URETHAN - A CARCINOGENIC AND CHEMOTHERAPEUTIC AGENT

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INTRODUCTION

The extensive use of chemical agents to produce tumors in experimental animals is one of the major advances in the study of precancerous and cancerous lesions. The ease with which tumors are produced by these agents enables a study of the mechanism of the production of tumors. As a result of these studies, more useful knowledge will be gained in the field of experimental oncology.

Salaman and Roe defined a complete carcinogen as a "substance capable of producing malignant tumors when applied in adequate dose(s) to susceptible tissue".[1]

Berenblum[2] applied a single dose of 9,10-Dimethyl-1,2-benzanthracene and produced irreversible changes to the backs of mice which resulted in tumors if a promoting agent such as croton oil was subsequently applied. He was able to wait up to six months before applying the croton oil and still obtain tumors. From these results Berenblum divided carcinogens into two distinct types - - complete and incomplete.[2] (Incomplete carcinogenesis will be discussed later.) Urethan, the ethyl ester of carbamic acid, was found to be a complete carcinogen for lung tissue.

In 1947, Larsen[3] tested the methyl, ethyl, n-propyl, isopropyl, n-butyl, and isoamyl esters of carbamic acid for carcinogenic potency on a quantitative basis. He used strain A mice because of their greater susceptibility to lung tumor induction. The ten to twelve week old mice were given intraperitoneal injections of 0.5 mg./gm. once a week for a total of thirteen weeks. The mice were sacrificed at six months of age. The results showed a lung tumor incidence of 16%, 100%, 59.5%, 90%, 12%, and 0%

respectively for these compounds when compared with the control groups. The conclusion drawn from these experiments was that urethan was the most potent carbamate in the production of tumors and that only three (ethyl, isopropyl, n-propyl esters of carbamic acid) of the total number of compounds tested displayed any carcinogenic potency.[3]

Nettleship and his coworkers[4] gave weekly intraperitoneal injections of 1 cc. of a 10% solution of urethan in distilled water/100 gm. of mouse to C3H female mice in an attempt to determine the minimal time of occurrence of these lung tumors. At intervals up to seven and one-half months, the mice were sacrificed and autopsies performed. These experiments demonstrated that there was some evidence of tumors after two months of treatment in animals receiving four or more weekly injections. When the period of treatment was extended, fewer weekly injections were necessary to produce tumors.[4]

Henshaw and Meyer[5] using strain A mice found the minimal number of anesthetic treatments of urethan necessary to produce lung tumors. They used 1.5 ml. of 10% solution/100 gm. body weight. Six groups of mice were used. Group #1 was the control group. The remaining five groups received

ceived one, two, three, four, and five intraperitoneal injections of urethan at weekly intervals. Four and one-half months later, the animals were killed and examined for lung tumors. They observed that a single injection of urethan was sufficient to induce an appreciable number of tumors and that more injections increased the multiplicity of tumors. These tumors appeared in all lobes of the lungs and in all locations of the parenchyma. It was found that urethan induced tumors always developed at sites distant from the place of innoculation and that usually no tissue injury preceded the appearance of lung tumors. These findings suggested to Henshaw and Meyer that urethan's carcinogenic activity had characteristics associated only with the viruses.[5]

Orr,[6] using C3H strain mice which normally showed a low incidence of pulmonary tumors, injected 0.25 ml. of 12% urethan solution intraperitoneal weekly for a period of eighteen weeks. At the end of this time period, he demonstrated multiple adenomatous nodules in all urethan mice.

In 1945, Henshaw and Meyer[7] administered urethan by routes other than intraperitoneal injection to determine if lung tumors could still be produced. Using strain A mice, they implanted 1 mg./gm. of mouse of crystalline urethan subcutaneously. They found a large number of lung

adenomata but no tumors at the site of implantation. When urethan was given orally in drinking water, they again found only lung tumors. It was further observed that with larger doses of urethan, more tumors were produced. This latter finding suggested to Henshaw and Meyer that the cells of the lung vary in susceptibility to urethan and that for a given dose, the pulmonary epithelium responds in a quantitative manner.[7]

In 1947, Larsen et al.[8] demonstrated that urethan crosses the placental barrier. Strain A pregnant mice were given intraperitoneal injections of 25 mg. of urethan. The offspring were sacrificed at six months of age and their lungs examined. Each of the young mice exhibited multiple lung tumors. From these experiments, Larsen postulated that some carcinogenic action had been started in the fetal lung tissue particularly in those mice whose mothers had been injected late in term.[8]

Klein,[9] in 1954, studied the relationship of the induction of lung adenomas in pregnant, newborn, and immature mice, Pregnant, newborn and forty-seven day old strain A albino mice were given one intraperitoneal injection of 2.5 cc. of a 10% solution of urethan in distilled water. Some of the fetuses were removed by Cesarean section and

placed with untreated foster mothers of the same strain. All of the mice were sacrificed six months later. The group of mice that were left in utero five minutes after injection and then transferred to foster mothers had a lung tumor incidence of 82% which indicated that effective amounts of carcinogen crosses the placenta. If the fetuses were left five hours in utero before being transferred to untreated mothers, the incidence increased to 100%. From this data, Klein suggested that because of the short time of exposure of the fetus to the compound, the whole molecule rather than a metabolite crosses the placenta. When newborn mice were injected with urethan, the average number of tumors per lung were less than those mice subjected to urethan in utero although the incidence of tumor formation was the same. In the forty-seven day old mice, there were more tumors per lung than in the newborn and fetus groups. Klein concluded that there was an increased susceptibility to lung tumors in older mice.[9]

Tannenbaum and Silverstone[10] recently proposed that urethan might be a multipotential carcinogen which both induced and augmented formation of various tumors in a number of different tissues. In their first experiment, they used three groups of CXH mice (hybrids of $C_{57}Bl$ females

and C3H males). Groups #1 and #2 received three drops to the interscapular area of a 20% solution of crystalline urethan in redistilled acetone two times per week (about 12 mg. urethan per dose). Group # 3 (control) were given acetone alone. Groups #1 and #3 were treated for eighteen months and Group #2 was treated for six months. The experiment was terminated when the mice were two years old. The mice in Group #1 that died prior to the termination of the experiment were autopsied and the following pathologic conditions were found: 1) benign lung adenomas; 2) blood cysts of the liver; and 3) mammary carcinoma. Of the mice that lived to the termination of the experiment, the following pathologic conditions were found: 1) mammary carcinoma; 2) pulmonary adenomas; 3) changes in the interscapular fat pad varying from hemorrhagic cysts to spongy, blood filled tumors; 4) blood cysts of the liver; and 5) intra-orbital lesions, multilocular papilliferous cystadenomas. The mice of Group #2 had fewer tumors of the breast, interscapular fat pad, and intra-orbital tissues. The lung and liver involvement were equal. From these latter results, Tannenbaum and Silverstone concluded that the lung tumors and liver blood cyst formation either required a shorter time for development or a lower dose of urethan.[10] In

Group #3, five mice developed pulmonary adenomas. Because of this finding, it was postulated that in mice treated with urethan the formation of lung adenomas was one of potentiation or enhancement of a spontaneous process. Mammary carcinoma had appeared spontaneously in the CXH mice; and therefore, the question arose whether treatment with urethan actually induced or just enhanced the formation of "normally expected" tumors.

In a second experiment, Tannenbaum and Silverstone[10] used two groups of DBA female mice (three months old) to demonstrate that urethan would enhance the formation of mammary carcinoma in more susceptible strains. The mice were given two applications per week of urethan (20% solution in acetone) for a total of sixteen months. At twenty-two months, the experiment was terminated. The results of the experiment showed that 41% of urethan treated mice developed mammary carcinoma in 60.7 weeks while 27% of the control group developed mammary carcinoma in 70.1 weeks. Of the urethan treated mice 12% developed hemorrhagic swelling of the interscapular fat pad, two of which were malignant tumors. No mesenchymal tumors developed in the control group. Lung adenomas and hepatic blood cysts developed in the urethan treated mice but none developed in the control group. No intra-orbital swellings developed in either group.

In a third experiment, Tannenbaum and Silverstone[10] used forty C3H female mice, two months old. The doseage applied to the interscapular area was the same as in the previous experiments. All the mice of both groups developed mammary carcinoma. In the urethan treated mice, the minimal time of occurrence of the mammary carcinoma was 45.7 weeks, as compared to 46.8 weeks for the control group. The number of tumors per mouse in the urethan treated group was 2.1 as compared with 1.5 tumors per mouse in the control group. All the other types of tumors previously mentioned were found in the urethan treated mice except the intraorbital tumors. Spontaneous mammary carcinoma appeared when the C3H mice were ten months old. In these mice, urethan increased the number of tumors per mouse. Spontaneous mammary carcinoma occurred when DBA female mice were seventy weeks old. In these mice urethan decreased the average time of occurrence of these tumors by ten weeks and augmented the incidence of tumor formation. In CXH female mice, there was a low incidence of spontaneous mammary carcinoma with three years being the average time of occurrence. In the CXH group of mice treated with urethan, the average time of occurrence of mammary carcinoma was 69 weeks.

In evaluating all three experiments, Tannenbaum and Silverstone[10] concluded that the blood cyst formation in the liver was due to a dilatation of the sinusoids followed by a pressure necrosis of the liver cords and not neoplasia. It was further thought that urethan induced only mesenchymal tumors and lacrimal tumors. From the results of these experiments, Tannenbaum and Silverstone doubt if the two-step concept of carcinogenesis (which will be discussed in the following section) can be applied to urethan. Their theory is that it is better to consider urethan as a multi-potential carcinogen that affects different tissues in varying degrees: the lung readily and potently, the interscapular fat pad with intermediate intensity and the skin to a mild degree. Urethan causes no overt inflammatory or progressive tissue changes in the skin but may cause subtle and yet unrecognizable alterations which can lead to carcinogenesis. Urethan has been postulated to act through an interference with the nucleic acid synthesis (which will be discussed later). Damage to the liver caused by urethan administration may result in a decreased inactivation of the estrogenic substances and increase the effective levels of estrogen on the target tissues. Estrogens have been known to evoke leukemia and

the formation of breast cancer. However, at present "there is no reason to assume that the mode of carcinogenic action of urethan must be the same for all tissues."[10]

Salaman and Roe[1] define an incomplete carcinogen as a substance which plays some part in tumor production but is incapable of producing malignant tumors when applied alone. Carcinogenesis consists of two phases - - initiation and promotion.[11] They are different and independent processes which in order to be effective must take place in this sequence.[11] They do not act by summation.[12] The number of tumors produced is a function of the initiating action while the latent period is a function of the promoting agent[13] (which will be discussed later).

Salaman and Roe[1] painted urethan on the backs of S strain albino mice for eighteen weeks. There were no tumors produced by two weekly doses of urethan (120 mg. each dose) alone. However, two weekly doses of urethan followed by weekly applications of 0.5% of croton oil produced a large number of tumors. From the results of these experiments, it was concluded that urethan was capable of initiating the process of carcinogenesis. Salaman and Roe[1] also concluded that urethan was capable of transforming normal tissue into preneoplastic tissue. A promoting agent (such as croton oil) which is necessary for the second step of carcinogenesis is capable of transforming the precancerous tissue into visible tumors.[12]

In 1954, Roe and Salaman[14] determined the effect produced on tumor formation by urethan in different solvents. Acetone as a solvent permitted urethan to be readily absorbed whereas urethan suspended in carbowax required a longer time period for absorption. Croton oil was applied twice weekly. However, there was no noted change in tumor incidence between the two groups. This indicated that the rate of absorption did not affect the efficiency of urethan as an initiator or carcinogenesis.[12]

Berenblum and Haran-Ghera[13] using female Swiss mice studied the systemic initiating action of urethan. A 5% solution ofurethan in distilled water was given by the oral, subcutaneous and intraperitoneal routes to respective groups of mice. A 40% solution in acetone was used for skin application. In the first group, single feedings of 1 mg. (0.2 cc.), 4 mg. (0.8 cc.), 16 mg. (3.2 cc.) and 64 mg. (12.8cc.) of urethan were followed by twice weekly applications of croton oil. An increased response in skin tumor production was noted with increased doseage. In the second group, urethan's action as a skin carcinogen was compared when it was given by the oral, subcutaneous and intraperitoneal (I.P.) routes. A total dose of 50 mg. (10 cc.) I.P. and 64 mg. (12.8 cc.) by the other routes were given. This was followed by twice weekly applications of

croton oil. The experiments demonstrated that there was a 100% tumor induction incidence in all groups. In the third group, a comparison of the results was made between the skin application of urethan with and without a plastic collar worn by the mice. The collar was used to prevent the mice from licking the urethan off their backs. The results of the experiments showed that both groups developed tumors; however, the group without the collar had a slightly higher incidence. A fourth group of experiments evaluated the influence of sex on tumor formation by urethan. It was shown that the female mice were more susceptible to skin and lung tumors. Their data was collected by pooling the results of their past experiments. The experiments demonstrated that regardless of the route of administration of urethan, skin and lung tumors were produced. Because urethan acted almost as effectively any way it was given, the changes in the gut and absorption through intestinal mucosa were not required for action.[13]

As previously mentioned, carcinogenesis is ordinarily considered a two step process - - initiation and promotion.[12] Promoting agents, such as croton oil which is one of the most potent co-carcinogenic agents on mouse skin,[15] are capable of producing tumors in tissues which have undergone the changes produced by an initiating agent.[1]

Berenblum[16] suggested that croton oil might exert its action by one of two possible ways. Either croton oil might be a weak carcinogen and a summation action might take place between the oil and the other compound used as an initiating carcinogen or croton oil was able to augment carcinogenesis without itself possessing any carcinogenic action. Berenblum[16] favored the latter explanation because in his experience only small warts were produced by croton oil which never became malignant and would disappear when croton oil treatment was stopped. However, Roe[17] found that papillomas appeared in almost all groups of mice after eighteen weekly applications of croton oil. When treatment was stopped, the tumors disappeared but reappeared when treatment was again started and in increased numbers. Recently Boutwell et al.[18] demonstrated on Sutter mice that croton oil was an effective agent for the production of both papillomas and carcinomas without the aid of a preceding small dose of carcinogenic hydrocarbon. Thirty mice were used each receiving 1/40 ml. of 1.5% croton oil in benzene applied to the skin of the backs of the mice two times per week for a total of sixty-two weeks. By the end of the twenty-eighth week more than 45% of the mice had tumors. The benzene was checked separately and shown not to have had initiating or promoting properties. A small

dose of DMBA applied prior to croton oil treatment produced rapid formation of papillomas in comparison with croton oil alone. From these experiments, it was concluded that croton oil was a weak carcinogen and a strong promoting agent. The acknowledgement of croton oil as a weak carcinogen did not discredit the concept of promotion of carcinogenesis nor the ability of croton oil to do so, but called for further evaluation of experiments where croton oil was assumed to be noncarcinogenic.[18]

Recently, Boutwell and Bosch[19] confirmed the carcinogenicity of creosote for mouse skin. They observed that mice which had lived in wooden cages treated with creosote (a wood preservative) developed papillomas readily when further treated with croton oil. They had assumed that croton oil was the carcinogen. However, further study revealed that carcinogenesis had been initiated previously by the use of creosote. ("Creosote oil is a loosely defined industrial distillate of coal tar or of petroleum residues and is of variable composition."[19]) Seven groups of female albino mice eight weeks of age were selected for these experiments. The first group received no initial treatment and one week later 25 µl, of undiluted creosote was applied twice weekly for the duration of the experiment.

The second group received an initial application of 75 µg. of DMBA per mouse followed one week later by twice weekly treatment with 25 µl. of benzene (solvent control). The third group got one application of DMBA followed by 25 µl. of undiluted creosote twice a week. The fourth group got one application of 75 µg. of DMBA and one week later had 25 µl. of 0.5% solution of croton oil in benzene applied twice a week for the remainder of the experiment. The fifth group received no initial treatment, and one week later 25 µl. of 0.5% solution of croton oil was put on the dorsal skin of the mice two times per week for four weeks, followed one week later by twice weekly applications of croton oil. Papillomas developed in Groups #1, #3, #4, #5, and #7 but not in Groups #2 and #6. Group #1 developed papillomas rapidly with a maximum of 5.4 tumors per mouse at the twenty-eighth week. Application of 75 µg. of DMBA shortened the time for appearance of papillomas by four weeks. Mice treated with croton oil only developed 0.1 papillomas per mouse at 28 weeks while mice which received creosote for four weeks prior to croton oil treatment developed 2.8 papillomas per mouse. The mice in Group #4 developed papillomas the most rapidily; six per mouse at sixteen weeks. From these experiments, Boutwell and

Bosch confirmed earlier reports that creosote was a complete carcinogen for the skin and had both the initiating and promoting properties. They also demonstrated the "unusual sensitivity" of mice previously exposed to creosote to the promoting effect of croton oil. Therefore, they concluded that mice exposed to creosote are "unsuitable for use in experimental oncology".[19]

Urethan is a colorless, odorless, crystalline compound which has a slightly salty or bitter taste.[20] The chemical structure for urethan is $H_2N - C(=O) - O - C_2H_5$. It has a melting point of 48°C and a boiling point of 184°C.[21] Urethan is rapidly absorbed from the gastrointestinal tract and hydrolyzed in the liver.[22] The radioactive carbon atom from the carbonyl labeled urethan which remains in the body after injection is fairly well distributed throughout most tissues without indication of consistent localization or tissue specificity.[23] The metabolism of urethan in the body can proceed by either of two pathways or both - - spontaneous or enzymatic hydrolysis.[24] The hydrolysis of urethan produces carbon dioxide, ethyl alcohol, and ammonia.[25]

Whether urethan itself or a metabolite is responsible for carcinogenesis has not been determined. Skipper et al.[26] found that almost immediately after injection, urethan began to break down with about 90% of the radioactive carbonyl carbon appearing in the expired air in the first twenty-four hours. Using metabolism cages, they were able to collect the expired gases of the mice as well as their excrement. They also found 5 to 10% of the C^{14} in the

urine and about 1% distributed throughout the body.[26] A portion of the tissue activity after administration of C^{14} urethan was due to $C^{14}O_2$ which was produced by urethan's metabolism.[23] The exact rate of hydrolysis of urethan is unknown but it appears to be complete within eighteen hours.[26]

In 1955, Rogers[27] showed by a series of experiments that an intermediate of urethan was involved in the process of carcinogenesis. By exposing fetal lung tissue in vitro to varying concentrations of urethan, he was able to show that no pulmonary adenomas were produced after waiting various periods of time up to twenty-four hours before transplantation of the lungs to thighs of unexposed mice. After an interval of two months, the lung tissue was inspected and as mentioned previously no lung tumors were found. In another group of experiments, pregnant mice, of eighteen to twenty weeks gestation, were used to study the effect of urethan in vivo on fetal lung. A 5% solution of urethan was injected by the intraperitoneal route and after a period of three hours, the lungs of one group were transplanted into the thighs of unexposed hosts; the second group was left in utero for a longer period of time before transplantation; and the third group was left

in utero. After an eleven week interval, the lungs of all three groups were excised and the results showed that the group left in utero had the most papillomas while the group left in utero for five hours before transplantation had the second greatest number of tumors. Another experiment consisted of exposure of fetal lung tissue in vitro to serum taken from rabbits previously injected with urethan and then transplanted to the thighs of unexposed hosts. In this group, Rogers found a large number of papillomas. It was concluded from these experiments that it was necessary for lungs to be exposed to an intermediary substance(s). When urethan is painted on the skin, enough is absorbed to cause a "narcotizing effect" and there is no reason to believe that an intermediate substance may not be necessary for the initiating of skin tumors.[27] The final answer as to whether urethan or a metabolite is the carcinogen depends on the isolation of this metabolite.

Many theories have been proposed to explain the mechanism of action of the carcinogenic compounds. The Millers[28] suggested a binding mechanism between carcinogens and protein. It was demonstrated that binding occurred between the azo dye and protein. They fed an azo dye (4-dimethylaminoazobenzene) to rats. Later the liver was removed and homogenized. Attempts to liberate the dye from the crude protein by mild procedures were unsuccessful. The removal of nucleic acids from the liver powder with hot trichloroacetic acid (TCA) did not significantly alter the content of bound dye in liver protein. By using colorimetric measurements in an acid solution, the amount of dye bound to liver protein was detected. From these experiments, the Millers concluded that, in vivo, a firm combination took place between the derivatives of the carcinogen and a cellular constituent of high molecular weight, probably protein. By using trypsin to digest the liver powders, it was found that the dye was released at a rate parallel to the destruction of protein. The dye was not bound to nucleic acid because upon their removal, the

dye was not liberated. It has been shown that a covalent type of linkage exists between the protein and the dye. It was unlikely that any other type of linkage could withstand the prolonged action of hot polar solvents, heat coagulation, detergent and hot TCA. The latter reagent disrupts both the salt and nonsalt linkage which exists between the nucleic acid and the protein.[28]

A further finding from the above experiments was the absence of bound dye in tumors. The Millers[28] interpretation was that a tumor, once initiated, did not need a carcinogen for its continued growth. They also suggested that tumor cells and normal cells differed with respect to certain proteins. These conclusions were strengthened by showing that paradimethylaminoazobenzene did not bind to tissues (small intestine, kidney, spleen, lung, heart, and skeletal muscle) which were resistent to the carcinogenic action of this drug.

In 1951, E. C. Miller[29] demonstrated the binding of derivatives of 3,4-Benzpyrene to epidermis of mouse skin. Using 0.2% solution of benzpyrene in benzene, four to six drops were applied to the backs of cleanly shaved female Sutter mice. The skin was collected, homogenized and precepitated with TCA. To determine if the dye was bound to protein, the protein extracts were washed with three 30 ml.

charges of boiling solvents over a ten hour period. If the derivatives had only been adsorbed to the protein, significant loses would have been detected by fluorescence. The experiments indicated that the derivatives of 3,4-benzpyrene were combined chemically in vivo with the epidermal protein. Derivatives of benzpyrene were differentiated from the parent substance by their different solubility properties and by the fluorescence of 3,4-benzypyrene being quenched to a greater degree by dissolved oxygen than its derivatives. The lack of extraction of derivatives of benzpyrene from protein by boiling solvents showed there was binding. Mrs. Miller concluded from these experiments that the possible action of a carcinogen is to alter or delete some of the proteins and that the cells which survived grew, but lost their capacity to respond to the growth controls of the body and became classified as tumor cells.[29]

Further work by the Millers[30] on the combination of carcinogens and tissue constituents strengthened the correlation between protein-binding and carcinogenesis. Livers of rats fed azo dyes were analyzed for both free and bound dyes. Tumors arising in these livers did not contain detectable quantities of dye although bound dye was present in the liver before evidence of these tumors were present. The tumors did contain marked levels of free dye "so lack

of penetration of the dyes into the tumors does not seem to be responsible for the lack of bound dye."[30] This finding suggested that the "carcinogenic dyes, through combination with certain proteins, might cause their (proteins) gradual deletion from liver cells."[30] It was also thought that cells might arise which had either complete absence or low levels of those proteins which were bound to the dye. A further result of this experiment showed that with the cessation of dye administration, the disappearance of the dye from the liver cells were correlated with the breakdown of normal liver protein. This work helps substantiate that protein binding is important in the mechanism of carcinogenesis.[30]

In 1953, Weist and Heidelberger,[31, 32] demonstrated quantitatively binding between 1,2,5,6-Dibenzanthracene-9,10-C^{14} and the protein of skin. A 1.0 ml. dose of 0.5% solution of DBA-9,10-C^{14} in benzene (.5 mg. of hydrocarbon or 6.5×10^5 cpm per mouse) was applied to the skin of the backs of ten Rockland female mice. The mice were sacrificed two hours, two, nine, eighteen and forty-two days after application of the compound. The skins were cleaned off with benzene to remove any adhering hydrocarbon and then shaved. The connective tissue and fat were removed by a

liquid air scraping technique[31] and the skins of three mice were combined and homogenized in isotonic potassium chloride. The homogenate was centrifuged to separate the tissue into particulate and soluble fractions, and the proteins were isolated from the two fractions by precipitation with TCA and washed with sodium hydroxide, ethyl alchol-ether mixture, benzene and purified dioxane.[31] The washing procedure was sufficient to remove all the adsorbed radioactive compounds. The results showed that mice sacrificed after two hours exposure to the radioactive hydrocarbon had 5 to 6% of the total radioactivity bound to skin protein. The maximum specific activity in the skin protein was highest after two days exposure to DBA-9,10-C^{14}. By using a control mouse and adding 0.15 mg. of DBA-9,10-C^{14} to the homogenate and treating the tissues with the same washings as described above, Weist and Heidelberger were able to demonstrate only slight in vitro binding of the hydrocarbon to protein. They further showed a covalent chemical bond between the radioactive compound and the tissue protein. The proteins were "redissolved in suitable solvents",[32] washed with organic solvents, reprecipitated and the specific activity again measured. A decrease in the specific activity would indicate dissociation of the C^{14} from protein. The results

showed that the reprecipitated protein had the same specific activity; and, therefore, ruled out any possibility of surface absorption of the radioactive hydrocarbon.[32] By extracting the protein for fifteen minutes with 5% TCA at 90°C, Weist and Heidelberger demonstrated the specific activity remained constant. This proved that the radioactive hydrocarbons were not bound to nucleic acids which would have degraded by this strong treatment. This tracer study method gave a more quantitative result than the fluorescent method of determining protein binding because the relative amount of binding could be defined.[32]

In 1955, Bhargava et al.[33] demonstrated that the metabolite(s) of DBA-9,10-C^{14} were bound to amino acids. Using Rockland female mice, they painted the backs of these mice with DBA-9,10-C^{14}. The mice were sacrificed at the end of two days at which time there was maximum binding. The skin was removed and the proteins precipitated, washed, extracted and plated. The proteins were then subjected to digestion by pepsin which degraded the proteins to the polypeptide level. The polypeptides were plated and no loss of specific activity was noted. The polypeptides were then subjected to alkaline hydrolysis which degraded them to amino acids. These amino acids were plated and counted

and again no decrease in specific activity was noted. The results of these experiments demonstrated that binding was not due to adsorption or occlusion of DBA on the protein. Also the extended washings to which the proteins were exposed showed that a covalent bond must be present between the DBA or its derivatives and the amino acids because ionic bonds could not survive the treatment to which the proteins were subjected.[33]

Bhargava and Heidelberger[34] in a subsequent paper demonstrated the structure of the carcinogen-protein complex. They had observed that about 25% of the protein-bound radioactivity in the skin involved the binding of 2-phenylphenanthrene-3,2'dicarboxylic acid (PDA) to the protein. If PDA were bound to protein through its carboxyl groups as an ester, amide or imide, "hydrazine treatment of the protein would result in cleavage of the peptide-metabolite bond and thus yield radioactivity". Carrier experiments were done using the hydrazides of derivatives of PDA on the first fraction of the peptide fraction (pepsin-insoluble large granular, pepsin-soluble large granular, pepsin-treated soluble). It was revealed that half of the radioactivity was due to the dihydrazide of PDA and the other half to the cyclic hydrazide of PDA.

When the PDA-protein bond was treated with hydrazine, the dihydrazide of PDA was formed. From the results of these experiments, it was concluded that PDA was bound to protein through a nitrogen (amide or imide) or oxygen (ester) bond or both. To further evaluate the possibility of an ester linkage, the peptide fractions were treated with lithium aluminum hydride. If an ester-linkage had been involved, there would have been a cleavage of PDA-protein bond with the formation of alcohol of PDA. However, when this procedure was done, no radioactivity was extracted. If binding involved an amide linkage, treatment of the PDA-protein bond with phosphorus pentachloride, stannous chloride in ethereal hydrochloric acid, and water, would result in a "mono-and/or dialdehyde of PDA which would be extractable in organic solvents". Bhargava and Heidelberger found radioactivity which was extracted with ethyl acetate after hydrazine treatment and concluded that the binding of PDA to mouse skin was through an "amide (both mono and di) linkage". This represented about 25% of the total bound radioactivity. However, it is not yet known whether this 25% "or the other 75% represents the structure of the complex" concerned with the initiation of cancer.[34]

Heidelberger and Moldenhauer[35] using a series of C^{14} hydrocarbons demonstrated a relationship between binding and carcinogenic action. They found that the non-carcinogenic compounds such as phenanthrene were weakly bound; weak carcinogens like 1,2-benzanthracene bound a little more; and potent carcinogens such as 3,4-benzpyrene and 20-methylcholanthrene resulted in peak specific activities. An interesting finding of these experiments was that 1,2, 3,4-DBA reported previously as a non-carcinogen was bound to the greatest degree. However, except for this latter compound, there was still an excellent correlation between the carcinogenic activities of the various hydrocarbons and the quantities bound to skin protein. Also of interest was the observation that a pre-application of 1,2,3,4-DBA which was non-carcinogenic but was highly bound, did not inhibit the binding of 1,2,5,6-DBA. This indicated that non-carcinogens and carcinogens might be bound to different receptor sites.[35]

In 1957, Rogers[36] attempted to explain the mechanism of action of urethan in the production of pulmonary adenomas. He pointed out that young, rapidly growing mice were more responsive to standard doses of urethan than were older and slower growing mice. There was also

a recognized correlation in the rate of synthesis of deoxyribosenucleic acid and the growth rate of the body at a given time. Therefore, Rogers[36] showed to what extent the number of tumors initiated by a single injection of urethan might be influenced by exposure of the animals to DNA, RNA, their chemical components, precursors and the substances influencing their rate of synthesis. A single injection of DNA hydrolysate prior to the exposure of mice to urethan greatly reduced the number of tumors initiated. Conversely, the administration of aminopterin which inhibited nucleic acid synthesis increased the carcinogenic activity of injected urethan. This increase could be prevented by the injection of a DNA hydrolysate. The various components of nucleic acids were tested and the pyrimidines were found to be the most active inhibitors of tumors produced by urethan. Orotic, dihydro-orotic acid and carbamyl aspartic acid were also found to exert profound inhibition upon carcinogenesis by urethan. In DNA treated mice, nineteen adenomas appeared after further treatment with urethan while forty-seven adenomas appeared in mice treated only with urethan. When mice were pretreated with aminopterin for several days, the number of adenomas increased. However, mice pretreated with aminopterin followed by treatment

with DNA hydrolysate showed greatly reduced numbers of adenomas. Orotic acid not only modified the response of animals to urethan, but varying the dose of either the urethan or orotic acid showed that these two substances acted as competitive antagonists. Dihydro-orotic acid also inhibited the formation of pulmonary adenomas by urethan. From these experiments, Rogers concluded that the mechanism of action of urethan in initiating pulmonary tumors was closely associated with nucleic acid synthesis. The experimental results indicated that urethan interfered with the normal DNA synthesis. Inhibition of adenine synthesis by aminopterin produced no effect on tumor formation by urethan, while interference with thyminesynthesis increased urethan's carcinogenic activity. Rogers further suggested that the carcinogen acted in the pathway of nucleic acid synthesis before the stage of synthesis of orotic acid; and, perhaps, at the level of ureidosuccinic acid because this was one of the first compounds in the chain to inhibit urethan's activity.[36]

Elion and her co-workers[37] demonstrated a tumor inhibition and uracil antagonism by certain compounds. These drugs (4 thiouracil, 6 azauracil and 6 azacytosine) which were uracil antagonists were tested against Lactobacillus

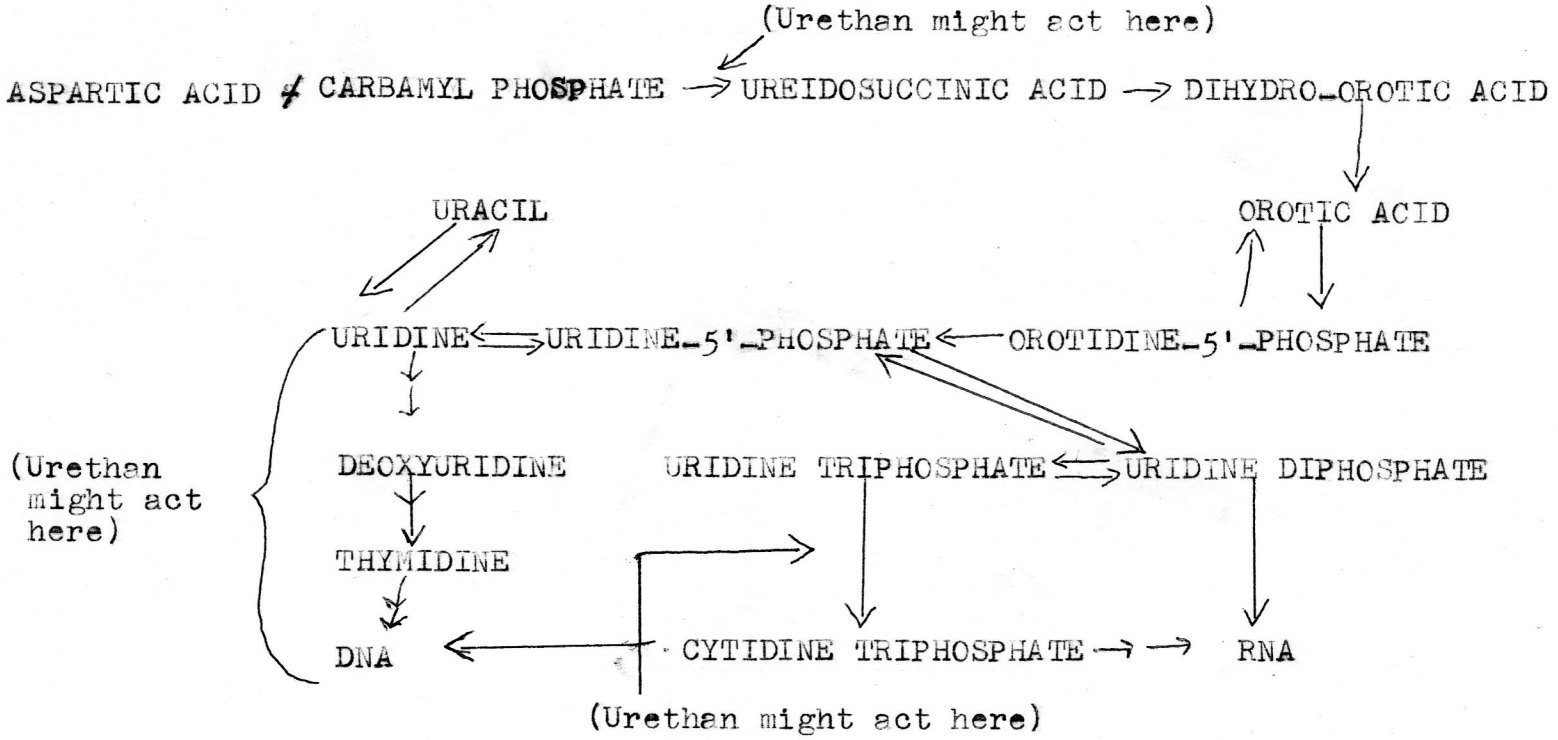
casei. This bacteria, grown on OFA media (a folic acid, amino acid, vitamin, glucose media, with small amounts of Mg, Mn and Fe), needed uracil for its growth even though it was capable of the production of its own uracil. Isobarbituric acid produced inhibition of this organism, but the inhibition was reversed by the addition of uracil to the media. All the compounds tested which produced inhibition of growth of the organism on the OFA media were tested in the presence of varying concentrations of uracil. These antimetabolites which were found to inhibit growth of this bacillus were then tested against Mammary Adenocarcinoma 755 in C57 Bl/6 male mice. Urethan was also used because of its possible effect on thymidine synthesis. The tumors were weighed, transplanted to the axilla of normal hosts, treated for seven days, removed and weighed again. Urethan showed good anti-tumor activity at a dose of 50 mg/kg and above. Urethan in combination with 6-azauracil and 6-azacytosine, produced similar anti-tumor effects at lower doseages than any of the compounds alone. The results demonstrated that 1/8 of the minimal effective dose (MED) of 6-azauracil and 1/4 the MED of urethan produced a better cure rate (tumor resorption) than with either drug used alone at its maximum tolerated dose. Urethan and 6-azacytosine

(25 mg/kg.) did not have much effect on tumor inhibition. Four thiouracil and urethan (½ MED, 25 mg/kg. of both) produced better inhibition of tumor growth than either of these two drugs alone.[37] The nucleosides, thymidine and cytidine, antagonized the effect of urethan on these tumors. Thymine overcame the effect produced by urethan but had only slight inhibition on the effects yielded by 6-azauracil alone or in combination with urethan. Orotic acid inhibited the effects of 6-azauracil but only partially reversed the effects of urethan alone or in combination with 6-azauracil. When 62.5 mg/kg. (½ MED) of 6-azauracil and 25 mg/kg. (½ MED) of urethan were used in combination, none of the metabolites effectively reversed inhibitions of tumor growth. Some further interesting findings were that the combination of 6-azauracil plus urethan was much less effective on older tumors. One-half of the MED of both compounds reduced the incorporation of uracil-2-C^{14} in all tissues studied (liver, gut, tumor). One-fourth of the MED of both drugs reduced the uracil uptake in tumors by 42% of the control figure.[37]

Combinations of anti-metabolites which affect systems related "sequentially or concurrently"[37] showed potentiation

of their effects with a great frequency. Urethan seemed to have had an effect on the pyrimidine biosynthesis and a synergistic action between urethan and some of the uracil antagonists appeared possible. Elion et al. postulated that the "loci of action" of 6-azauracil and urethan might be closely related. The reversal of urethan's carcinogenic effects by ureidosuccinic and orotic acids although incomplete and the lack of reversal by uracil and uridylic acid suggested to these workers that urethan did not interfere with carbamyl phosphate. Urethan might interfere with the methylation of the uracil moiety to the thymine moiety. Abnormal mitoses in Walker tumors produced by urethan were prevented by thymine but not uracil.[37]

In summary, the chart[37] on the following page demonstrates some of the possible biochemical loci of action of urethan.



Further research must be done before the location of urethan's action can be definitely established.

In 1946, Haddow and Sexton[38] demonstrated urethan's ability to inhibit cell division in both the Walker Rat carcinoma and white blood cells. In the same year Patterson and co-workers[39] demonstrated clinically that there was a marked fall in leukocytes in both chronic myelogenous and lymphocytic leukemia. She also noted a decrease in the size of the spleen and lymph nodes and an increase in the hemoglobin. Guyer and Claus,[40,41] using corneal epithelium to study mitotic effects, noticed that urethan abolished all mitotic stages for eight to twelve hours. This mitotic inhibition was found to be selective, acting principally in leukemia on early myeloid cells. However, the extent of this inhibition was of such a nature as to lead to agranulocytosis and even death.[20]

In rats with Walker 256 tumors treated with urethan, Haddow[42] found that histologically, the tumors had changed from a cellular structure to that of a fibrous structure with an abundant stroma. This change was much like that seen after roentgen therapy.[43]

Engstrom et al.[43] transplanted myelogenous chloroleukemia to mice which subsequently developed leukemia.

About six to eight weeks after innoculation, the spleen was palpable; there was a high white count with immature forms and enlarged lymph nodes. Urethan in an aqueous sol was given by intraperitoneal injection (.004 to 1 mg/gm. of body weight). The results showed a decrease in the white blood count from 100,000 to normal, the spleen and lymph nodes decreased in size and mature leukocytes appeared in the peripheral blood. The mice lost weight and attempts at reducing the dose resulted in a lesser degree of lowering of the white blood count and only a partial reduction in size of the lymph nodes and spleen. The white blood cells were, however, more mature than those present without treatment. Bone marrow studies showed a decrease in marrow activity in leukemic mice but not in normal mice.[43] Watkins,[44] in 1949, observed that no benefit was arrived in treating acute human leukemias with urethan.

Skipper and his coworkers[45] found that labeled urethan was hydrolyzed more slowly in cancerous mice than in normal mice. The hydrolysis was enzymatically catalyzed and appeared to be interfered with by certain tumors or leukemia. However, the mechanism of action of urethan on cancer cells might be due to the increased rate of metabolism and division

of these malignant cells and the greater preference of urethan for these cells. In earlier experiments, Skipper and his group[46] demonstrated that the combination of urethan and nitrogen mustard inhibited the incorporation of formate into the nucleic acids. Upon injection of sodium formate C^{14} into mice, the amount of formate incorporated in the nucleic acids in control groups was higher than those treated with nitrogen mustard and urethan. Skipper et al.[47] tested a series of anti-leukemic compounds in various combinations to determine their joint effects. The combination of urethan and nitrogen mustard was the only compounds and agents tested which exhibited any synergistic activity.

Urethan is active in one other neoplastic condition - - multiple myeloma. Multiple myeloma of a diffuse nature represents a form of aleukemic plasma cell leukemia.[48] Webb et al.[48] concluded that urethan reduced the localized pain, retarded the growth of the myelomatous marrow cells, diminished the abnormal serum globulins and Bence-Jones proteinuria, changed electrophoretic serum patterns, permitted some repair of bone lesions, reduced fever and maliase and provided general symptomatic improvement.[48]

Side effects of urethan therapy were nausea and vomiting. There was a general suppressive effect on all the cellular elements of the blood. The usual doseage was from two to four gms. per day for several months depending on the side effects. The patients were carefully studied with frequent hemoglobin, hematocrit, white blood count, and platelet studies. If a depression of these elements occurred, the drug was discontinued until the levels returned to normal. Relapses following administration of urethan might be due to the myeloma cells becoming adapted to the drug. Treatment of multiple myeloma is only partially satisfactory; and, unfortunately, there is no consistent and favorable response to urethan administration. It temporarily influences the disease but does not provide a cure. The more chronic the disease, the better the results with urethan may be. Webb et al.[48] report that the combination of 1.0 gm. of urethan and 50 mg. of nitrogen mustard daily may offer a chance of longer remissions.

The toxicity of urethan has not been fully worked out but there is good evidence that it can cause pulmonary adenoma formation in mice.[22] In rats, pulmonary adenoma formation has also been found and with prolonged administration of urethan malignant hepatomas have been produced.[22]

In man, the toxicity generally consists of nausea, vomiting and in rare instances hepatitis.[48]

Although much has been learned about urethan, its mechanism of action has not been established. The two major theories proposed which may be related but not firmly substantiated are the interference of nucleic acid synthesis and the binding of carcinogens to protein. In fact, using Rogers' work as a basis, we can not even be sure if urethan or its metabolite is the carcinogenic agent. However, it is well-documented that urethan or its metabolite is the carcinogen both of the complete and incomplete type[2] and, perhaps, multipotential.[10] Urethan passes through the placental barrier, induces lung adenomas,[8] and is more effective on older female mice.[10] Urethan causes the formation of skin papillomas and lung adenomas no matter by what route it is given.[13] Henshaw and Meyers'[5] results demonstrate that one anesthetic treatment is all that is necessary to induce an appreciable number of lung tumors in strain A mice.

Urethan is also able to induce blood cysts of the liver, mesenchymal tumors and intraorbital tumors if given for a varying period of time. The question, however, also arises if the adenoma formation is just one of enhancement

by urethan of expected tumors in mice.[10]

Urethan has been used as an anti-tumor drug since 1946.[39] Treatment of chronic myelogenous and lymphatic leukemia with urethan has been extensively used for palliation, but, unfortunately the palliative effects are neither satisfactory not lasting.[22,39] Urethan has no effect or benefit in acute leukemia.[44] The action of urethan on the leukemic process simulates the effects of x-ray.[39,43]

Urethan is still being extensively used for the treatment of multiple myeloma. In this condition symptomatic relief as well as abatement of some of the signs and symptoms does occur.[48] However, relapses do occur eventually, but this is no deterent for not using this drug to provide relief from multiple myeloma as long as possible.

This review is an attempt to summarize current facts and opinions concerning the carcinogenic action, the mechanism of action and the chemotherapeutic effects of urethan.

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