be reversible although in one or two cases the damage was more severe.

In general it could be stated that the observed organ changes caused by TSN therapy were more pronounced than those observed in case of TSN-TSN animals.

Experiments with Smaller Doses of TSN. The experiments employing a smaller dose of TSN (8 doses of 150 mg/kg for eight consecutive days started on the 7th day after implantation) revealed a reduced chemotherapeutic effect on tumor growth (Table V, Figure 8). On the 10th and 13th days after implantation the differences between the mean values of control and treated animals were statistically significant but on the 15th day the observed differences were not significant. With this decreased dosage schedule there was a marked drop in the death rate in the treated group (one death out of 15 treated animals, no deaths in control group). The changes in body weight of treated animals after the chemotherapy did not show significant difference from that observed with control animals (control initial weight - 170 gm., final - 205 gm; treated initial weight - 163 gm, final 190 gm). Both groups showed approximately the same weight gains.

TABLE V

THERAPY OF WALKER 256 TUMOR BY TSN

| Days after Implantation | Tumor Size scm (Product of 3 diams.) Control, m±SE** | TSN, m ± SE** | $SE_D$** | t** p |
|---|---|---|---|---|
| 7 | 2.68 ±.12 n*=10 | 2.19 ±.21 n=15 | .26 | 1.9 >0.05 |
| 10 | 10.0 ± .50 n=10 | 7.46 ±.48 n=15 | .70 | 3.7 <0.01 |
| 13 | 18.6 ±1.87 n=10 | 12.5±1.38 n=15 | 2.4 | 2.6 <0.05 |
| 15 | 22.8 ±3.72 n=10 | 15.4 ±1.90 n=10 | 4.2 | 1.7 >0.10 |

* n refers to the number of animals with tumors in each case
** See Appendix for the formulae used in statistical analysis

The experimental series where even smaller TSN dose was employed (6 X 125 mg/kg every day) showed no statistically significant difference between the mean values of control and treated groups on any time after the therapy was started.

From the results obtained with TSN on Walker 256 tumor one may conclude that the observed cancerostatic effect with larger doses of this compound might be partially explained by the general toxicity of the compound. However, some cancerostatic effect unassociated with severe toxicity, seems to be demonstrated in the case where smaller doses of the compound were administered (Table V, Figure 8).

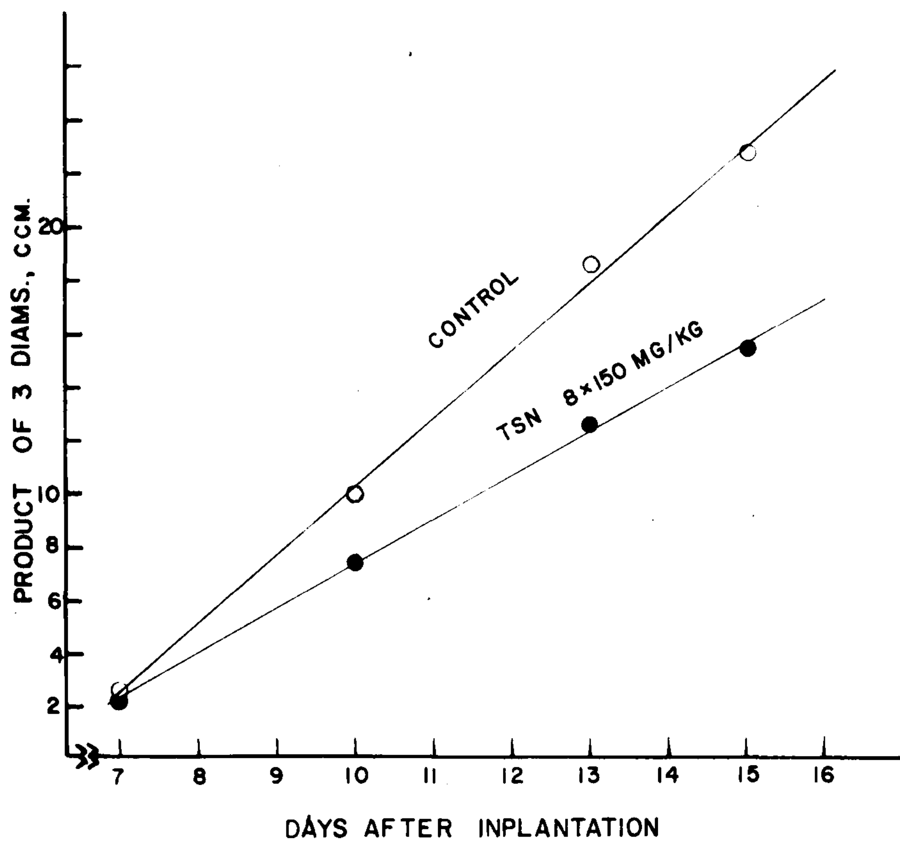



FIGURE 8. THERAPY OF WALKER 256 TUMOR BY TSN

(See Table V)

Histological Study of Walker 256 Tumor after Chemotherapy.

In the course of growth studies on Walker 256 tumor it was established that some forms of chemotherapy by TSN and TSN-TSN effected a reduction in the size of tumor. In order to complete the study the histological examination of tumor slides stained by hematoxylin eosin technique was undertaken.

Since the Walker 256 tumor is a typical malignant growth (carcinosarcoma) attempts were made to ascertain whether or not our chemotherapeutic experiments had any effect on the malignant characteristics of this particular tumor. The following criteria were used in the determination of the degree of malignancy: 1) - mitotic activity expressed as a number of the mitotic figures in one high power field (H.P.F.); 2) - size, form and staining characteristics of cell nuclei; 3) - ratio between plasma and nucleus volume; 4) - development of the intercellular supporting structures (stroma), and 5) - histodifferentiation of the tumor tissue i.e. formation of definite structural elements by tumor cells.

In general it can be stated that there were no striking differences observed between the histological organization of control and treated tumors and of tumors which underwent different types of chemotherapy. The histological picture was similar to that given by Earle (1935) in his classical description of the Walker 256 tumor.

All tumors examined showed a tendency for central necrosis, while the tissue located in subcapsular areas was a rind of healthy growing tumor tissue. This healthy portion of the tumor was used for the microscopical examination. No significant difference in the mitotic activity of treated and control tumors

was observed. In all cases the cell nucleus was large and somewhat vesicular, and varied in shape from ovoid to almost sperical plemorphism. It showed a prominent nucleolus. In two treated tumors (TSN and $B_{12}$ group) there was observed a good uniformity of nuclei in some selected areas. The hyperchromatic nuclei were not numerous in all groups of the tumors. The ratio between the mass of plasma and nucleus of neoplastic cells was not altered considerably from that observable in normal cells.

The stroma showed great variation in the individual tumors both treated and untreated. In some cases it was extremely scanty and delicate and the tumor was made almost solely of epithelial cells. No correlation could be found between the tumor size and the degree of stroma development. One common observation was that the region of tumor tissue with better developed stroma showed less tendency for necrosis. The analogous picture was observed in areas where striated muscle fibers were present. The cells located near muscle fibers showed a more uniform size.

The tumor epithelial cells lay in a disorganized fashion (i.e., small masses, nests or singly) in the matrix of the stromatous fibers. In individual tumors (control and treated) there were observed a few duct-like structures closer to the periphery of the tumor. In one treated tumor these structures were situated more centrally. The cells around these "ducts" were smaller in size and showed more uniformity in shape. The occurrence of these duct-like structures suggested that even after many transplantations there may appear structures which were present in the original spontaneous tumor (adenocarcinoma).

B. Preliminary Study of the Enzymatic Release of $H_2S$.

Since there was observed some cancerostatic effect by the TSN compounds, it became of interest to ascertain whether or not these compounds might exert any effect on some enzymatic system. The selection of an enzyme system concerned with the enzymatic release of $H_2S$ from the cysteine molecule was somewhat arbitrary and was made on a basis of the assumption that the TSN compounds, containing sulfur as a structural component, might somehow affect the sulfur metabolism. Any implication that the observed cancerostatic effects might be due to the effect on this particular enzyme system should be considered premature and speculative in view of the fact that numerous enzyme systems could be affected by these compounds. The second reason for choosing this particular enzyme lies in the fact that a new polarographic method was developed for determination of $H_2S$. Therefore, it appeared of interest to determine whether or not this procedure could be applied for the measurement of the $H_2S$ which is released enzymatically.

Determination of Precision. In order to determine the reproducibility of the $H_2S$ determination with identical enzymatic mixtures four samples of the same liver homogenate were run at two different substrate concentrations. The results are summarized in Table VI.

TABLE VI

DETERMINATION OF THE PRECISION OF THE ENZYMATIC $H_2S$ RELEASE BY LIVER I

| Experiment Number | Cysteine HCl 5 mg. | Cysteine HCl 10 mg. |
|---|---|---|
| | micromoles S | |
| 1 | 1.4 | 3.4 |
| 2 | 1.4 | 3.5 |
| 3 | 1.8 | 3.2 |
| 4 | 1.6 | 2.8 |
| Mean ± SE | 1.6 ± 0.10 | 3.2 ± 0.15 |
| Range | 1.4 - 1.8 | 2.8 3.5 |

3.0 ml. 1:2 homogenate; 1.0 ml. 1-cysteine HCl solution (neutralized); 1.0 ml. saline; 1.0 ml. 0.5 M Tris buffer, pH 7.4; incubation time - 50 min.; C.S. - 0.060 mu a/mm.

Table VII reveals the results obtained when duplicate determinations of enzyme activity were run for varied concentrations of cysteine. Two different homogenate sources were used to determine the precision obtained for each of several given concentrations of cysteine. The maximum difference in duplicate determinations obtained was 0.5 micromoles of S (at the 15 mg. level of cysteine).

As a result of these precision determinations a difference between two values smaller than 0.7 micromoles of S released was considered insignificant at current sensitivity of 0.060 mu a/mm.

TABLE VII

DETERMINATION OF THE PRECISION OF THE ENZYMATIC $H_2S$ RELEASE BY LIVER II

| Cysteine HCl mg. | Micromoles S Produced in Duplicates | |
|---|---|---|
| 5 | 1.6, 1.2 | 1.8, 2.0 |
| 10 | 2.4, 2.5 | 4.8, 5.0 |
| 15 | 2.3, 2.4 | 4.9, 5.4 |
| 20 | —— | 8.7, 9.0 |
| 30 | 4.0, 4.4 | —— |

3.0 ml. 1:2 homogenate; 1.0 ml l-cysteine HCl solution (neutralized); 1.0 ml. saline; 1.0 ml. 0.5 M Tris buffer, pH 7.4; incubation time - 50 min.; C.S. - 0.060 mu a/mm.

Effect of Ionic Strength and Incubation Time.

The effect of the incubation time on the enzymatic activity was determined by the preincubation of the enzymatic reaction mixture for 60 minutes and then adding cysteine HCl 20 mg. and incubating for 50 minutes (the reaction mixture was the same as indicated in Table VII). The amount of the $H_2S$ released was not significantly affected by this treatment: the control value - 3.9 micromoles S, the 60 min. preincubation values - 3.8 and 3.4 micromoles S.

The effect of varying ionic strength and of the presence of ammonium ions on the level of liver CD activity is summarized in Table VIII.

TABLE VIII

THE EFFECT OF IONIC STRENGTH AND AMMONIUM IONS ON THE ENZYMATIC $H_2S$ RELEASE BY LIVER

| | Micromoles S Produced |
|---|---|
| $H_2O$ | 4.0 |
| 0.15 M NaCl | 3.9 |
| 0.30 M NaCl | 3.7 |
| 0.45 M NaCl | 3.7 |
| 5 micromoles $NH_4Cl$ | 4.2 |
| 10 micromoles $NH_4Cl$ | 3.9 |
| 20 micromoles $NH_4Cl$ | 3.6 |
| Mean and SD | 3.9, 0.2 |

3.0 ml. 1:2 homogenate; 1.0 ml. cysteine HCl, 20 mg. (neutralized); 1.0 ml. 0.5 M Tris buffer, pH 7.4; 1.0 ml. $H_2O$, or NaCl solution (conc. as shown), or $NH_4Cl$ in saline (0.15 M NaCl); incubation time - 50 min.; C.S. - 0.060 mu a/mm.

It appears that the differences in ionic strength and the addition of ammonium ion did not demonstrate any marked effect on the level of the enzymatic activity at the concentrations used.

Determination of the Substrate Saturation Level. The effect of varying the substrate concentration was most prounced at lower cysteine levels (5 to 10 mg.). With increasing substrate concentration the leveling of the enzymatic activity was more pronounced although a perfect horizontality could not be obtained with the substrate concentrations used. On the basis of the results obtained it would appear safe to conclude that the substrate region between 10 and 20 mg. of cysteine HCl might be considered as the

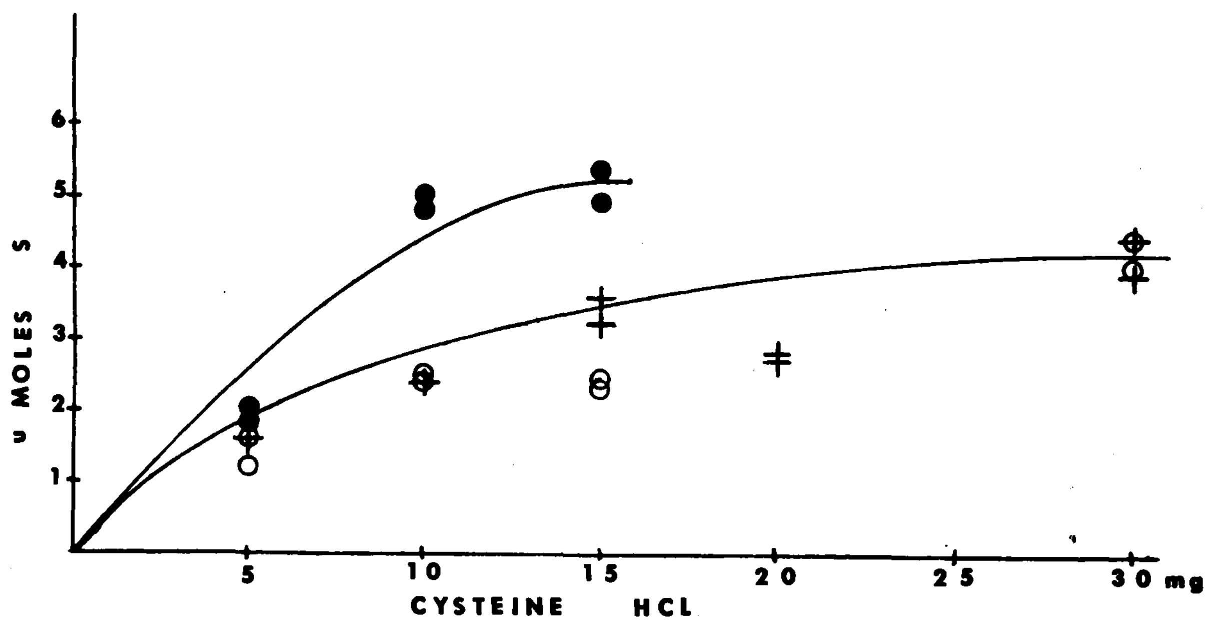



FIGURE 9. SUBSTRATE SATURATION STUDIES

Each Symbol Represents Values Obtained with an Individual Liver (see Table IX)

saturation level (Table IX, Figure 9).

TABLE IX

SUBSTRATE SATURATION STUDIES

| Cysteine HCl mg. | Micromoles S Produced* 1 | 2 | 3 |
|---|---|---|---|
| 5 | 1.4 (1.6; 1.2)** | 1.6 ( - ) | 1.9 (1.8; 2.0) |
| 10 | 2.4 (2.5; 2.4) | 2.4 ( - ) | 4.9 (4.8; 5.0) |
| 15 | 2.4 (2.3; 2.4) | 3.4 (3.5; 3.2) | 5.2 (4.9; 5.4) |
| 20 | - | 2.8 (2.8; 2.7) | 8.8 (9.0; 8.7) |
| 30 | 4.2 (4.0; 4.4) | 4.2 (4.4; 3.9) | - |

3.0 ml. 1:2 homogenate; 1.0 ml. l-cysteine HCl solution (neutralized; 1.0 ml. saline; 1.0 ml. 0.5 M Tris buffer, pH 7.4; incubation time - 50 min.; C.S. - 0.060 mu a/mm.

* Each column represents results obtained with one liver.
** Numbers in parentheses indicate duplicate values.

<u>Time and Activity Relationship.</u> On a basis of the substrate saturation studies it was decided to employ 20 mg. of cysteine HCl as a substrate concentration in the time and activity studies where the effect on the $H_2S$ production by several compounds was tested. The compounds used were alpha-ketoglutaric acid, mixture of alpha-ketoglutaric acid, pyridoxine and biotin, and TSN. Previous work with bacterial cysteine desulfhydrase (Kallio, 1951; Delwiche, 1951) suggested that alpha-ketoglutarate, biotin and pyridoxal $PO_4$ might increase the $H_2S$ production from cysteine. Therefore, it became of interest to see whether or not such an analogous phenomenon could be observed on the liver enzyme at the substrate saturation level in a time course study (Table X, Figures 10, 11, 12).

TABLE X

RELATIONSHIP BETWEEN TIME AND ENZYMATIC $H_2S$ RELEASE BY LIVER

| Time min. | Control, Micromoles S 1* | 2 | 3 | Mean | Activators, Micromoles S 1 | 2 | 3 | Mean |
|---|---|---|---|---|---|---|---|---|
| 30 | 5.9 | 5.3 | 4.7 | 5.3 | 5.9 | 5.2 | 6.0 | 5.7 |
| 60 | 10.2 | 10.7 | 8.2 | 9.7 | 10.7 | 12.4 | 9.4 | 10.8 |
| 90 | 12.1 | - | 10.5 | 11.3 | 13.5 | 15.8 | 13.1 | 14.1 |
| 120 | 17.0 | 16.2 | 14.8 | 16.0 | 15.9 | 18.3 | 16.1 | 16.8 |

3.0 ml. 1:2 liver homogenate; 1.0 ml. l-cysteine HCl. 20 mg.; 1.0 ml 0.5 M Tris buffer, pH 7.4; controls received 1.0 ml. saline; activated samples 1.0 ml. of activators - 1) 5.0 mg. alpha-ketoglutaric acid; 2) 5.0 mg. TSN; 3) KPB mixture - 5.0 mg. alpha-ketoglutaric acid, 1.0 mg. pyridoxine HCl, 2.5 mg. biotin; C.S. - 0.060 mu a/mm.

* Vertical columns represent three individual livers; S values are single determinations.

The comparison of the control and experimental activities indicated that the observed differences were of small magnitude and could be considered as insignificant. This is clearly indicated by the results obtained in the first 60 min. The rate of the production of $H_2S$ in the last 60 min. seems to be changed and there was observed a tendency for the leveling of the enzymatic activity. Therefore, the observed slight differences in the $H_2S$ yields by TSN and KPB in 120 minute period could not be considered too significant. A comparison of the averages of the activity of the control and activated samples at various time intervals points out that the effects by test compounds were insignificant.

Some introductory remarks are pertinent to the interpretation of the following series of experiments. The factors which tend to limit conclusive

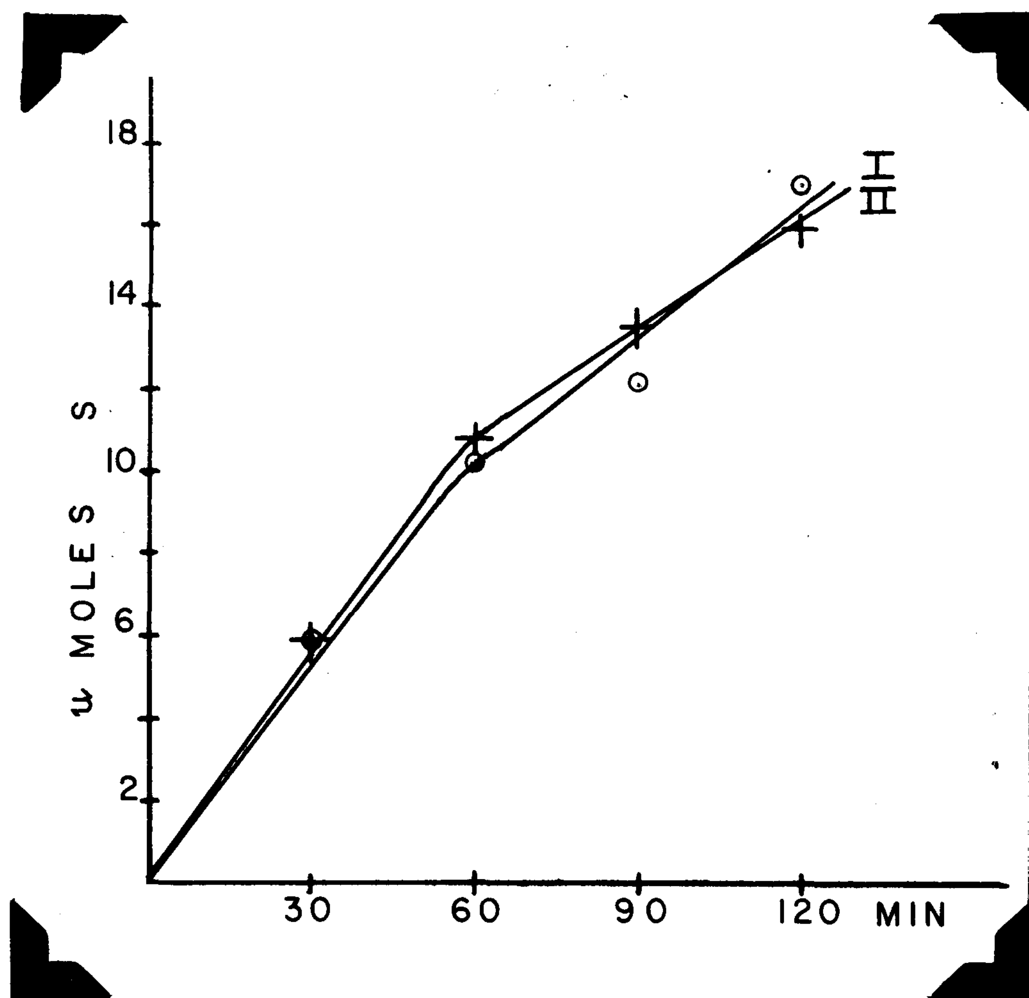



FIGURE 10. RELATIONSHIP BETWEEN TIME AND ENZYMATIC ACTIVITY I (see Table X)

I. Control
II. Alpha-ketoglutarate

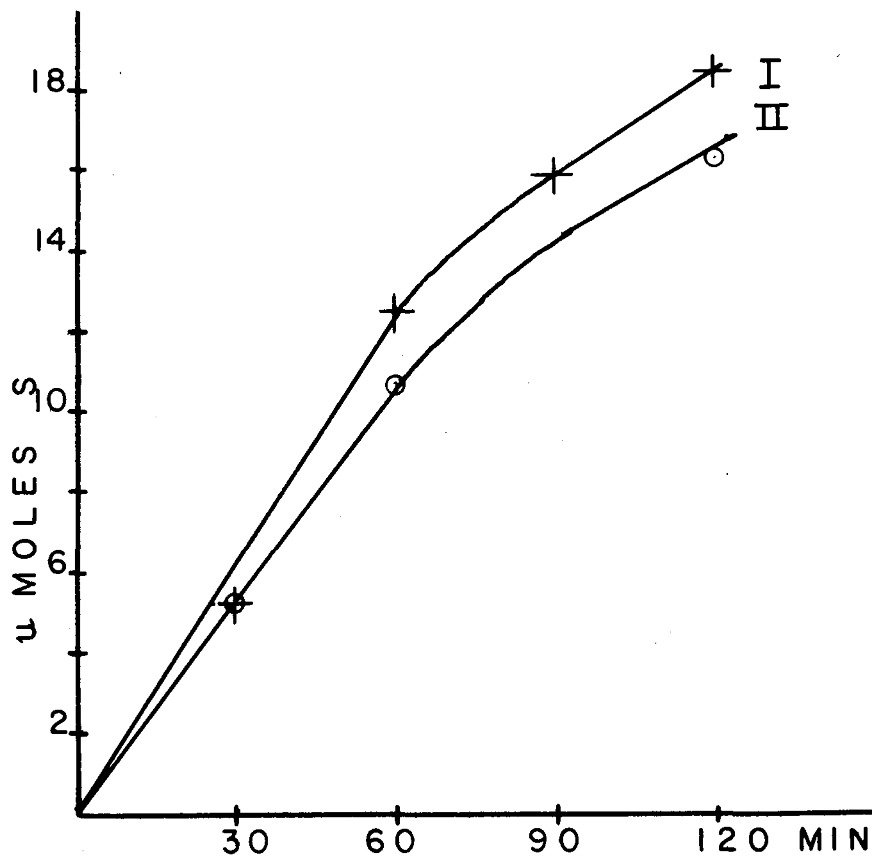



FIGURE 11. RELATIONSHIP BETWEEN TIME AND ENZYMATIC ACTIVITY II (see Table X)

I. TSN
II. Control

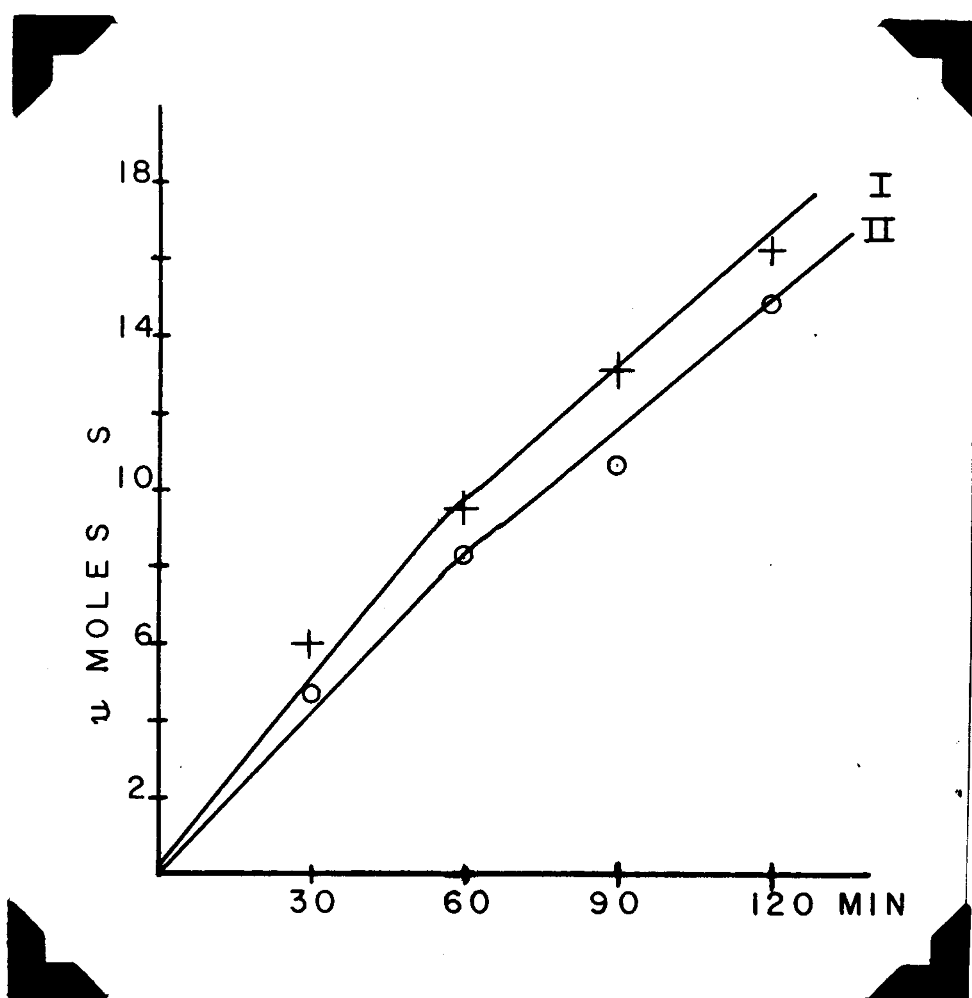



FIGURE 12. RELATIONSHIP BETWEEN TIME AND ENZYMATIC ACTIVITY III (see Table X)

I. KPB
II. Control

evaluation of the results might be summarized in the following manner: 1) the cysteine concentrations used were lower than the substrate saturation level, 2) a prolonged incubation period of 120 minutes might result in a partial loss of the linear relationship between the enzymatic activity and time, 3) the use of crude homogenate as an enzyme source might give results which are only indirectly related to the enzyme system under consideration.

These limitations apply particularly to the experiments where various compounds with a potential augmenting or depressive effect on the $H_2S$ production were studied. The same remarks might be applied but to a lesser degree to the study of the enzyme distribution effect on the liver injury on the level of enzymatic action, and to the attempts to localize the $H_2S$ producing enzyme system, with cysteine serving as a substrate in the Walker 256 tumor, although in the latter case the overall production of $H_2S$ over a longer time period might be more safely used as an indication of the enzymatic activity.

Organ Distribution. The liver tissue possessed the most pronounced CD-like activity of the organs examined. Kidney (no differentiation was made as to medulla and cortex) demonstrated approximately one-tenth of the liver activity. The other organs (brain, spleen, small intestine) did not show any measurable $H_2S$ release under the experimental conditions used. It is interesting to note that the homogenate of small intestine did not show any $H_2S$ production. This was true even though it might be expected to have possible residual bacterial contamination. It would appear that a careful washing of the intestinal tissue by saline effected the removal of the bacterial contamination.

TABLE XI

ORGAN DISTRIBUTION OF CYSTEINE DESULFHYDRASE-LIKE ENZYME

| Organ* | S Micromoles Produced | | |
|---|---|---|---|
| Liver | 3.40 | 4.70 | 6.0 |
| Kidney | 0.30 | 0.45 | 0.85 |
| (Spleen, Brain, Small Intestine) | No Activity | | |

3.0 ml. 1:3 homogenate; 1.0 ml. - 5 mg. l-cysteine HCl (neutralized); 1.0 ml. 0.5 M Tris buffer, pH 7.4; incubation time - 120 min.; C.S. - 0.030 mu a/mm.

* Determined on three different rats.

Liver Injury and the Enzymatic $H_2S$ Release by Liver.

Before describing the effects on CD-like activity caused by liver injury and the subsequent regeneration process of liver tissue, a few remarks may be pertinent concerning the method of injury. The abdominal cavity was opened, liver located, and a part of an individual liver lobe was injured either by crushing with forceps (second day postoperative series) or by making a few incisions with scissors (fourth and sixth day postoperative series), whereupon the peritoneal cavity was closed. The lobes which were left uninjured were control lobes whose $H_2S$ production was considered as the control level of CD-like activity. Only healthy portions of the injured lobe, adjacent to the site of trauma, were used for enzymatic study, and their $H_2S$ production was labeled as the "injured" value of CD-like activity. Keeping in mind the differences in the method of injury in the first group of animals from the other two series

(fourth and sixth days postoperative) one still could observe a definite trend in the enzymatic activity during the postoperative period (Table XII).

TABLE XII

LIVER INJURY AND CYSTEINE DESULFHYDRASE-LIKE ACTIVITY

| 2nd Day Postoperative | | 4th Day Postoperative | | 6th Day Postoperative | |
|---|---|---|---|---|---|
| Control | Injured | Control | Injured | Control | Injured |
| | | Micromoles S | | | |
| 11.5 | 5.5 (48%) | 2.4 | 0.5 (20%) | 4.5 | 5.8 (128%) |
| 5.0 | 1.6 (32%) | 1.7 | 1.2 (71%) | 3.6 | 4.2 (117%) |
| 8.2 | 2.5 (30%) | 4.9 | 3.6 (73%) | 3.8 | 4.4 (116%) |
| 6.1 | 3.0 (50%) | 3.9 | 2.4 (62%) | 2.6 | 2.3 (88%) |
| | | 2.6 | 2.4 (93%) | 3.6 | 3.9 (103%) |
| | | 5.2 | 5.8 (112%) | 2.1 | 1.9 (90%) |
| Mean: | | | | | |
| 7.7 | 3.2 (42%) | 3.4 | 2.6 (76%) | 3.4 | 3.8 (112%) |

2nd Day - 3.0 ml. 1:2 homogenate; 1.0 ml. l-cysteine HCl (neutralized); 1.0 ml. 0.5 M Tris buffer, pH 7.4; C.S. - 0.060 mu a/mm.
4th and 6th days - 3.0 ml. 1:3 homogenate; 1.0 ml. l-cysteine HCl, 5 mg. (neutralized); 1.0 ml. 0.5 M Tris buffer, pH 7.4; C.S. - 0.030 mu a/mm.
Incubation time - 120 min.

On the second day postoperatively, there was observed a marked decrease in the CD-like activity in the injured lobe, reaching about 40 per cent of control level. On the fourth day, there was some recovery of CD-like

activity, but it appeared to be below the level of control activity. After six days, the enzyme activity was fully recovered, and in some cases, it showed an activity higher than that observed with normal lobes. From these results, one may conclude that a definite waiting period was required for complete recovery of CD activity in the tissue adjacent to the trauma site. The level of enzymatic activity of normal lobes of rats with injured liver did not change during recovery period, as observed on the fourth and sixth days postoperatively. Therefore, it would seem that the effect of injury on CD-like activity was restricted to the tissue adjacent to trauma site.

Alpha-Ketoglutarate and the Enzymatic $H_2S$ Release by Liver.

In the presence of alpha-ketoglutarate increased $H_2S$ production from cysteine by liver homogenate could be observed at lower substrate levels (cysteine HCl 5 mg.) and prolonged incubation time (120 min.); see Table XIII, Figure 13. The augmenting effect was more pronounced in cases which showed a relatively weak control activity. The analogous situation was observed employing higher substrate concentration (cysteine HCl 10 mg.), see Table XIV. But with the substrate levels at a definite saturation concentration (20 mg.) no increased $H_2S$ production by alpha-ketoglutarate could be observed (see Table X and Figure 10).

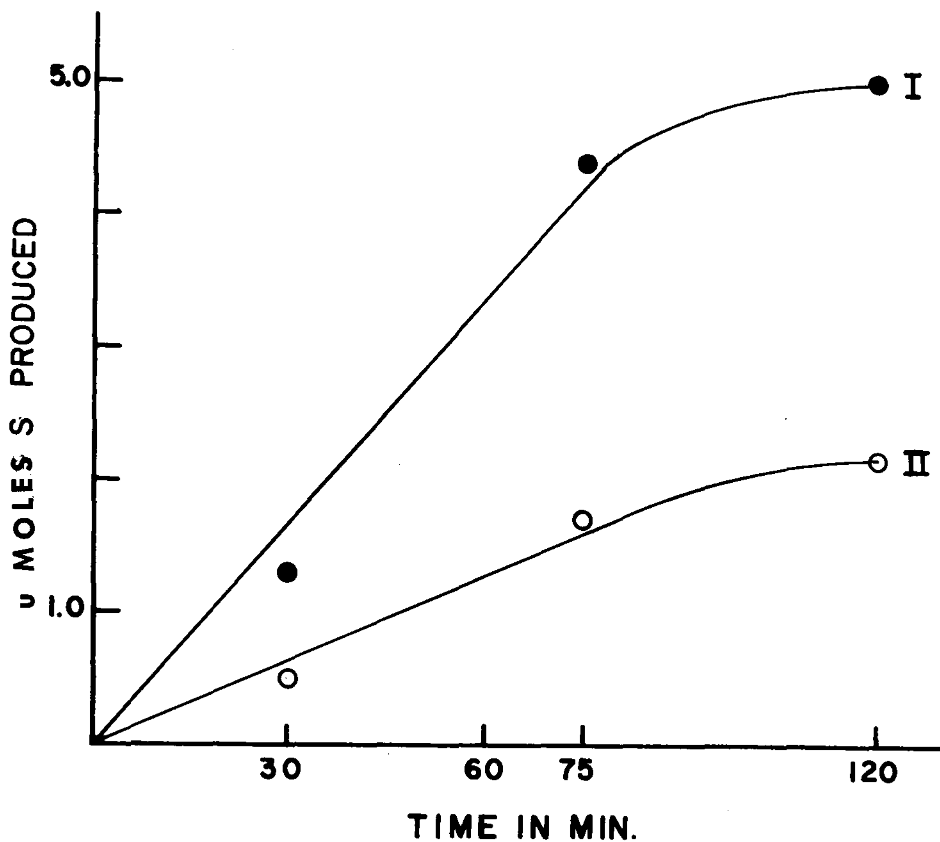



FIGURE 13. RELATIONSHIP BETWEEN TIME AND ENZYMATIC ACTIVITY IV

3.0 ml. 1:2 liver homogenate; 1 ml., 5.0 mg. l-cysteine HCl (neutralized).

I. 1.0 ml., 5 mg. alpha-ketoglutaric acid (Na salt).

II. 1.0 ml. saline.

TABLE XIII

LIVER CYSTEINE DESULFHYDRASE-LIKE ACTIVITY AND

ALPHA-KETOGLUTARATE

| Experiment Number | Control<br>Micromoles S | Alpha-Ketoglutaric Acid 5 mg.<br>Micromoles S |
|---|---|---|
| 1 | 1.9 | 4.0 |
| 2 | 3.1 | 6.0 |
| 3 | 6.3 | 6.4 |
| 4 | 7.1 | 9.1 |
| 5 | 2.6 | 5.2 |
| 6 | 2.0 | 6.6 |
| 7 | 3.1 | 4.3 |
| 8 | 4.5 | 8.2 |
| 9 | 3.6 | 5.5 |
| 10 | 3.8 | 6.0 |
| 11 | 5.0 | 7.7 |
| 12 | 4.3 | 3.3 |
| Mean ± SE | 3.9 ± 0.46 | 6.9 ± 0.59 |
| t = 4.3 p = 0.01 | | |

3.0 ml. 1:2 homogenate; 1.0 ml. l-cysteine HCl (neutralized), 5 mg.; 1.0 ml. alpha-ketoglutaric acid (Na salt), 5 mg. or 1.0 ml. of saline (control); incubation time - 120 min.; C.S. - 0.060 mu a/mm.

The results obtained with l-glutamate, an $NH_2$ analogue of alpha-ketoglutarate, are of interest, since these two compounds showed antagonistic effects on the $H_2S$ production from cysteine (Table XIV). It would appear probably that the equilibrium state between these two compounds might be a factor influencing the control levels of CD-like activity.

TABLE XIV

LIVER CYSTEINE DESULFHYDRASE-LIKE ACTIVITY AND EFFECT

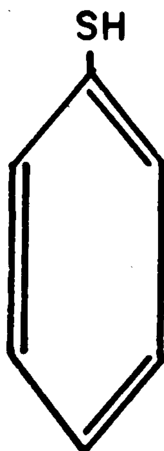
OF ALPHA-KETOGLUTARATE AND L-GLUTAMATE

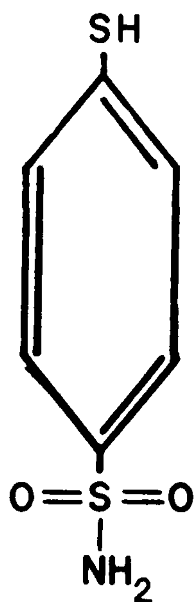
| Experiment Number | Control | Alpha-Ketoglutaric Acid-5 mg. | L-Glutamic Acid-5 mg. | Alpha-Ketoglutaric Acid - 5 mg. L-Glutamic Acid 5 mg. |
|---|---|---|---|---|
| | Micromoles S | | | |
| 1 | 17.1 | 18.2 | 14.6 | 15.8 |
| 2 | 16.3 | 17.7 | 12.3 | 16.5 |
| 3 | 6.8 | 12.4 | 4.4 | 10.4 |
| 4 | 5.0 | 10.1 | 3.6 | 8.6 |
| Mean | 11.3 | 14.6 | 8.7 | 12.8 |

3.0 ml. 1:2 homogenate; 1.0 ml. l-cysteine HCl (neutralized), 10 mg.; 1.0 ml. saline (controls) or 1.0 ml. alpha-ketoglutaric acid or l-glutamic acid (Na salts), 5 mg., alone or combined; 1.0 ml. Tris buffer, pH 7.4; incubation time - 120 min.; C.S. - 0.060 mu a/mm.

Structure and Activity Relationship (SAR) Among TSN Analogues

(Figure 14). The effect obtained with equimolar 0.004 M solutions of TSN and sulfanilamide on the CD-like activity in liver suggested that the latter compound proved to be slightly inhibitory. This effect is just the opposite to the observed with TSN. (No enzymatic breakdown of TSN with release of $H_2S$ in liver homogenates occurs.) It would appear that these compounds might possess a qualitatively different effect on the enzymes involved in the $H_2S$ release from cysteine. But on the other hand a possibility stands out that the observed effects might be caused by the inhibition of other enzymes involved in the cysteine breakdown, thus creating different substrate levels for the CD-like



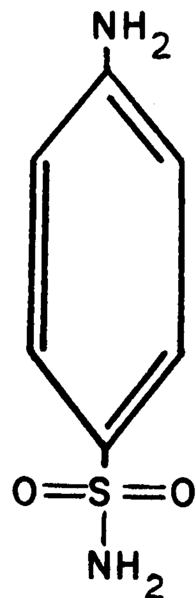




FIGURE 14. STRUCTURAL ANALOGUES OF TSH COMPOUNDS

A. Thiophenol
B. TSH
C. Sulfanilamide

enzymes. Such a reasoning has support in view of the fact that the substrate levels used were below the saturation point.

TABLE XV

STRUCTURE AND ACTIVITY RELATIONSHIP AMONG TSN ANALOGUES

| Control | | $4 \times 10^{-3}$ M TSN | | $4 \times 10^{-3}$ M Sulfanilamide | |
|---|---|---|---|---|---|
| Micromoles S | | Micromoles S | | Micromoles S | |
| 5.1 | 100% | 8.5 | 167% | 3.3 | 35% |
| 8.4 | 100% | 11.0 | 131% | 7.6 | 90% |

3.0 ml. 1:2 homogenate; 1.0 ml. = 5 mg. l-cysteine (neutralized); 1.0 ml. 0.5 M Tris buffer, pH 7.4; 1.0 ml. test solution (Na salts); incubation time = 120 min.; C.S. = 0.060 mu a/mm.

The presence of thiophenol ($4 \times 10^{-4}$ or $1.3 \times 10^{-4}$ Molar) in liver homogenates caused an observable increase in the enzymatic production of $H_2S$ with cysteine serving as a substrate (Table XVI). The use of thiophenol at concentrations equivalent to those of TSN and sulfanilamide proved to be impractical, because thiophenol (due to its vapor pressure) would leave reaction mixture and combine with Cd ions thus interfering with $H_2S$ determination. At concentrations used there was no interference by thiophenol, because a homogenate blank (homogenate, buffer and thiophenol) was equivalent to the reagent blank (saline, cysteine, buffer).

TABLE XVI

LIVER DESULFHYDRASE-LIKE ACTIVITY AND THIOPHENOL

| Control | Thiophenol | | | |
|---|---|---|---|---|
| | $4 \times 10^{-3}$ M | $2 \times 10^{-3}$ M | $4 \times 10^{-4}$ M | $1.3 \times 10^{-4}$ M |
| Micromoles S | | | | |
| 4.5 | -* | -* | 7.0 | 7.0 |

3.0 ml. 1:2 homogenate; 1.0 ml. - 5 mg. l-cysteine HCl (neutralized); 1.0 ml. 0.5 M Tris buffer, pH 7.4; 1.0 ml. thiophenol solution; final volume 6.0 ml.; incubation time - 120 min.; C.S. - 0.060 mu s/mm.

* liver homogenate containing only thiophenol gave high blank value due to its combining power with Cd.

TSN Effect on the Enzymatic $H_2S$ Release by Liver.

The presence of TSN in the form of the sodium salts appeared to increase the $H_2S$ production from cysteine at concentrations below the substrate saturation level (there was no enzymatic breakdown of TSN with release of $H_2S$ as determined in preliminary experiments). This augmenting effect by TSN appeared to be statistically significant at this substrate concentration. The analogous action of TSN at a substrate saturation level (20 mg. cysteine HCl) was not significant as revealed by the time and activity curves (Table X, Figure 11).

TABLE XVII

LIVER CYSTEINE DESULFHYDRASE-LIKE ACTIVITY AND TSN

| Experiment Number | Control Micromoles S | TSN 5 mg. Micromoles S |
|---|---|---|
| 1 | 1.4 | 6.5 |
| 2 | 1.6 | 6.6 |
| 3 | 4.2 | 9.6 |
| 4 | 5.5 | 7.7 |
| 5 | 6.6 | 10.7 |
| 6 | 10.5 | 14.0 |
| Mean ± SE | 5.0 ± 1.4 | 9.2 ± 1.2 |
| $t = 2.3$ | | |
| $p = 0.05$ | | |

3.0 ml. 1:2 homogenate; 1.0 ml. l-cysteine, 10 mg. (neutralized); 1.0 ml. 0.5 M Tris buffer, pH 7.4; 1.0 ml. test solution or 1.0 ml. saline; incubation time - 120 min.; C.S. - 0.060 mu a/mm.

Thioacids and the Enzymatic $H_2S$ Release by Liver.

It was of interest to compare the effects of some aliphatic thioacids on the enzymatic production of $H_2S$ by liver with cysteine serving as the substrate. The earlier evidence by Lawrence and Smythe (1943) indicated that thioglycolate and thiobutyrate inhibited the enzyme system, cysteine desulfhydrase, which produces $H_2S$ from cysteine. The results summarized in Table XVIII indicated that mercaptopropionic and mercaptosuccinic acid had an observable inhibitory effect which was more pronounced at lower homogenate (enzyme) concentrations (no enzymatic breakdown of these compounds with resulting $H_2S$ release), see Figure 15. It is difficult to say anything regarding the type of the inhibition, because we are dealing with an impure enzyme preparation, and secondly, there is no linear relationship between the amount of $H_2S$ produced and enzyme

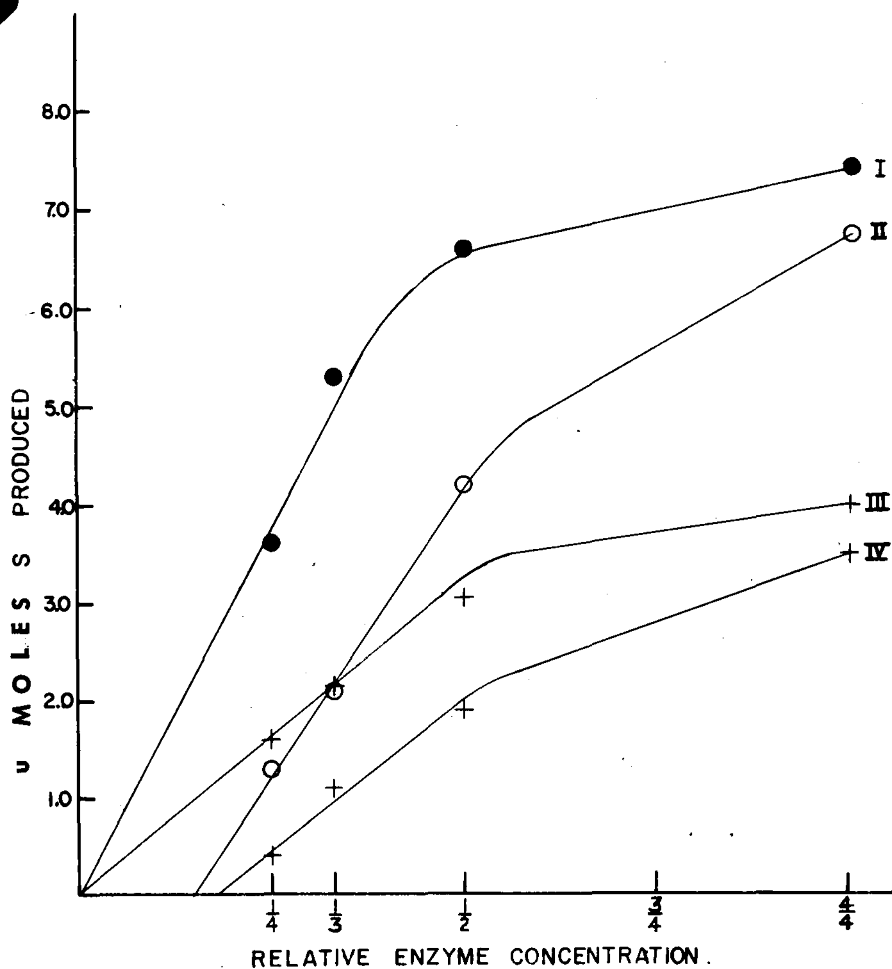



FIGURE 15. INHIBITION OF LIVER CYSTEINE DESULFHYDRASE-LIKE ACTIVITY BY THIOACIDS
(see Table XVIII)

I. Control of II.
II. $2.64 \times 10^{-3}$ M mercaptosuccinic acid.
III. Control of IV.
IV. $3.10 \times 10^{-3}$ M mercaptopropionic acid.

concentration with higher homogenate concentrations.

TABLE XVIII

THIOACIDS AND CYSTEINE DESULFHYDRASE-LIKE

ACTIVITY IN LIVER

| Homogenate Concentration | Control | Mercapto-succinic $2.64 \times 10^{-3}$M | Control | Mercapto-propionic $3.10 \times 10^{-3}$M |
|---|---|---|---|---|
| | Micromoles S | | | |
| 1:1 | 7.4 | 7.0 (94.6%) | 4.0 | 3.5 (87.5%) |
| 1:2 | 6.6 | 4.2 (63.7%) | 3.1 | 1.9 (61.3%) |
| 1:3 | 5.3 | 2.1 (39.6%) | 2.2 | 1.1 (50.0%) |
| 1:4 | 3.6 | 1.3 (36.1%) | 1.6 | 0.4 (25.0%) |

3.0 ml. homogenate; 1.0 ml. l-cysteine HCl, 5 mg. (neutralized); 1.0 ml. 0.5 M Tris buffer, pH7.4; 1.0 ml. test solution (Na salts) or 1.0 ml. saline; incubation time - 120 min.; C.S. - 0.030 mu a/mm.

Enzymatic $H_2S$ Production and Walker 256 Tumor.

The results of preliminary experiments designed to determine the enzymatic production of $H_2S$ from cysteine by Walker 256 tumor suggested a complete absence of this activity in tumor tissue. From this experimental series it appeared that the analogous enzymatic activity in liver was not affected to any considerable degree by the presence of tumor growth, as was found by earlier workers (Greenstein, 1954).

TABLE XIX

CYSTEINE DESULFHYDRASE-LIKE ACTIVITY
IN LIVER AND WALKER 256 TUMOR

| Experiment Number | Liver* Micromoles S | Tumor* Micromoles S |
|---|---|---|
| 1 | 3.3 | 0.0 |
| 2 | 7.6 | 0.0 |
| 3 | 7.1 | 0.0 |
| 4 | 7.9 | 0.0 |
| 5 | 5.1 | 0.0 |
| Average | 6.2 | 0.0 |

3.0 ml. 1:2 homogenate; 1.0 ml., 5 mg. l-cysteine HCl (neutralized); 1.0 ml. 0.1 M phosphate buffer, pH 7.4; final volume - 5.0 ml.; incubation time - 120 min.; C.S. - 0.060 mu a/mm.

* Liver and tumor tissues came from the same animal in each experiment.

The experiments with Walker tumor employing the supernatants of homogenates of higher tumor tissue concentration are summarized in the following table. In addition, the mixture of activators was used in effort to stimulate any residual enzymatic activity which could not be detected using lower tissue homogenates. The activators (biotin, pyridoxin, alpha-ketoglutaric acid) used were those which have been found to be effective in raising the level of the activity of the similar enzyme system of bacterial origin. The observed $H_2S$ production (9 times out of 14 tumors) employing these activators was of a very small magnitude and the amounts produced approached the limit of the sensitivity of method. A possibility that these minute amounts of $H_2S$ might be the result of bacterial contamination could not be

ruled out completely. Nevertheless, attempts were made not to use any tumor showing superficial necrotic processes through which the bacterial invasion could occur. Secondly, with each tumor the reagent blank control was run which did not indicate any $H_2S$ production and gave similar values to those of tumor samples which did not show any residual activity.

TABLE XX

WALKER 256 TUMOR CYSTEINE DESULFHYDRASE-LIKE ACTIVITY AND ACTIVATION

| Experiment Number | Control | KPB |
|---|---|---|
| | Micromoles S | Micromoles S |
| 1 | 0.75 | 0.75 |
| 2 | 0.80 | 0.66 |
| 3 | 0.0 | 0.55 |
| 4 | 0.0 | 0.55 |
| 5 | 0.0 | 0.50 |
| 6 | 0.0 | 0.45 |
| 7 | 0.0 | 0.45 |
| 8 | 0.0 | 0.45 |
| 9 | 0.0 | 0.40 |
| 10 - 14 | 0.0 | 0.0 |

3.0 ml. 1:1 homogenate; 1.0 ml. l-cysteine HCl, 5 mg. (neutralized); 1.5 ml. KPB solution (5 mg. alpha-ketoglutaric acid (Na salt), 1 mg. pyridoxine HCl (neutralized), 2.5 mg. biotin (Na salt)); or 1.5 ml. saline; incubation time - 120 min.; C.S. - 0.030 mu a/mm.

# CHAPTER V

## DISCUSSION

### A. Chemotherapy of Walker 256 Tumor.

The chemotherapeutic experiments designed to show the effect of TSN-TSN and TSN on the Walker 256 tumor yielded results indicating that these compounds might possess some cancerostatic properties. The results obtained will be discussed with respect to some of the complicating factors (toxicity, effects on tumor histology, etc.) which may limit the degree to which definite conclusions can be reached, and secondly, an attempt will be made to compare the effects of these compounds with the effects obtained by others using different aromatic and aliphatic sulfur compounds.

The preliminary experiments with TSN-TSN (Table II) showed a marked TSN-TSN cancerostatic effect, but the death rate among the treated animals was considerable at the dosage schedule used. In the second experimental series where reduced dosage was used and therapy was started one day after transplantation (7 x 250 mg./kg. TSN-TSN every second day) there was observed a marked reduction of the tumor volume among treated animals (Table III, Figure 6). The differences in the tumor size were found to be statistically significant. But there were some complicating factors which can be summarized in following manner: 1) presence of general toxicity as shown by the increased mortality rate of treated animals (six treated animals died out of 24 versus two control deaths out of 16 animals). Nevertheless, the histological examination of the

organs (liver, kidney) did not reveal any significant changes which would appear to be irreversible in nature. 2) Absence of any effect upon the establishment of tumor growth. 3) Occurrence of metastatic growth in two treated animals. 4) Absence of any clearly defined histological effect upon tumor tissue (eosin hematoxylin stain). A difference between the control and treated group was apparent in that the treated animals showed a tendency for the accumulation of edematous fluid in the peritoneal cavity. This was probably caused by the presence of drug which failed to be absorbed from the intraperitoneal injection site. It did not appear probable that this edema could have been caused by kidney damage, since the kidney changes observed histologically were of minor degree.

No direct comparison of the potency of TSN-TSN with the potency of the aromatic sulfur compounds studied by others is possible, because of the differences in tumors, animals, and route and schedule of administration used in the course of the therapy. The compound with closest structural similarity to TSN-TSN tested by Boyland (1946) on a basis of the nature of the linkage between two phenyl groups was 2:2'-diaminodiphenyldisulfide. This compound was administered at a dose of 2 mg. per mouse (approximately 100 mg./kg.) twelve times, and its cancerostatic effect was observed only in the case of the spontaneous mouse tumor, while it did not exhibit any effect on transplanted sarcomas. The results obtained with TSN-TSN suggested that it had cancerostatic properties on transplanted Walker 256 tumor in the rat. It is premature to say anything about the spectrum of the cancerostatic properties of these two compounds, but on a basis of the available evidence it is conceivable that they might have

different spectra.

A more detailed analysis of the structural formulae of these compounds points out the differences both in the nature and the relative position of the substituent on phenyl radical. The $NH_2$ group in the TSN-TSN molecule is not directly attached to the phenyl radical, but rather is included in the sulfonamide radical in para position to the disulfide linkage as opposed to the direct amino substitution to phenyl in 2:2' positions in Boyland's compound (2:2'-diaminodiphenyldisulfide). It is possible that these differences in structure might contribute to the nature of the chemotherapeutic effect. Boyland (1938) found that compounds with para $NH_2$ groups (e.g., 4:4'-diaminodiphenylsulfoxide) had some cancerostatic effect on transplanted Crocker 180 sarcoma. Further, it was demonstrated that compounds with 4:4'-$NH_2$ substitution were more effective as cancerostatic agents than those with 4:2'-$NH_2$ substitution against spontaneous mouse tumors, although they did not have any significant effect against the transplanted MCBI sarcoma in the mouse (Boyland, 1946).

The cancerostatic effect of the effective compounds tested by Boyland (1938, 1946) on spontaneous mouse tumors was limited to the time of drug administration, because after cessation of therapy the treated tumor showed the same growth rates as untreated ones. In that respect the results obtained with TSN-TSN on Walker 256 tumor are qualitatively similar, because it does not seem from the results obtained that the effect obtained could be of longer duration, since the growth was observable even during the period of drug administration (Figure 6).

The therapy of Walker 256 tumor by TSN at two different dose levels (Tables IV, V) yielded results suggesting that this compound possessed some

cancerostatic properties under the experimental conditions employed. The drug administration was started in both cases seven days after implantation (when tumors were palpable). The results with a higher daily dose (200 mg./kg. for eight consecutive days) revealed that such a form of therapy inhibited the tumor growth rate significantly, and it appeared that the cancerostatic effect was more evident at the earlier stages of tumor growth as seen from the growth curves. The growth rate in later stages was somewhat increased, but stayed below that of the control tumors (Figure 7). The final size of treated tumors was considerably smaller than that of control tumors and the difference appeared to be significant statistically (Table IV, Figure 7). The death rate was higher in treated group (five deaths out of 20 animals, while no death occurred in the control group during aperiod of 16 days). The weight differences between the two groups after therapy were insignificant. The histological examination of kidney and liver tissues revealed some damage, but it did not appear that the extent of damage was irreversible in nature in the majority of cases. Nevertheless, it would appear that the general toxicity of the compound might adumbrate its real cancerostatic action.

For reasons outlined in the preceeding paragraph an experimental series using smaller dosage schedules was undertaken (8 daily doses 150 mg./kg. TSN, therapy started on 7th day after implantation). This therapeutic regimen showed decreased cancerostatic effects. The observed differences in tumor size were statistically significant only at 10 and 13 days after implantation, while on 15th day the differences were insignificant (Table V, Figure 8). The death rate among treated animals was reduced (one death out of 15). There was no

initial inhibition of the growth rate observed as in previous experimental series (Figure 7 and 8). These results would suggest that a more pronounced cancerostatic effect by TSN with larger doses might be partially explained by the general toxicity of the compound, although some effect might be demonstrated with no significant toxicity as shown by the last series of animals. At any rate the cancerostatic effects observed did not appear to be permanent, because the growth of tumors continued even at the time of drug administration.

The chemotherapeutic attempts were made with a combination of TSN (200 mg./kg.) and vitamin $B_{12}$ (5 micrograms per each administration of TSN as outlined before). The reasons for using such a combination are as follows. Woolley (1953) found that spontaneous tumors were able to synthetize vitamin $B_{12}$, while none of the transplanted tumors which he tested possessed this characteristic. Secondly, the work by Boyland (1938) indicated that several aromatic sulfur compounds (para-aminobenzenesulfonamide, p-aminobenzenesulfonate, Na-parahydrazinobenzenesulfonate, and para-heptylaminobenzenesulfonate) had some effect against spontaneous tumors, while their action on transplanted Crocker sarcoma 180 was either considerably weaker or completely absent. On a basis of these findings it was of interest to ascertain whether or not the combination of TSN and vitamin $B_{12}$ might have a different effect than TSN alone. The results obtained did not suggest any chemotherapeutic improvement over the simple TSN administration. The cancerostatic action was somewhat decreased (Table V, Figure 7), but the mortality of the TSN plus vitamin $B_{12}$ treated animals was apparently reduced in comparison to TSN group (TSN group - 5 deaths out of 20; TSN plus vitamin $B_{12}$ group - 2 deaths out of 20; no deaths

in control group out of 13 animals). The changes in the organs examined (kidney and liver) were similar to those seen in the TSN animals. No histological effect on the tumor structure was observed.

The comparison of the chemical structure of TSN and other organic sulfur containing compounds (Boyland, 1938) would suggest that the replacement of para-$NH_2$ group in sulfanilamide molecule by SH did not cause a disappearance of cancerostatic properties of the original molecule. In fact it would appear on a basis of the comparison of the size of doses of TSN and the other compounds used by Boyland (1938) that TSN might be more potent. It is interesting to note that the doses of compounds containing only one phenyl radical were considerably larger than those of the diphenyl counterparts. A similar difference apparently does not exist between the size of effective doses of TSN and TSH-TSN.

The author's search of literature for the systemic toxicity of para-thiocresol did not yield any information on this point. Therefore, nothing definite can be said regarding the comparative toxicity of TSN and para-thiocresol. But in view of the fact that para-thiocresol was not used for any systemic administration as therapy and that, in general, the phenolic compounds have higher systemic toxicity one might postulate that TSN would have lesser toxicity than para-thiocresol.

The cancerostatic results with TSN are also of interest insofar as the results obtained with it differ from those obtained by administration of some aliphatic compounds with SH groups (thiomalic and thioglycolic acids). The aliphatic SH compounds cause slight stimulation of transplanted rat tumor

(Brunschwig et. al., 1946). It would appear that the aromatic SH radicals might have a different functional role than that of the aliphatic SH group, although it is premature to make this statement in any absolute manner, since nothing definite is known about the metabolic pathways of the TSN-like compounds.

B. Preliminary Studies on the Enzymatic $H_2S$ Release.

In the course of this work a method has been developed for the determination of $H_2S$. It is based on the quantitative determination of free residual cadmium ions after an excess of Cd has reacted with $H_2S$ to form CdS. Use of this method has been made in following the enzymatic degradation of cysteine with resulting release of $H_2S$. Therefore, it appeared appropriate to discuss other available methods used for following cysteine degradation. They fall into three general groups: $H_2S$, $NH_3$ and pyruvate determinations. From a survey of the used it seems that the pyruvate determination is least commonly employed because Smythe (1942) has shown that pyruvate amounts yielded were not quantitatively proportional to the $H_2S$ or $NH_3$ evolved from enzyme reaction mixtures containing unpurified enzyme preparations. The use of $NH_3$ determination by Nessler's procedure appears to be more common perhaps because of the widespread general use of this method. But one drawback of this technique might be caused by the fact that sometimes there occurs a considerable production of endogenous $NH_3$ by crude homogenate preparations (Greenstein and Leuthard, 1945-46). This factor might interfere with the accuracy of cysteine $NH_3$ determination in cases where $NH_3$ production from cysteine is low. Secondly, it is conceivable that in studying the effects of various compounds on cysteine degradation on basis of $NH_3$ measurement might be rather inaccurate, because the ammonia produced might

enter into some secondary enzymatic reactions. It was shown by Cammarata and Cohen (1950) that cysteine is involved in the transamination reactions with alpha-ketoglutarate. On a basis of these considerations it would appear that the $H_2S$ determination might be a more specific indicator of cysteine degradation. Smythe (1942) found that there is no measurable endogenous production of $H_2S$ and the $H_2S$ produced by CD activity can be recovered quantitatively under anaerobic experimental conditions.

The conventional methods of $H_2S$ measurement can be divided into two groups: 1.- spectrophotometric determination of metal sulfides, e.g., ZnS or PbS (Fromageot, 1951; Delwiche, 1951), 2.- iodometric determination of S ion (Masters, 1938; Smythe, 1955). From these methods the spectrophotometric is considerably more sensitive - minimal detectable amount is 3 micrograms of sulfur in a range of 3 to 100 micrograms of sulfur. A larger minimum value of 150 - 200 micrograms of S is obtained with the iodometric technique (titration of the excess of iodine with thiosulfate after iodine has been reduced to iodide by S ions). The minimal detectable amount of S employing our method (with higher and lower sensitivity on polarograph) appeared to be 10 and 20 micrograms of S (0.3 and 0.7 micromoles), see Figures 4, 5 and page 28. The maximal amount of S which might be determined could be approximately 20 micromoles of S on a basis of the Cd ions available. From these considerations it would appear that our method is less sensitive to spectrophotometric method, but more sensitive than the iodometric technique. But the range of $H_2S$ determined is considerably broader in our case than that of the spectrophotometric method (Delwiche, 1951). Our polarographic method could be applied convenient-

ly in cases where Warburg vessels are used for trapping $H_2S$ by Cd ions in the center well and determining free Cd ions after an excess of cadmium has reacted with $H_2S$ to form insoluble CdS. Thus our method might find application employing the standard incubation vessels as they are used with iodometric technique. The advantage afforded over the iodometric method is a greater sensitivity of our method.

The applicability of our method for biological work is demonstrated by results in Tables VI and VII, where the reproducibility of the $H_2S$ measurement from four identical samples of liver homogenate was determined at two different substrate concentrations (5 and 10 mg. cysteine HCl). The results obtained under these experiental conditions were as follow: cysteine HCl 5 mg. = 1.6 $\pm$ 0.10 micromoles S, range 1.4 - 1.8; cysteine HCl 10 mg. = 3.2 $\pm$ 0.15 micromoles S, range 2.8 - 3.5. The determination of $H_2S$ production by duplicate samples demonstrated a maximum difference of 0.5 micromoles S between two enzyme mixtures. As a result of these studies a difference in the $H_2S$ production by two samples smaller than 0.7 micromoles of S is considered as insignificant.

On a basis of the above criteria a study of the effect of ionic strength and presence of $NH_3$ ions on the liver CD-like activity was undertaken. The results indicated that the small variations in ionic strength and presence of $HN_4$ ions as they could occur during incubation of enzyme mixture did not demonstrate any effect on the $H_2S$ production (Table VIII). Previous work by Lawrence and Smythe (1943) indicated that a similar enzyme system was sensitive to the variation of ionic strength if higher concentrations are used.

A selective sensitivity of this enzyme system was shown toward ammonium ions at such high concentrations.

The studies varying substrate concentration have revealed that the definite substrate saturation region lies at cysteine HCl concentration of 20 mg. (Table IX, Figure 9). These studies might be critical for several reasons. Since we are dealing with a crude enzyme preparation it is probable that the available cysteine can be utilized by other enzymes so that a substrate limiting situation is created for the enzyme under consideration. Thus the actual amount of cysteine available to the enzyme system under examination might affect the yields of the end products one is measuring. Therefore, critical studies concerned with the enzyme activation or inhibition have to be performed under such conditions. Additionally, the measurement of the $H_2S$ production should be performed at different time intervals in order to have a proof that there exists a linear relationship between the enzymatic activity and time course. A preliminary study of this type was performed with activators (biotin, alpha-ketoglutarate, pyridoxine) or their combinations which have been reported to be active in a similar enzyme system of bacterial origin (Delwiche, 1951; Kallio, 1951). The results obtained indicated that the enzymic $H_2S$ production from cysteine was not affected in any significant way. An analogous picture was seen in the case when TSN was present in the enzymatic reaction mixture (Table X, Figures 10, 11, 12). This was demonstrated in a conclusive manner especially in the first 60 min. period where a good linear relationship between time and enzyme activity exists. The true reason for change of the slope of enzyme activity - time course curve cannot be given, because of the lack of experiment-

al evidence. Some factors which could be eliminated as possible causes are as follows. From the results obtained from the preincubation experiments it does not appear that enzyme becomes inactive. The production of $NH_3$ could not have had an effect in the amounts of $NH_3$ released in the enzymatic reaction mixture (Table VIII). It does not appear on a basis of the $H_2S$ produced that the substrate could have been used up. The possibility of reaching the equilibrium state is remote, because of the constant escape of $H_2S$ as one of the reaction products. A partial answer to this problem could be given by the determination of the cysteine levels in the reaction mixture.

Before proceeding into the discussion of the following results one should keep in mind that these experiments were performed at the cysteine concentration below the substrate saturation level and secondly, the incubation time was extended to 120 min. On a basis of these limitations any conclusive interpretation of the results is rather difficult especially in cases where the inhibitory or augmenting effects on the $H_2S$ production were studied. Therefore results will be summarized in a concise manner and briefly related to the findings reported in literature.

The effect of alpha-ketoglutarate on the enzymatic $H_2S$ release from cysteine proved to be augmenting under the following experimental conditions: cysteine below substrate saturation level and incubation time - 120 min. (Table XIII). A similar finding was reported by Delwiche (1951) on the analogous enzyme system of bacterial origin. It is interesting to note that there was observed some antagonistic action between l-glutamate and alpha-ketoglutarate under our experimental conditions (Table XIV). This finding perhaps

might throw some light on the variations encountered in the control levels of enzymatic $H_2S$ production (see Appendix). It is conceivable that there might occur varying ratios between these two compounds in organs <u>in vivo</u>, e.g. La Page (1950) found variations in the content of alpha-ketoglutaric acid in the rat livers. Another contributing factor to the variation of the level of enzymatic activity could be a possibility of a variance in the organ level of pyridoxine in the test animals. There is some experimental evidence in literature pointing in that direction (Dietrich and Shapiro, 1953; Meister et. al., 1953; Sherman, 1954).

The augmentory effects of TSN on the $H_2S$ production (cysteine below substrate saturation level, incubation time - 120 min.) are rather interesting in view of the fact that some aliphatic thiol compounds, as shown by Lawrence and Smythe (1943) and our experiments, exhibited inhibitory action (Tables XVII, XVIII). But the activation observed under the experimental conditions used might be caused by some unspecific action. It is conceivable that the inhibition of other enzymes concerned with the cysteine metabolism can afford more substrate for the enzymatic reaction in question so that the observed differences in the level of activity are caused by variations in substrate concentration. This view is partially supported by the finding when cysteine was at the substrate saturation where TSN did not afford any significant elevation of $H_2S$ production (Table X, Figure 11).

In a consideration of the structures of TSN, thiophenol and sulfanilamide it is obvious that only the first two compounds have aromatic SH groups (Figure 14). Contrasting the effects on the enzyme caused by thiophenol and

TSN on the one hand and that of sulfanilamide on the other hand (Tables XV, XVI) would suggest that the presence of the SH on phenyl radical might be the chemical moiety responsible for the increased $H_2S$ production (cysteine below substrate saturation level, incubation - 120 min.). As opposed to the findings with aromatic SH group there was observed an inhibitory effect by the aliphatic thioacids on $H_2S$ production from cysteine. These findings would support a view that there might exist differences in the enzymatic properties between aliphatic and aromatic SH groups under the experimental conditions used.

Our findings on the liver CD-like activity indicated that there occurred a decrease in the $H_2S$ production in the injured liver lobe 2 days after the operation. The recovery of this particular enzyme system occurred between 4 to 6 days after operation (Table XII). The previous reports on the effects of partial hepatectomy on the enzyme called exocystine desulfhydrase (enzyme very similar to that under our consideration) did not indicate any observable changes in the level of the activity, although this enzyme was less active in fetal liver than in normal liver (Greenstein, 1954). Our findings on injured liver (perhaps due to the different surgical procedure employed and variations of the observation times) and those on fetal liver would suggest that there is an inversely proportional relationship between the enzymes concerned with cysteine (cystine) degradation and the extent of the protein synthesis. The activity of the transaminase is also lowered in regenerating tissues (Cohen and Hekhuis, 1941). The parallelism in the activities of the transamination and desulfuration enzyme would suggest a postulate that both systems might have something in common. Carrying this reasoning further, one

would suspect that tumor tissue where the protein synthesis is occurring at a high rate should possess low levels of these two enzymes. This was found to be true in the case of transaminase (Cohen and Hekhuis, 1941) and cysteine desulfhydrase.

The attempts to localize the CD-like activity in Walker 256 tumor employing higher homogenate concentrations and various activators (Table XX) did not produce any positive evidence regarding the presence of this enzyme system. A similar enzyme system, exocystine desulfhydrase, was reported to be absent in almost all tumors except mouse hepatomas (primary and secondary) induced by chloroform (Greenstein, 1954).

The question might arise as to the mechanism of action of TSH as the cancerostatic agent. It can be stated that it is too early to formulate any mechanism of action on a basis of these preliminary enzymatic data. If one would compare the effects on tumor by TSH and the compounds containing aliphatic SH radicals it would appear that the character (aromatic or aliphatic) of the sulfhydryl groups might be a factor determining the action on the tumor tissue. Additionally, it was observed that TSH and aliphatic thioacids have opposing effects on the enzymatic $H_2S$ production from cysteine. Further, it has been shown that the lack of cysteine in the diet can cause inhibition of spontaneous carcinoma or prevents appearance of leukemia induced by carcinogen (White and Andervont, 1942-43; White et. al., 1942-43, 1943-44). On a basis of the difference in the effect of TSH on CD-like activity and those observed with aliphatic thiol compounds, the role of cystine (cysteine) in the tumor development, and the cancerostatic effect of TSH as opposed to the cancerogenic action by

aliphatic thioacids, one might formulate a hypothetical mechanism of action of TSN as a cancerostatic agent. One might suspect that the increased cysteine catabolism possibly caused by TSN might be one of the factors. But on the other hand it was demonstrated by Boyland (1938) that sulfanilamide possessed some cancerostatic properties, while its effect on CD-like enzyme system as observed in our hands was slightly inhibitory. This finding would suggest that it might not be practical to limit a possible mechanism of action to effects of an agent on one particular enzyme system.

# CHAPTER VI

## CONCLUSIONS

1. The results of chemotherapeutic experiments with TSH-TSH (4:4'-sulfonamidodiphenyldisulfide) and TSH (para-thiolbenzenesulfonamide) on the Walker 256 tumor indicated that these compounds might possess some cancerostatic properties as evidenced by the final size of treated tumors.

2. The observed cancerostatic effect with larger doses of these compounds might be partly associated with the general toxicity of the test agents, since the inhibition of tumor growth with smaller doses of TSH which did not show any significant toxicity phenomena was proportionally less than in cases where such toxicity was evident.

3. The observed cancerostatic effects caused by TSH-TSH and TSH appeared to be limited to the period of drug administration. The results with TSH would suggest that the cancerostatic effect is primarily restricted to the initial phases of the tumor growth.

4. A procedure has been developed for $H_2S$ determination. The method utilizes polarographic determination of free $Cd^{++}$ ions after excess of $Cd^{++}$ ions has been allowed to react with the $H_2S$ to from CdS. This polarographic method is more sensitive than the iodometric technique of S determination, but is less sensitive than the spectrophotometric methods.

5. A new method for the determination of cysteine desulfhydrase-like activity has been evolved. The method utilizes the polarographic

determination of $H_2S$ just referred in order to estimate the enzymatic degradation of cysteine. Application of this method has been demonstrated in a preliminary study of factors which might influence the enzymatic production of $H_2S$ from cysteine in liver homogenates. Results of studies made to ascertain whether or not the influence of TSN and selected aliphatic thiol compounds on the enzymatic $H_2S$ release could be correlated with their effects on tumor growth failed to afford data from which definite conclusion concerning their mechanism of action might be drawn.

6. No definite proof of the enzymatic $H_2S$ production from cysteine by homogenates of the Walker 256 tumor could be obtained employing activators which appeared to function in the analogous enzyme system of bacterial origin.

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## APPENDIX

### VARIATIONS IN CONTROL VALUES OF THE CYSTEINE DESULFHYDRASE-LIKE ACTIVITY IN LIVER

| Range Micromoles S | Number | Activity of Individual Livers Micromoles S |
|---|---|---|
| 0.0 - 0.9 | 1 | 0.9 |
| 1.0 - 1.9 | 4 | 1.0; 1.3; 1.6; 1.9; |
| 2.0 - 2.9 | 7 | 2.1; 2.2; 2.2; 2.4; 2.6; 2.8; 2.9; |
| 3.0 - 3.9 | 7 | 3.1; 3.1; 3.4; 3.6; 3.7; 3.8; 3.9; |
| 4.0 - 4.9 | 4 | 4.3; 4.5; 4.7; 4.9; |
| 5.0 - 5.9 | 4 | 5.0; 5.1; 5.7; 5.9; |
| 6.0 - 6.9 | 2 | 6.2; 6.5; |
| 7.0 - 7.9 | 1 | 7.1; |
| 8.0 - 8.9 | 2 | 8.6; 8.6; |
| 9.0 - 9.9 | 0 | |
| 10.0 -10.9 | 0 | |
| | | Mean ± SE 3.9 ± 0.36; |
| | | SD = 2.0; n = 32; |

3.0 ml. 1:2 homogenate; 1.0 ml. - 5 mg. l-cysteine HCl (neutralized); 1.0 ml. saline; C. S. -0.060 mu a/mm; incubation time - 120 min.

## FORMULAE USED IN STATISTICAL ANALYSIS

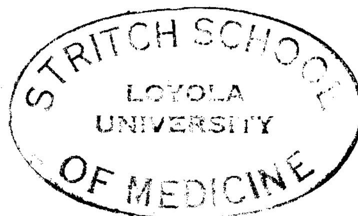
SD (standard deviation) $= \sqrt{\dfrac{\Sigma (x-\bar{x})^2}{n - 1}}$

$SE_m$ (standard error of mean) $= \dfrac{SD}{\sqrt{n}}$

$SE_d$ (standard error of difference) $= \sqrt{\dfrac{(SD_1)^2}{n_1} + \dfrac{(SD_2)^2}{n_2}}$

t $= \dfrac{mean_1 - mean_2}{SE_d}$

For the determination of P values a table of the t distribution was used (Burn et. al., 1950).



APPROVAL SHEET

The dissertation submitted by Jonas A. Gylys has been read and approved by the members of the Department of Pharmacology.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

9-6-56
Date

Charles E. Jacobs
Signature of Advisor