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POTENTIAL NEOPLASM INHIBITORS II

A Thesis

Presented to

the Faculty of the Department of Chemistry

University of Richmond

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Ronald Lee Floyd

August 1966

W. Allan Powell

J. Stanton Pierce

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VIRGINIA

For her patience, understanding, and encouragement,

I dedicate this thesis to my beloved wife,

Patricia Reardon Floyd.

Acknowledgement

The author is deeply indebted to Dr. J. Stanton Pierce, Professor of Chemistry, for his leadership and direction in this research project. I feel very grateful for the opportunity of having been associated with this dedicated scientist and shall always have a special place set aside for Dr. J. Stanton Pierce.

The author is also indebted to Mr. Richard Corbin for his great assistance in preparation of these compounds and to Mr. Franklin Pinchbeck for his assistance in literature searching and help in determining the purity of compounds. Thanks also is due to Mr. Dallas Pinion for his help in development of good research techniques used in this project.

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INTRODUCTION

1. Cancer

"Cancer has been defined by Willis[93] as an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of normal tissues, and persists in the same excessive manner after the cessation of the stimuli which evoked the change."

"Cancer is a disease of the cell that is transferred to the descendants of the cell. The disease is recognized by the behavior of a population of abnormal cells within a normal tissue, as manifested by varying degrees of morphologic disorientation, aggressive growth and invasion, with ultimate destruction of the normal cell population."[1]

"Cancer is a generic term for a variety of malignant neoplasms, due to unknown and probable multiple causes, arising in all tissues composed of potentially dividing cells, in man and other animals, and resulting in adverse effects on the host through invasive growth and metastases. It is one of the major causes of death throughout the world. In the United States, deaths from cancer are second only to cardiovascular and renal diseases. Wherever malnutrition and other socioeconomic factors, as well as infectious diseases, etc., result in a short average span of life, cancer is not an important health problem, in spite of increase in population. Conversely, an increase of population that is associated with

an increase in life expectancy does bring a progressive increase in the occurrence of cancer. Thus, the inevitable paradox: as public health improves, cancer becomes a greater problem.[2]

The progressive increase in the number of reported deaths from cancer is, to a large extent, only apparent, for more cases are being diagnosed accurately as the public and the medical profession become cancer conscious and as more accurate methods for diagnosis become available. The mortality from cancer is also due to an increase in the numbers of individuals reaching advanced age. In the United States there were 13 million persons 45 years of age or older in 1900; by 1948 there were 39 million persons in that age group and in 1958 there were 50 million. In view of the fact that cancer is prevalent among aged individuals, cancer cases may continue to increase in the United States for a number of years on the basis of these facts alone. In addition, there appears to be a true increased incidence of certain forms of cancer.

The outstanding recent changes in cancer morbidity and mortality are as follows: (1) a threefold increase in cancer of the lung among males, (2) a twofold increase in leukemias and "lymphomas," (3) a 30% decrease in cancer of the stomach, and (4) a 30% decrease in cancer of the cervix. Also, the gap among the greater probability of cancer in females and the lesser one in males has been closing due mainly to the increase in pulmonary cancer in men. More than half of the

cancer risk in men is due to cancer of the skin, prostrate, lung, stomach, and intestine, whereas more than half of the cancer risk in women is due to cancer of the breast, intestine, skin, and cervix uteri. During a lifetime one in every four women and one in every five men may be expected to develop cancer. The increased risk is due in part to the decline of noncancerous causes of death."[3]

2. Chemotherapy of Neoplastic Disease

"Although great strides have been made in the chemotherapy of neoplastic disease, the sobering fact must be realized that, after the diagnosis has been made, only 20 per cent of all patients are salvaged for five years by currently available therapeutic measures. Inasmuch as surgery and radiotherapy have been developed to a high degree of perfection, it is obvious that major advances must be sought in other fields. Methods for early diagnosis and effective chemotherapy could obviously make great contributions.

It should be stated at the onset that, with the exception of early childhood leukemia (which can be cured by folic acid antagonists), no chemical compound has yet been found which is capable of curing any form of cancer. However, various drugs and hormones are available which can induce temporary remissions in certain types of neoplastic disease. Other agents are of value in that they appear to support the host against the ravages of the disease. Drugs capable of inducing temporary

remissions can be divided into three classes. (1) Cytotoxic agents which exert their actions by virtue of their chemical properties: as a group these compounds lack selectivity of action in that they are toxic to normal as well as malignant cells. However, they are of some therapeutic value inasmuch as the vulnerability of cells appears to be related to their rate of mitotic activity. (2) Cytotoxic agents which exert their actions by virtue of their physical properties: this group comprises the radioactive isotopes, which exert their effects through ionizing radiation. Radioactive isotopes also lack selectivity of action, but in a few instances advantage can be taken of the selective distribution of a particular element in the body to attain relatively high concentrations at a desired site. (3) Agents which exert their action by altering the environment of the cancer cell: this group comprises certain hormones, notably estrogens, androgens, and adrenocortical steroids. Altering the hormonal environment with respect to estrogens and androgens is of some value in the control of neoplasms of the secondary sex organs. Induced hypercorticism provides an unfavorable environment for certain malignant growths involving hematopoietic tissue."[45]

Purpose and Scope of the Research

In August, 1965 Andrew Bachmann presented a thesis based on the fact that indole was selectively absorbed in the cancer tissue. This paper is similar in that indole is used as the means of transport to concentrate the alkylating agents in the tumor. The alkylating agents presented in this paper are epoxides and methane sulfonates.

The historical portion of this paper is designed to show the value of epoxides and methane sulfonates in chemotherapy. The experimental portion is composed of the synthesis of these compounds, related intermediates, and a new color test which is a potential method for the detection of indole in a tumor.

HISTORY

Evidence for the Association of Indole With Tumor Cells

"Indole has been tested for its anti-cancer properties and found lacking. However, it was found that lipotropic materials such as amytol, phentothal, Br_3CCH_2OH, chloretone and indole, which greatly inhibit brain respiration, also markedly inhibit the respiration of Erlich Ascites tumor cells. These compounds inhibit the intake of glycine into these cells under anaerobic conditions in the presence of glucose, the extent of inhibition being greater than that of anaerobic glycolysis. It is suggested that respiratory mechanisms and the transport system of the whole cell depend for their activities on the lipid components of the cell membrane structures with which these lipotropic agents become associated, thereby decreasing these activities."[15]

Review of Related Work from Potential Neoplasm Inhibitors I

A suspension of indole containing radioactive C^{14} was administered to a female C3H mouse by intraperitoneal injection by Bachmann and the following data were collected. Bachmann found about a 17:1 concentration ratio of indole in cancer tissue as opposed to similar muscle tissue. The indole concentration remained at a high level for about twelve (12) hours.

Then over the next seventy-two (72) hours, the indole appeared to be removed and detoxified by the liver.

The fact that the indole content of the corresponding muscle tissue was always so small indicated that the pH induction of the tumor had indeed been used to cause a selective deposition. To Bachmann's knowledge, this was the first instance that such an effect had been observed.

"Nitrogen mustards and alkyl methanesulfonates will react with the hydrophobic constituents of a living cell in considerably less than twelve (12) hours, which is the time limit for the maximum concentration of indole. Therefore, the indole moiety should act as an effective transport molecule for alkylating substituent should combine with the cell wall, if indole concentrates there, and change its character enough to inhibit cell respiration, thereby killing the cell through respiration failure. If indole concentrates elsewhere in the cell, the alkylating substituent should interrupt metabolic pathways resulting in the death of the cell."

Bachmann also suggested that if a gamma ray emitter were placed upon an indole molecule, the resulting molecule might serve as a locator for tumors. To investigate this idea, 5-iodoindole was synthesized which contained iodine-125. Though encouraging, data on this experiment was not conclusive.[16]

Influence of pH on Reactivity:[17,51]

"In Warburg's studies on tumors, the observation was made that, whereas both normal and tumor tissues produced lactic acid from glucose or glycogen in the absence of oxygen, practically only cancer tissue showed the ability to produce lactic acid from glucose in the presence of oxygen.

The respiration ratio of cancer and of normal tissues appeared to be very nearly of the same order of magnitude, and to account for the high aerobic glycolysis in tumors Warburg postulated that the respiratory mechanism is such tissues was damaged as a result of the neoplastic transformation. High anaerobic glycolysis appeared to be a general property of growing or multiplying tissues, since it was found in embryo tissue and testes, but in these normal tissue glycolysis was largely abolished by the presence of oxygen. In most tumors, the high anaerobic glycolysis was relatively little reduced when oxygen was admitted to the system.

That lactic acid is actually produced from tumors was shown by several investigators who observed that the venous blood leaving a tumor contained less glucose and more lactate than did venous blood in a comparable tumor-free site in the same animal.

Since lactic acid is a relatively strong organic acid, its accumulation within the tumor should produce a lowering of the pH, provided that the rate of glycolysis exceeds both

the diffusion of buffers from the arterial circulation into the cells and provided that the rate of supply of the glucose to the cells is sufficiently high.

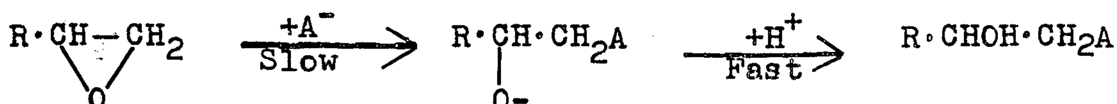
By inserting electrodes directly into the tumor mass of the living animal, Voegtlin *et al* demonstrated that the administration of glucose, whether subcutaneous or intraperitoneal, was followed very quickly by a drop in pH of 0.4 to 0.6 unit. The pH of the tumor may drop from 6.9 to 6.3 without the occurrence of any pulmonary respiratory symptoms indicative of systematic acidosis, thus indicating that the increased acid production is largely confined to the tumor and that the excess lactate carried off in the venous circulation is quickly metabolized by the normal tissues.

Kahler and Robertson compared the hydrogen ion concentration of the liver and of a transplanted heptoma in fasted rats. The pH of the liver was 7.4, that of the tumor 7.0. When the rats were supplied with excess glucose, the pH of the liver remained unaltered whereas that of the heptoma dropped to 6.4"

Alkylating Agents

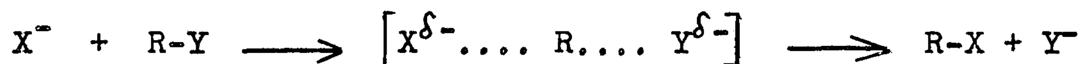
1) Epoxides[91]

There are two (2) possible mechanisms by which an epoxide can react with a nucleophilic centre (A−) in neutral solution.



The first is a unimolecular process which would only be favored if the terminal carbon atom were substituted, and the second is a typical Sn2 substitution as shown above.[74]

The Sn2 mechanism may be explained more clearly by the following:



The complete separation of R and Y does not occur, but a transition state occurs in which R is loosely combined with both X and Y. The attainment of the transition state is also assisted by solvation of the polar complex and in the subsequent process desolvation, charge transfer, and bond formation are involved. Effectively a carbonium ion R^+ is again transferred from Y to X but in this case it is at no time free in the sense of being detached from Y, for the bond forming and bond breaking processes occur simultaneously. In the rate determining formation of the transition complex two molecules are undergoing covalency change. A feature of the Sn2 reaction is that the rate is dependent on the concentration of the displacing group X^- and upon

its affinity for the electrophilic R^+. The driving force is the attack of the nucleophilic group X^- on a carbon atom in the R group of the alkylating agent and the extent of the reaction will depend on the relative nucleophilic capacities of groups X^- and Y^-.[72]

Epoxides are relatively slow to react: the half-life of diepoxybutane is about 100 hours at 37°C in purely aqueous solution. The reaction rate will be increased by the presence of reacting groups, since for terminally unsubstituted derivatives the Sn2 mechanism operates. Actually, only terminally unsubstituted compounds are of interest biologically. We thus have a group of moderately reactive alkylating agents which are effective under biological conditions and which are transported as neutral molecules; the rate of reaction will depend on the concentration of reacting centres.

The reagents which react by the Sn2 route can be removed quickly at sites of high nucleophilic potential. The virtue of the slower reacting epoxides may be that they survive long enough to alkylate target sites at distant regions. Henry, Rose, and Walpole (1950) suggested that cytotoxic alkylating agents polymerize *in situ*, giving a structure containing reactive groups which are spaced at approximately 7.6Å apart.

This repeat distance is similar to that of the side chains in a fully extended protein or the internucleotides in a nucleic acid. Multipoint attachment of the polymer to either of these macromolecules in a chromosome structure was thought to be

responsible for the induced mitotic abnormalities.[75]

Effect of pH on Reactivity of Epoxides

Some simple epoxides have a high rate coefficient for acid catalysed type of reaction. This type of compound would be expected to react more readily with nucleophilic centres in regions where the acidity is relatively higher. For those groups with pKa somewhat removed from physiological pH, such as phosphate groups, carboxyl groups, and relatively weak basic groups, the effect of the limited pH change on ionization will not be significant.

A careful study of certain basic diepoxide derivatives by Gerzon _et al_. (1959) has shown how another structural modification may be introduced in order to obtain acid-enhanced reactivity. Ross (1950) had shown that the incorporation of a charge onto the nitrogen atom of the methiodide of N,N-di-(2,3-epoxypropyl)-p-anisidine enhances the reactivity of the epoxide ring. This effect is independant of the rate enhancing effect of pH change alone. Some correlation between basicity, rate of reaction with phosphate ion, and antileukemic activity was established by Gerzon _et al_. (1959) and it would seem profitable to extend the study of these basic epoxides to the treatment of solid tumors, especially after pretreatment of the host with glucose.[77]

Effects on Protein Synthesis and on Respiration

Butadiene dioxide inhibited the in vitro incorporation of glycine-1-C^{14} into the proteins of Ehrlich ascites carcinoma cells but had no effect upon the rate of respiration.[33]

Reactions With Proteins and Compounds Containing Functional groups That Occur in Proteins

It has been shown that epoxides can react with various proteins including casein,[19] egg albumin,[43] bovine serum albumin,[4,6,7,8] B- lactoglobulin,[43] wool, and human hair.[30] Tests for functional groups of the proteins showed that alkylation of the following types of groups occurred: Carboxyl,[5,7] phenolic,[43] amino,[6,7,43] imidazole[6,7] and sulfhydryl.[6] Epoxides reacted with esters of amino acids[58] to yield disubstituted amino compounds[58] and lactones.[59] Reaction with thioamides and thiols yielded 2-hydroxy-alkyl sulfides[34] and reaction with pectic acid resulted in esterification.[36] Thus, reaction can occur with a variety of groups occurring in proteins, and if polyepoxides are used, cross-linking of proteins may occur.[4,8,42] Very little deactivation of cholinesterase occurred, however.[27]

Cytologic and Mutagenic Effects

Glycidol caused chromosomal bridging and breakage in cultured S-180 cell[20] and the administration of butadiene

dioxide to male mice interfered with spermatogenesis and caused chromosome breaks.[66] Epoxides have been shown to be effective mutagens in a variety of biological systems including *Drosophila Melanogaster*,[22,23] *Neurospora crassa*,[57,61] *Penicillium Chrypogenum*,[54] *Escherichia coli*,[87] and tomatoes.[41]

Reactions With Nucleic Acids and Nucleic Acid Moieties

Bifunctional epoxides reacted with nucleoproteins in solution to cause the formation of a gel, and the alkylation occurred exclusively with the DNA portions of the molecules.[7,11,12] This gel formation was probably the result of cross-linking of DNA molecules. There is some evidence that alkylation of both nucleoprotein[7] and DNA[9] occurred at the phosphate groups with the resulting formation of esters. These esters might in turn alkylate the ring-nitrogen atoms of the purines, which resulted eventually in scission of the ribose-phosphate chain of the DNA.[9] Treatment of guanosine with ethylene oxide and propylene oxide in unbuffered solution at 37°C yielded a product that was spectrally similar to 7-methylguanosine.[63] Treatment of tobacco mosaic virus RNA with ethylene oxide or propylene oxide caused deactivation of the reconstituted virus,[44,45] when as few as 1-3 molecules of the epoxide had reacted with one molecule of the RNA, and it appeared that the guanine moieties were the chief sites of reaction.[44]

Effects on Fertility

Fahmy and Fahmy (1958) have made related studies on the action of different alkylating agents on different stages of cell development in the germinal tissue of Drosophila males. The adult males were injected with the agent and the germ line was fractionated by repeated matings to a succession of virgin females. The mutation rate fluctuated in successive broods. It is believed that since the sperm used in the sequential matings time of treatment, the variation in brood mutation response probably reflected the relative susceptibility of the various cell stages to the action of the mutagenic alkylating agents.

Diepoxides and most simple mustards produce a maximum effect on early spermatids and little action on spermatocytes and spermatagonia.[78]

2) Methanesulphonates[73,87]

Esters of alkanesulfonic and arenesulfonic acids are alkylating agents because in reaction the alkyl-oxygen bond undergoes fission. Methyl esters react with nucleophilic centres by a bimolecular mechanism previously discussed in this paper.[82] Therefore, the chemical reactions of the sulfonic esters are generally similar to those of the epoxides.

In the series $CH_3SO_2O(CH_2)_nOSO_2CH_3$, the chemical reactivity is low in compounds (n=2 and 3) due to the mutual deactivating effect of the methanesulfoxy groups. From n=4 upwards the

chemical reactivity is higher and more uniform.[76]

Distribution and Fate of Administered Sulfonic Esters

The compound, S^{35} - labeled tetramethylene ester of methanesulfonic acid was administered to rats, mice and rabbits and the data were collected. In the rat there was some selective concentration of the S^{35} in the spleen and bone marrow, but 32 hours after injection, 95 per cent of the S^{35} was present in the urine. Small amounts of S^{35} were associated with the liver proteins. Following the administration of this agent to humans the S^{35} disappeared rapidly from the blood and 45-69 per cent of the S^{35} was present in the urine in 48 hours.[68,69]

Tetramethylene ester of methanesulfonic acid labeled with C^{14} in the 1 and 4 positions was also injected into rats and the data were collected. The specific activities of the kidneys, lungs, and liver were higher than those of the other tissues examined, and in the liver only small amounts of radioactivity were associated with proteins, fats and sodium nucleates. Within 24 hours 12-14 per cent of the injected C^{14} was excreted as carbon dioxide, 22-36 per cent was in the urine, and 3-8 per cent was in the feces.[83,89]

Effects on Glycolysis and Respiration

1,9-Di-(methanesulfonxy) nonane depressed the respiration of ascites tumor cells and promoted aerobic glycolysis 60-80

per cent but had no influence or anaerobic glycolysis.[39] Several other methanesulfonyl esters acted similar. Kinetic studies with enzymes led to the conclusion that the inhibition of respiration by beta-chloroethyl methanesulfonate was chiefly due to physical effects of this agent upon the hydrogen transport chain of the cell.[25]

Effects on Protein Synthesis

The majority of the esters of methanesulfonic acid had little or no significant effect on protein synthesis.

Deactivation of Enzymes

Several methanesulfonic esters had very little anticholinesterase activity,[27] but tetramethylene ester of methanesulfonic acid inhibited crystalline triosephosphate dehydrogenase.[55]

Reactions With Proteins and With Compounds Containing Functional Groups that Occur in Proteins

Evidence has been obtained that tetramethylene ester of methane-sulfonic acid can react with the protein portion of nucleoproteins,[28] and several methanesulfonic esters reacted with the mercapto groups of denatured egg albumin and with carboxyl and imidazole groups but not the amino groups of bovine serum albumin.[6,7] The reactions possibly cause dethio-

lation of proteins, but the biological significance of such dethiolation is not yet known.

Antimitotic, Cytologic, and Mutagenic Effects

Tetramethylene ester of methanesulfonic acid inhibited the mitosis of cultured fibroblasts,[31,32,46] and 1,4-dimethyltetramethylene ester of methanesulfonic acid inhibited cultured leukemia cells.[11] Experiments with tetramethylene ester of methanesulfonic acid and tritiated thymidine showed that synthesis of SNA by fibroblasts could occur even when the mitotic activity was reduced.[31,32] Among the cytologic effects noted for the tetramethylene and 1,4-dimethyltetramethylene esters of methanesulfonic acid on mammalian cells were giant cell formation,[10] nuclear enlargement,[46] multinucleation[32,35] chromosome breaks,[46] and chromosome bridges.[46,37] Concerning the mutagenic effect, it is of interest that radioactivity was detected in the testicles of mice following the injection of 1,4-dimethyltetramethylene ester of methanesulfonic acid.

Effects in Synthesis of Nucleic Acids

Tetramethylene ester of methanesulfonic acid had only a slight or no inhibitory effect upon the *de novo* synthesis of nucleic acid purines. The lack of inhibition of the synthesis of nucleic acids is consistent with the observation that the incorporation of tritiated thymidene into the nuclei of cultured

cells in the presence of tetramethylene ester of methanesulfonic acid occurred even after mitotic activity was greatly reduced.[31,32]

Reaction With Nucleic Acids and Nucleic Acid Moieties

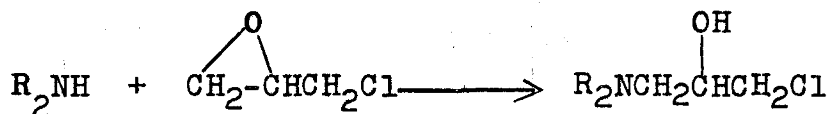
After treatment with tritiated tetramethylene ester of methanesulfonic acid, tritium was found localized in the nuclei of the root tips of *Vicia faba*, whereas no tritium was detected in the cytoplasm.[67]

There is also evidence that sulfonic esters can react with DNA *in vitro*. Alkylation of DNA with methyl methanesulfonate and with ethyl methanesulfonate caused an immediate decrease in viscosity of the solution, perhaps because the DNA molecules become more highly coiled. It was suggested that the initial site of alkylation might be the phosphate groups of the DNA with subsequent alkylation of the purine-ring nitrogen atoms by the tri-esters; alkylation of the purines could then result in expulsion of the quaternized purine and rupture of the desoxyribosephosphate chain.[9]

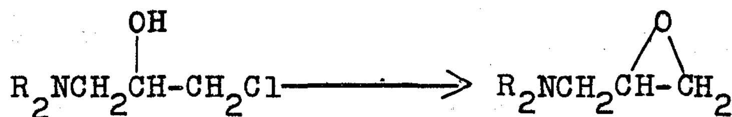
Preparations and Reactions of Related Compounds

1. Reactions of Epichlorohydrin with Secondary Amines[38,18,40]

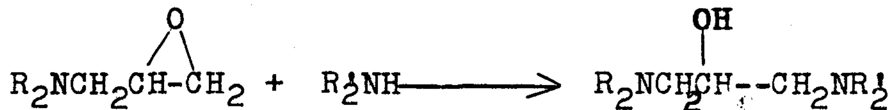
   (1) Secondary amines react readily with excess epichlorohydrin to yield mainly amino chlorohydrin as indicated below:



With alkali, the epoxy ring is closed to yield an epihydrinamine as shown below:



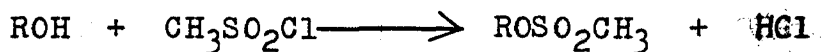
The latter products condense with another secondary amine to yield unsymmetrical 1,3-diamino-2-propanol according to the following equation:



2. Preparations of Methane Sulfonates

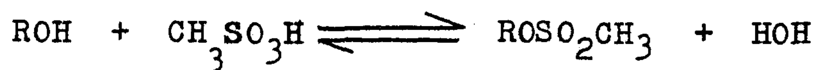
There are three general methods for the preparation of methanesulfonates.

   (1) Refluxing methane sulfonyl chloride with an alcohol, or reacting in pyridine.[79]



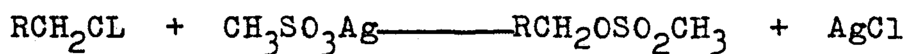
$$ROH + CH_3SO_2Cl \longrightarrow ROSO_2CH_3 + HCl$$

(2) Refluxing an alcohol with methane sulfonic acid to give poor yields.[13]



$$ROH + CH_3SO_3H \rightleftharpoons ROSO_2CH_3 + HOH$$

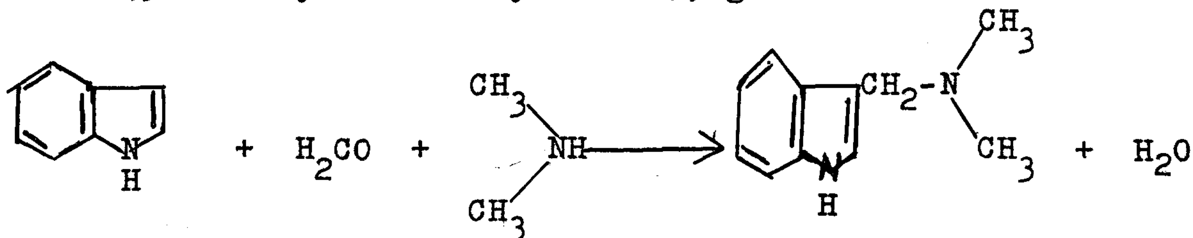
(3) Refluxing the silver salt of methane sulfonic acid with an alkyl chloride.[47,86]



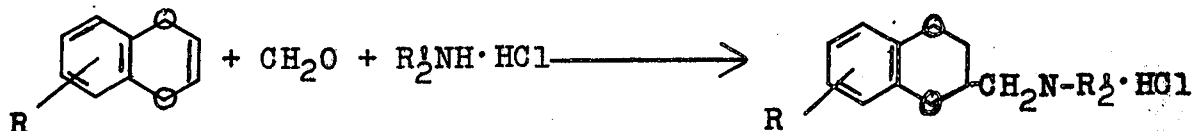
$$RCH_2CL + CH_3SO_3Ag \longrightarrow RCH_2OSO_2CH_3 + AgCl$$

3. Mannich Reaction

(1) The beta or 3-position in indole is very reactive and with formaldehyde and dimethylamine in the presence of acetic acid undergoes the Mannich reaction to form the (3-dimethylaminomethylindole), gramine.[29]



(2) The study of reaction of chromones--with formaldehyde and a secondary amine hydrochloride by the Mannich reaction.[92]



R=methoxy, alkyl, halogen or hydrogen

R' = Alkyl

4. Schiff Base[49,50]

(1) The conversion of a primary amine into an aldimine by condensation with an aldehyde.

RNH_2 + RCHO ⟶ R·N=CHR + H_2O

R = Phenyl and Aliphatic

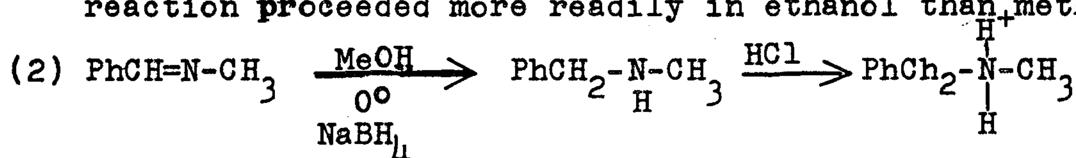
(2) Preparation of a ketimine by reaction of a primary amine with a ketone.

5. Reduction of Schiff Bases with Sodium Borohydride[21,56]

(1) $Ar\text{-}CH{=}N\text{-}Ar^1 \xrightarrow{NaBH_4} Ar\text{-}CH_2\text{-}NH\text{-}Ar^1$

Where Ar and Ar^1 may be a phenyl group or a nitro-, chloro-, methoxy-, or hydroxyphenyl group.

The yields range from 81-90% and the reduction condition aren't too critical. It was found, however, that the reaction proceeded more readily in ethanol than methanol.



The mixture comes to room temperature and is then heated for two (2) hours at 50°. The methanol is removed and product extracted with ether. The hydrochloride is formed by treating with HCl gas.

Previous Color Methods for the Detection of Indole

Several color tests have been developed for the detection of indole derivatives. These include a test which employs a color reaction between indole and a ceric nitrate reagent by Hartough.[52] Anger and Ofri describe a reaction test, yielding a red color, by reaction of an indole derivative with pyridyl-pyridinium chloride.[14] Sawicki obtained a blue color by reaction of 3-methyl-2-benzothiazolone with indole.[83] The indole ring in reserpine was detected by Haycock and Mader by reaction with dilute nitrous acid.[53] When heated with Arreguine's reagent (glacial acetic acid, concentrated hydrochloric acid, and xanthydrol) a color reaction is given by indole.[84] A test using a chloroform solution of piperonal, phosphorous pentachloride, and trifluoroacetic acid has also been used.[71]

Discussion and Experimental

In the present study it was found that butyl nitrite, acidified with concentrated hydrochloric acid, gave a color reaction test for indole and many indole derivatives.

Early in this work it was found that the test is sensitive for indole in the presence of freshly dissected liver, stomach, and kidney of a normal mouse. Repetition of the test with a cancerous mouse showed that after twenty (20) hours the indole was concentrated in the tumor, and it was not detected in the liver or kidney.

The procedure using normal mice is described below:

To a solution of 6.5ml of polyethylene glycol (PEG-300) was added 100 mg of indole and 5.5 ml of sterile glucose solution. A 0.5 ml sample was taken from this solution and injected into the abdomen of each of two (2) normal mice which had been previously starved for two (2) hours.

One of the mice became a little sick and was sacrificed (chloroform) after one-half hour. The liver, stomach, and kidneys were removed, placed in three (3) separate beakers, each containing 50 ml of ether. The tissues were crushed, removed from the extract, and the ether evaporated to near dryness by means of a hot-water bath. The test for indole was made by dissolving the residue in 5 ml of ethanol and adding two (2) drops each of butyl nitrite and concentrated hydrochloric acid.

Results of Test on First Mouse:

Liver - Negative, no color was observed.

Stomach - Negative, no color was observed.

Kidney - Negative, no color was observed.

When no test was given for indole, the remainder of the mouse was placed in a beaker and extracted with 100 ml of ether. The ether was evaporated and the color test was repeated.

Results:

Body cavity of the mouse --- A positive test (red color) was obtained.

Two (2) hours after the injection, the second mouse was sacrificed (chloroform) and dissected. The kidney, liver, and stomach were removed and placed in three (3) separate beakers, each containing 50 ml of ether. The tissues were crushed and removed from the ether extract. The ether was then evaporated to near dryness on a hot-water bath and the residue dissolved in ethanol. The solution was tested using butyl nitrite and concentrated hydrochloric acid.

Results:

Liver - Positive, a red color was observed.

Kidney - Positive, a red color was observed.

Stomach - Positive, a red color was observed.

Since the test showed that indole can be found in the dissected organs of a normal mouse, it was decided to repeat the test with cancerous mice. The procedure is described

as follows:

To a solution of 6.5 ml of polyethylene glycol (PEG-300) was added 100 mg of indole and 5.5 ml of sterile saturated aqueous solution. A 0.5 ml sample was taken from this solution and was administered to cancerous C3H mouse which had been previously starved for two (2) hours. After a period of thirty (30) minutes, another cancerous C3H mouse was injecetd using the same procedure as described above.

The first mouse died approximately one-half hour after injection. The mouse was dissected; the liver, kidney, tumor and corresponding muscle tissues were removed, extracted with ether, and tested using the butyl nitrite-hydrochloric acid test. The results are shown below:

Tumor - Negative, no color was observed.

Kidney - Negative, no color was observed.

Liver - Negative, no color was observed.

Muscle - Negative, no color was observed.

After a period of twenty (20) hours, the second mouse was sacrificed (chloroform) and dissected. The kidney, liver, tumor, and corresponding muscle tissue were removed, extracted, and tested using the butyl nitrite-hydrochloric acid test. The results are shown below:

Tumor - Positive, an orange-red color was observed.

Kidney - Negative, no color was observed.

Liver - Negative, no color was observed.

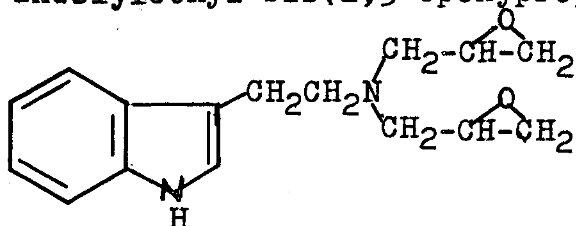
Muscle tissue - Negative, no color was observed.

Since indole was shown, by the butyl nitrite-hydrochloric acid test, to concentrate in cancerous tissue in mice, several derivatives of indole and of related compounds were purchased or prepared for this study.

Initially, it was planned to test each compound purchased or prepared in a cancerous mouse, and a few were tested. However, the butyl nitrite-hydrochloric acid test offered such a rapid, convenient. and inexpensive method to test indole and related compounds, in vitro, it was employed as a guide to predict whether or not these particular compounds should be used in the animal test.

The following compounds were prepared for injection into cancerous mice.

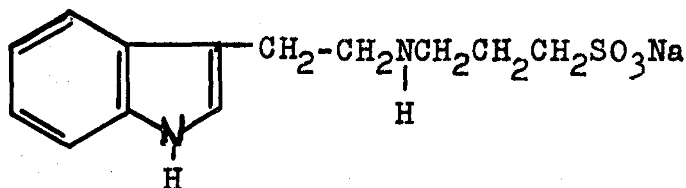
3- Indolylethyl-bis(2,3-epoxypropyl)amine



To 3.92 grams (0.02 mole) of tryptamine hydrochloride was added 50 ml of water and 20 ml of 95% ethanol. The reaction mixture was stirred, warmed and made distinctively basic by the addition of a saturated solution of potassium carbonate. The oily layer which separated was extracted into 40 ml of ether. The ether solution was added dropwise to 3.79g (0.042 mole) of epichlorohydrin in 20 ml of 95% ethanol. The mixture was allowed to stand overnight.

The reaction mixture was diluted with 250 ml of water, then treated with 100 ml of ether and 10 ml of glacial acetic acid. The upper layer was extracted with 100 ml of 10% acetic acid, and the aqueous acetic acid portions were combined and treated with excess 6N sodium hydroxide solution. The oily layer which separated was taken up in 100 ml of ether. The ether extract was filtered and treated with approximately 12 grams of sodium hydroxide pellets. After three (3) days in the refrigerator and the addition of 125 ml of ether, the solvent was filtered, the ether evaporated on a hot-water bath, and the residue was left overnight in a flask in a vacuum desicator. The yield of clear oil was 1.70 grams (33% yield). Calculated for $C_{16}H_{20}N_2O_2$: titrateable N, 5.50%. Found, 4.9%.

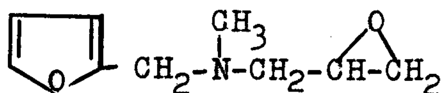
Sodium 3 2(3-indoly)ethylamino propane sulfonate.



To 1.81 grams (0.01 mole) of tryptamine hydrochloride was added 20 ml of water and 10 ml of 95% ethanol. The reaction mixture was warmed, stirred, and made distinctively basic by the addition of a saturated solution of potassium carbonate. The oily layer which separated was extracted into 40 ml of ether. The ether and ethanol were removed by means of a rotary evaporator, the residue dissolved in 5 ml of absolute methanol and added to 0.61 gram (0.005 mole) of propane sultone dissolved in 10 ml of absolute methanol. The reaction mixture was allowed

to stand at room temperature for three (3) days. The ether and ethanol were removed by means of a rotary evaporator. To the product (3.6 grams) was added 10 ml of methanol, and the mixture was warmed. Then, 14.5 ml of 0.1N sodium methoxide (0.115 g of sodium metal in 50 ml MeOH) was added; the mixture was swirled and cooled in an ice-water bath. A crystalline product could not be obtained; therefore, the mixture was triturated with ether. An oily, sticky product was obtained. The ether and water were decanted off, and the product was dried in a flask in a vacuum desicator. The yield of the oil was 1.22 grams (40% yield). Calculated for $C_{13}H_{17}NO_3SNa$: tritrateable N, 4.60%. Found 4.2%.

Methyl-2-furylmethyl-2,3-epoxypropyl amine

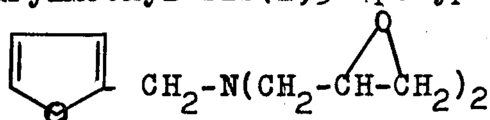


To a three-necked liter flask with a thermometer and mechanical stirrer attached was added 27.75 g (0.3 mole) of epichlorohydrin. The flask was placed in an ice bucket and cooled to 4°C. From a dropping funnel 33.3g (0.3 mole) of N-methylfurfuryl amine was added slowly, keeping the temperature below 30°C. To catalyze the reaction, approximately 0.5ml of water was added. The reaction mixture was stirred, and the temperature kept below 30°C for one and one-half hours. The mixture was then cooled to 10°, and 16 g (0.4 mole) of sodium hydroxide in 25 ml of water was added batchwise, keeping the

temperature below 25°. The reaction mixture was then extracted with three (3) 150 ml portions of ether, the three (3) ether portions combined, placed over sodium hydroxide pellets, and left to stand in the refrigerator overnight.

The mixture was filtered by gravity and the ether removed by means of a rotary evaporator, without heat. The mixture was then distilled under reduced pressure (35mm), bp 145-80°. The yield of clear oil was 30.88g (61.4% yield). Calculated for $C_9H_{13}NO_2$: tritrateable N, 8.38%. Found 8.22%.

2-Furylmethyl-bis(2,3-epoxypropyl)amine



To a three-necked liter flask with a thermometer and mechanical stirrer attached was added 55.5g (0.6 mole) of epichlorohydrin. The flask was placed in an ice bucket and cooled to 4°. From a funnel 29.1 g (0.03 mole) of furfuryl amine was added slowly with stirring and cooling, keeping the temperature below 30°C. Approximately 0.5ml of water was added to catalyze the reaction. The mixture was stirred overnight and left to stand over the week-end.

The mixture was stirred again, cooled to 10°C, and 32g (0.8 mole) of sodium hydroxide in 60 ml of water was added batchwise, keeping the temperature below 20°. After one hour of mixing, the reaction mixture was diluted with 100 ml of water and extracted with three 150 ml portions of ether. The three portions of ether were combined, placed over sodium

hydroxide pellets, and left to stand overnight in a liter flask in the refrigerator.

The mixture was filtered by gravity and the ether removed by means of a rotary evaporator without heat. The mixture was then distilled under reduced pressure (0.1 mm), bp 143-6°. The yield of clear oil was 11g (17.6% yield). Calculated for $C_{11}H_{15}NO_3$: titrateable N, 6.69%. Found, 6.6%.

The four (4) synthesized compounds were tested in cancerous C3H mice as previously described. The following results were observed.

(1) 3-Indolylmethyl-bis(2,3-epoxypropyl)amine

Tumor - Positive, but only a slight color was observed.

Liver - Positive, but only a very slight color was observed.

Muscle - Negative, no color was observed.

(2) Sodium-3-2(3-indolyl)ethylamino propane sulfonate

Tumor - Positive, a beautiful red color was observed.

Liver - Negative, no color was observed.

Muscle - Negative, no color was observed.

(3) Methyl-2-furylmethyl-2,3-epoxypropyl amine

Tumor - Negative, no color was observed.

Liver - Negative, no color was observed.

Muscle - Negative, no color was observed.

(4) 2-Furylmethyl-bis(2,3-epoxypropyl)amine

Tumor - Negative, no color was observed.

Liver - Negative, no color was observed.

Muscle - Negative, no color was observed.

The procedure for dissection and testing these compounds previously described proved rather tedious. Therefore, the following compounds were purchased and tested, _in vitro_, by the butyl nitrite-hydrochloric acid test with the results indicated below.

The following compounds gave a positive test _in vitro_:

Gramine

Indole-3-carbinol

Sodium-N-tryptamine-gamma-propane sulfonate

2-Methylindole

3-Methylindole

7-Methylindole

N-Beta hydroxyethyl-2,3,4,5-tetramethylpyrrole

The following compounds gave slightly or doubtful tests:

3-Indolebutyric acid

3-Indolepropionic acid

5-Bromoindole

Tryptophol

5-Hydroxyindole

5-Aminoindole

2-Indoline Sodium Sulfonate

1-Acetyl-2-Indoline Sodium sulfonate

Pyrrole

The following compounds gave a negative test:

2-Indolecarboxylic acid

3-Indolecarboxylic acid

1-Indoleacetic acid

3-Indoleacetic acid

3-Indolecarboxaldehyde

5-Hydroxy-3-indoleacetic acid

The basis of this present study is the possibility of getting alkylating agents selectively in the cancer cell. As was shown by A.G. Bachmann in 1965 (Potential Neoplasm Inhibitors), indole and certain indole derivatives are selectively absorbed in cancerous tissue at a pH slightly lower than that of a normal cell.

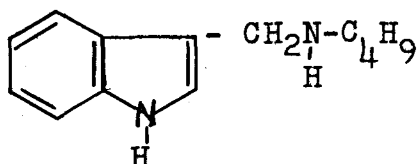
The desired derivatives of indole were epihydrinamines, beta-chloroethylamines, and methanesulfonates. To make the epihydrinamines and beta-chloroethylamines as intermediates, it was necessary to have basic primary amino groups or basic secondary amino groups, preferably the latter. For the methanesulfonates, alcohols were the intermediates.

In some cases satisfactory preparations were not obtained for the products which are potential carcinolytic agents. The synthesis of the potential intermediates are given, however, as a guide to future work. Also, the chemical activity of some of the potential carcinolytic agents was so great that isolation

of the compound was not successful. In such cases derivatives were made which required as intermediates the desired active chemical.

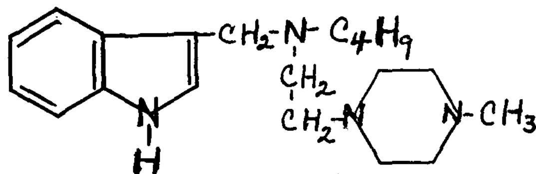
The following intermediates were prepared:

3-Indolylmethylbutyl amine



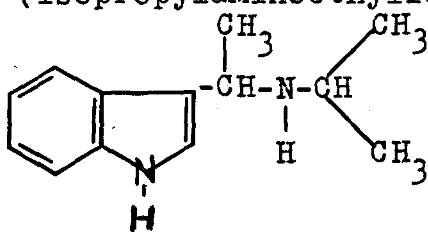
To a solution of 23.4g (0.2 mole) of indole-3-carboxaldehyde dissolved in 25 ml of methanol was added 16.7g (0.22 mole) of *n*-butylamine. The mixture was heated over a hot-water bath for approximately 10 minutes. To this mixture was added slowly a solution of 16g sodium borohydride dissolved in 200ml of methanol, made basic with sodium hydroxide. The resulting mixture was allowed to stand overnight. The mixture was refluxed gently for one hour, cooled, and diluted to 500 ml with water. An oil formed which settled to the bottom of the container. The solution was acidified and the oil dissolved in the acidic layer. The solution was then extracted with two 250 ml portions of ether. The acidic layer was made basic with 6*N* sodium hydroxide and an oil which separated was extracted with 30 ml of ether. The ether was removed on a hot-water bath. The yield of the oil was 12.2g (30% yield). Calculated for $C_{13}H_{18}N_2$: titrateable N, 6.93%. Found, 7.09%.

n_-Butyl-3-indolylmethyl-2-(4-methylpiperizino)ethyl amine



To a solution of 12.2g (0.06 mole) of 3-Indolylmethylbutyl amine in 5 ml of methanol was added very slowly with swirling 17.4g (0.11 mole) of beta-chloroethyl methanesulfonate, and it was left to stand overnight. To this mixture was added slowly with stirring 5g (0.05 mole) of N-methylpiperazine. The reaction mixture was heated on a hot-water bath near 80° for about 20 minutes. Approximately 125 ml of 10% acetic acid was added and the mixture treated with two 50 ml portions of ether. The aqueous-acid layers were treated with 6N sodium hydroxide solution, and a deep red oily layer separated. On treatment with 25 ml of ether, the product crystallized. The solution was filtered; the crystalline product was dissolved in dilute acetic acid and extracted with 6N sodium hydroxide and extracted with ether. The crystalline product which precipitated was 3.7g (23% yield). Calculated for $C_{20}H_{32}N_4$: titrateable N, 12.80%. Found, 12.28%.

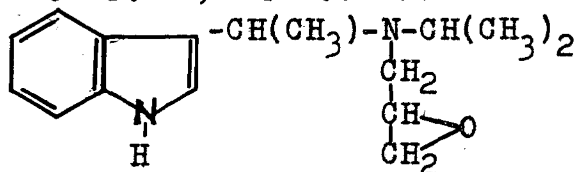
3-(Isopropylaminoethylidene)-indole[85a]



A solution of 46.9g (0.40 mole) of indole in 240 ml of glacial acetic acid was chilled and stirred while 26.4g (0.44 mole) of isopropylamine was added. To this cold solution was added a solution of 18.6g (0.416 mole) of acetaldehyde in 80 ml of benzene. The mixture was allowed to stand at 0°.

The reaction mixture was poured into 800 ml of ice-water and 50 ml of ether. The ether layer was separated and extracted with two 125 ml portions of 1M potassium bisulfite. The combined aqueous solutions were washed with two 50 ml portions of ether, then made basic with 10N sodium hydroxide; during addition of the alkali, the temperature was kept below 25°. To promote crystallization, 100 ml of methylcyclohexane was added, and the mixture was allowed to stand. The precipitate which formed was filtered and recrystallized using methylcyclohexane. The yield of the crystalline product was 35.0g (33% yield), mp 112-4°. Calculated for $C_{13}H_{18}N_2$: titrateable N, 6.93%. Found, 6.8%.

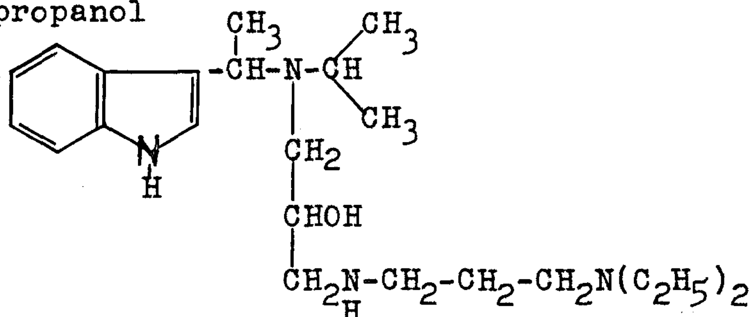
Isopropyl-2,3-epoxypropyl∝(3-indolylethyl)amine



To 12.4g (0.13 mole) of epichlorohydrin was added 10g (0.05 mole) of 3-(Isopropylaminoethlidene)-indole, and the mixture was allowed to stand over hot water (60-75°) for one-half hour. The reaction mixture was then stored in the

refrigerator for approximately four weeks. The viscous substance was acidified with 50 ml of 10% acetic acid. The acetic acid solution was extracted with 50 ml of ether. The aqueous portion was treated with excess 6N sodium hydroxide. The oily layer which separated was taken up in 25 ml of ether. To the mixture was added 10 ml of acetic anhydride, and it was allowed to stand about 15 minutes. An oil, the acetate of the amine, came out of solution and was drawn off. The solution was made strongly basic with 50% sodium hydroxide. An oil which rose to the top was extracted with 10 ml of ether. The ether was removed by means of a rotary evaporator and a clear oil remained. Calculated for $C_{16}H_{22}NO$: titrateable N, 5.43%. Found, 5.9%.

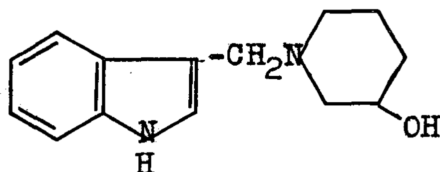
1-[3-Indolylethylisopropylamino]-3(3-diethylaminopropyl)-2-propanol



A mixture of 12g (0.13 mole) of epichlorohydrin and 10g (0.05 mole) of 3-Indolylethylisopropylamine was heated in a hot-water bath (75°) for half an hour. The resulting red solution was dissolved in 50 ml of ether and extracted with 100ml of 15% acetic acid. The aqueous layer was made basic with 6N sodium hydroxide. The oil which separated was extracted with 25 ml of ether. To this solution was added 6g (0.046 mole) of diethylaminopropylamine and the resulting solution allowed

to stand for several minutes. The ether was evaporated and the residue was treated with excess dilute acetic acid. The solution was extracted with 50 ml of ether, and the ether layer was made basic with 6N sodium hydroxide, and the oil which separated was treated with dilute acetic acid. The pH of the solution was adjusted to 7, and the solution was extracted with 20 ml of ether. The aqueous layer was made basic with 6N sodium hydroxide, and the oil which separated was dried and analyzed. Calculated for $C_{23}H_{40}N_4O$: titrateable N, 10.82%. Found, 11.2%.

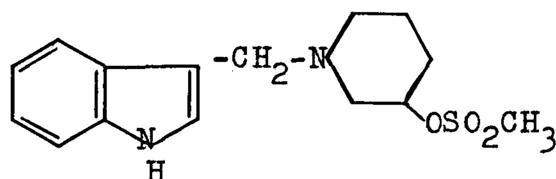
3- [3-(hydroxy)piperidinomethyl indole]



To a solution of 4.5g (0.05 mole) of 3-hydroxypiperidine dissolved in 15 ml of acetic acid was added 7.5g (0.075 mole) of indole dissolved in 10 ml of acetic acid, keeping the temperature below 20°. To the cooled mixture was added slowly 10 ml of formaldehyde keeping the temperature below 10°. The mixture was allowed to stand at room temperature for one hour and then poured slowly with swirling into 250 ml of ether. The mixture was poured into three (3) different 250 ml volumes of ether. Each time the ether was discarded. The aqueous layer was diluted with 100 ml of water and extracted again with 150 ml ether. Excess sodium hydroxide was added to the

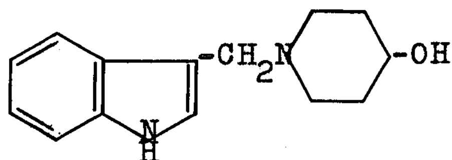
aqueous layer, and a viscous oil appeared. The mixture was stirred well, but the product did not crystallize. The water was decanted off. The product was dissolved in ether, and the ether was vacuum evaporated. The residue crystallized. Calculated for $C_{14}H_{18}N_2O$: titrateable N, 6.09%. Found, 6.09%.

3-[3-(methanesulfonoxy)piperidinomethyl indole]



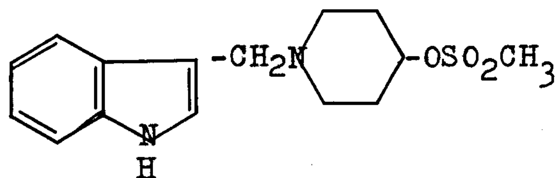
To 2.05g (0.009 mole) of 3-[3-(hydroxy)piperidinomethyl indole] in 10 ml of pyridine was added 11.5g (0.01 mole) of methanesulfonyl chloride, keeping the temperature at 0-10°. After addition was complete, the reaction mixture was allowed to come to room temperature and was poured into 200 ml of ice-water containing 6 ml of 6N sodium hydroxide. An oil separated. The ice-water was decanted, and the oil was extracted with 40 ml of ether. The ether was removed with suction under vacuum. A hot-water bath (95°) was used to speed up the evaporation. These conditions were too drastic, and the compound polymerized. In another similar run there was obtained an oil which gave the following analysis. Calculated for $C_{15}H_{20}N_2O_4S$: titrateable N, 4.54%. Found, 4.6%.

3-[4-(hydroxy)piperidinomethyl indole]



To a solution of 4.5g (0.05 mole) of 4-hydroxypiperidine dissolved in 15 ml of acetic acid was added 7.5g (0.075 mole) of indole dissolved in 10 ml of acetic acid, keeping the temperature below 20°. To the cooled mixture was added slowly 10 ml of formaldehyde keeping the temperature below 10°. The mixture was allowed to stand at room temperature for three (3) hours and then poured into two (2) different 250 volumes of ether as the flask was swirled. Each time the ether was discarded. To the oily product was added 250 ml of water and 25 ml of acetic acid. The mixture was extracted with three (3) 125 ml volumes of ether, each time discarding the ether. The aqueous layer was made basic, and the oil which separated was extracted with 50 ml of ether. The ether was filtered, evaporated, and an oily product was obtained. Calculated for $C_{14}H_{18}N_2O$: titrateable N, 6.09%. Found, 6.39%.

3-[4-(methanesulfonoxy)piperidinomethyl indole]



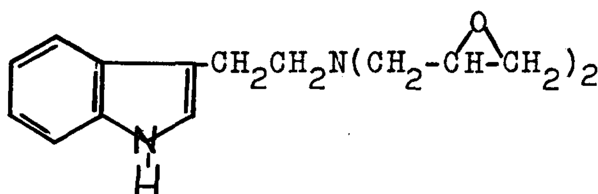
In a similar manner in the run with 0.52g (0.0023 mole) of 3-(4-hydroxypiperidino indole) in 10ml of pyridine and

0.34g (0.003 mole) of methanesulfonylchloride, the product polymerized. In another similar run there was obtained an oil which gave the following analysis. Calculated for $C_{15}H_{20}N_2O_4S$: tritrateable N, 4.54%. Found, 4.3%.

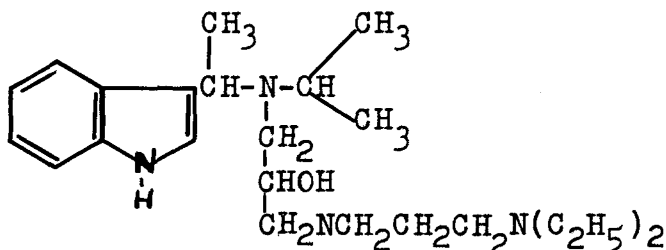
Analytical

Titration Method

The purity of all of the compounds prepared in this work was determined by titration of the basic nitrogen atom(s) using glacial acetic acid as solvent and 0.0903N perchloric acid in glacial acetic acid as the titrant.[85] The following compounds, for example, would contain:



one titrateable nitrogen atom



three titrateable nitrogen atoms

Equipment: Beckmen Glass Electrode pH meter

Reagents: Reagent grade glacial acetic acid, 0.0903N perchloric acid in glacial acetic acid.

Procedure: A sample ranging from 0.05g-0.1g is weighed into a 250 ml beaker. The sample is dissolved in 35-50 ml of glacial acetic acid. Using a pH meter to follow the change in potential of the solution, the sample is titrated with perchloric acid in glacial acetic acid. Increments of 0.10 ml

are added to the solution until the endpoint is approached. The perchloric in glacial acetic acid is then added in increments of 0.05 ml and the endpoint is indicated by the greatest change in potential per 0.05 ml increment of acid.

Calculations:

$$\text{Molecular Weight} = \frac{\text{A x wt. sample x 1000}}{\text{ml acid x N of acid}}$$

$$\%\text{Nitrogen} = \frac{\text{A x 14.0 x 100}}{\text{Molecular weight}}$$

A = Number of titrateable nitrogen atoms in both cases

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