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PROGRESS REPORT

TO

THE UNITED STATES ATOMIC ENERGY COMMISSION

CONTRACT AT(11-1)-3521

BIOLOGICAL EFFECTS OF RADIATION AND RELATED BIOCHEMICAL

AND PHYSICAL STUDIES

Proposal No. 5:	Chemical and Biological Studies on Nucleic Acids and Derivatives
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DISCLAIMER

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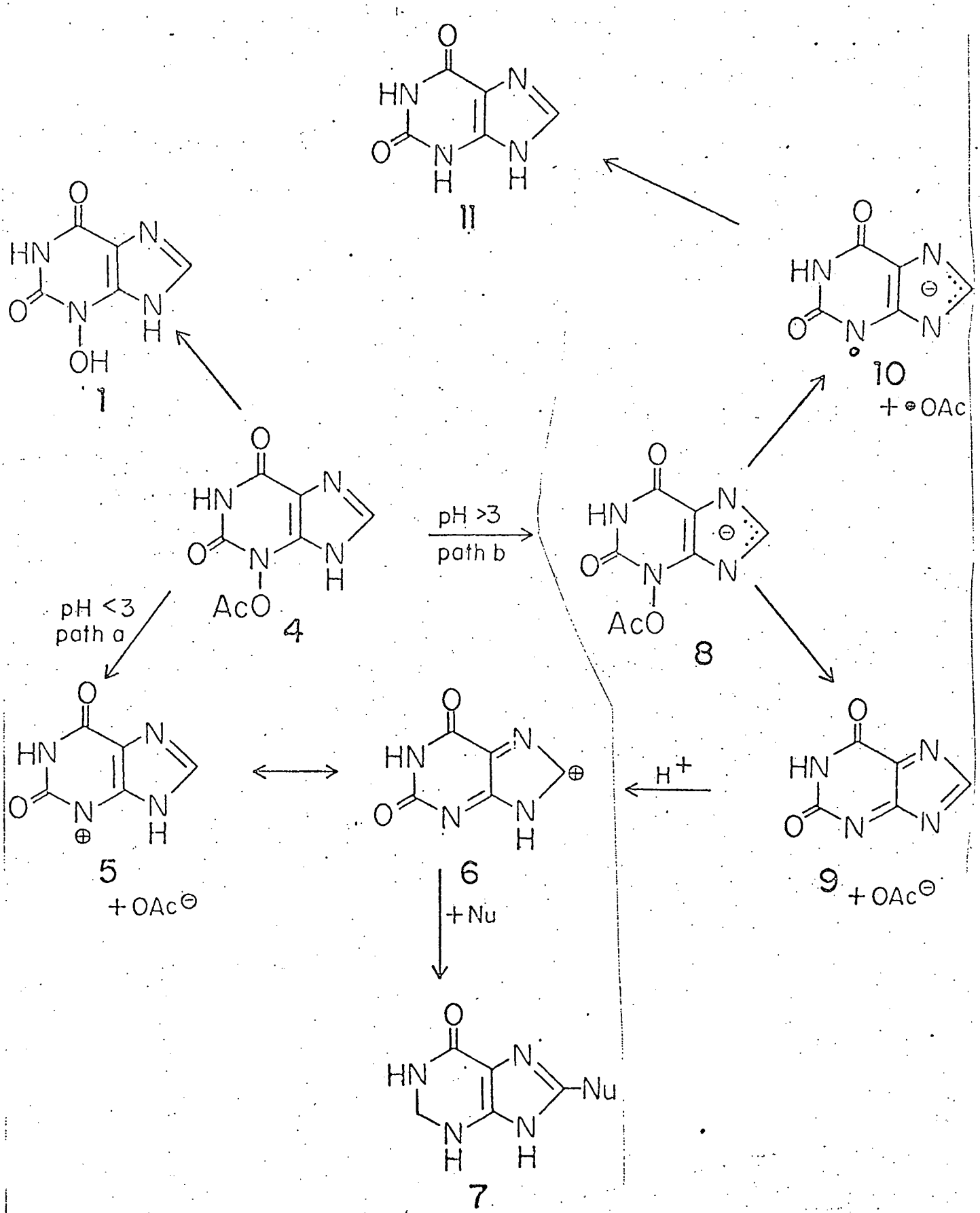
ONCOGENIC PURINE N-OXIDE DERIVATIVES

Correlations between Chemical Properties and Oncogenicity - A recent manuscript[18] discusses the correlations between the background of known chemical properties and the results of recent assays of a series of purine N-oxide derivatives. In brief, the chemical reactivities of various methyl derivatives substantiate "path b" of our proposal for the mechanism of the "3-Acyloxypurine 8-substitution reation," and the assay results correlate with the operation of that pathway in vivo during the process of induction of cancer by these compounds. Thus the ionization of the imidazole proton to yield the ester anion 8 (Scheme 1), is an essential intermediate step in the "fast" path b reaction, and prevention of formation of such an anion [as in 3-hydroxy-7-(or 9)methylxanthine (or guanine)] abolishes oncogenicity. The H on N-1 does not participate in this reaction, and replacement of it by a methyl [as in 3-hydroxy-1-methylxanthine (or guanine)] does not decrease the oncogenicity.

The anion ester 8, (the acetoxy derivative in vitro,[5,6,7] or the sulfate in vivo[8,18] leads to either the 8-substitution reaction, via 6 to 7 (Scheme 1), or to the reduction to xanthine, 11, via the radical ion intermediate, 10 (Scheme 1). In vitro the reaction can be diverted to ~100% reduction by radical scavengers (I^-, Vit. C, hydroquinone, HSO_3^-, etc.), or it can be diverted to ~80% 8-substitution with highly effective nucleophiles. In water alone at pH ~7 about 1/3 proceeds via the radical pathway to xanthine, about 1/3 to uric acid by 8-substitution (by water), and about 1/3 goes to the yet uncharacterized "blue compound"[6,7] (and previous reports). In related work it has been found that uric acid "disappearance" by air (or radical?) oxidation accounts for the decreased uric acid recovery above pH 5, as plotted in Figure 4 of Birdsall et al.,[7] or see Figure 3, page 5 of the 1970 Report. No deductions regarding operation of the radical pathway in vivo can be drawn from the fact that xanthine is produced in vivo from 3-hydroxyxanthine[21] (and guanine from 3-hydroxyguanine[22]), since those can also arise by the reductive action of xanthine oxidase.[23]

This further evidence for the parallel formation of two highly reactive intermediates, the carbonium ion, 6, and the radical, 10, reinforce our interest in investigating which one may be

SCHEME 1



more important in the initiation of the cancer process.

The specific investigations of the character of radicals photochemically induced in purine N-oxide derivatives, the mechanisms of the photochemical reduction of such derivatives, and the influence of structure on the course of the photochemically induced reactions, are described below. In parallel we are studying structural variations which influence the direction of the reaction in solution, i.e., whether via the 8-substitution or the radical pathways.

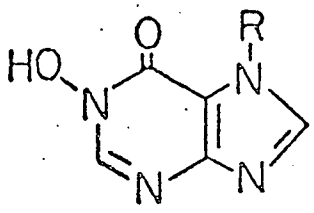
For instance, in 3-hydroxy-8-azaxanthine and 3-hydroxy-8-methylxanthine, the usual 8-substitution reaction is blocked. The oncogenicity of 3-hydroxy-8-azaxanthine is about 3 orders of magnitude less than that of 3-hydroxyxanthine, but it is real since 9 tumors were produced in 20 rats at a dose of 1 mg 3 x/wk for 22 weeks. During the methanolysis of 3-acetoxy-8-azaxanthine, there is a small amount of reduction, presumably via a radical intermediate.[46]

The 3-hydroxy-8-methylxanthine undergoes some reactions similar to those of 3-hydroxyxanthine. Substitution by water occurs on the 8-methyl group to yield 8-hydroxymethylxanthine.[14] No substitution occurs with nucleophiles other than water. This compound is not highly oncogenic,[18] but a "severe" assay such as that with 3-hydroxy-8-azaxanthine is not yet complete.

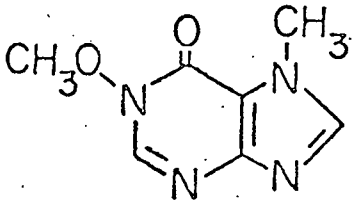
Further studies of these compounds, as well as of the 8-phenyl analog, are in process. A possible photochemical synthesis of the 8-trifluoromethyl analog is mentioned below. These integrated chemical and biological studies are directed toward obtaining a compound with a preferential chemical reactivity via either the radical or the ionic substitution reaction.

I. UV Irradiation of N-Hydroxypurines in Solution

A. The photochemical studies of 1-hydroxyhypoxanthine (1) and selected methyl derivatives of it have been completed and are in press.[15] The study of 1-hydroxy-7-methylhypoxanthine (2) with triplet sensitizers in our last progress report was expanded to include m-methoxyacetophenone, a high energy triplet sensitizer that does not induce reduction by ketyl radicals, and was extended to 1-methoxy-7-methylhypoxanthine (3), a derivative of 1 constrained in the 6-oxo-1-OR tautomer. This compound



1, R=H
2, R=CH_3



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provided UV spectral data which confirmed that 1 and 2 exist as the N-hydroxy tautomer in the neutral species. It was also hoped that it would provide a model compound for the photochemical reactivity to be expected of the N-hydroxy tautomer in the absence of a tautomerizable hydrogen. From the photochemical studies of 1 and 2 at selected pH's, it was concluded that deoxygenation is the primary reaction of the neutral N-hydroxy species, while rearrangement occurs from an excited state of the enolate anions of these 1-hydroxyhypoxanthines. The sensitizer studies of 2 (Table I) demonstrated that in dioxane photoreduction of the neutral species of 2 occurs through the triplet state, while in acetonitrile photoreduction of it takes place by a combination of triplet energy transfer and chemical sensitization. 1-Methoxy-7-methylhypoxanthine, 3, did not prove to be an ideal model for the N-hydroxy species, since the sensitizer studies indicated that photoreduction of it occurs from the excited singlet, although photoreduction can also be accomplished by chemical sensitization with aromatic ketones. It was also found that the enolate anions of 1 and 2 can be photoreduced with aqueous acetone as sensitizer. In addition, another photoinduced rearrangement product, 6,8-dihydroxypurine, was isolated and identified from the photolysis of 1. It is formed under conditions that favor the N-hydroxy tautomer and is not a photoproduct of the enolate anion. The pK_a of the excited singlets (pK_a*) of 1 and 2 were calculated to be 2.7 and 1.2, respectively, and are thus 3 to 4 pH units below the ground state pK_a's. Hence, strongly acidic solutions (pH 0 or below) are required to inhibit completely ionization of the excited N-hydroxy species.

TABLE I

Sensitization Studies[a]

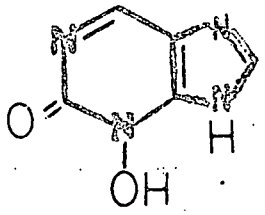
Expt. No.	Solvent / Sensitizer[b]	1-Hydroxy-7-methyl-hypoxanthine				1-Methoxy-7-methyl-hypoxanthine			
		Time[c]	9	2	Recovery	Time[c]	9	6	Recovery
33	Acetonitrile	30	5%	92%	97%	30	4%	95%	99%
34	+ Acetophenone	30	65	0	65	30	75	0	>75[d]
35	+ m-CH_3O-Acetophenone	30	53	47	100	30	6	92	98
36	+ Benzophenone	40	87	4	91	30	33	67	100
37	+ Benzophenone					120	63	12	75
38	+ Fluorene	30	8	92	100	30	9	91	100
39	Dioxane	30	6	92	98	30	1	99	100
		240	26	35	61				
40	+ Acetophenone	30	48	30	78	30	68	0	>68[d]
41	+ m-CH_3O-Acetophenone	30	49	32	81	30	17	72	89
42	+ Benzophenone	30	9	88	97	30	4	96	100
43	+ Fluorene	30	3	80	83	30	4	96	100
44	Acetone (neat)	30	78	0	78	30	65	0	65

a/ Pyrex filter, b/ 0.2 M in sensitizer, c/ Minutes,

d/ One additional unknown noted, λ_{max} pH 0 and 11: 247 nm.

In 3 N CF_3CO_2H (pH ~ 0) the yield of 6,8-dihydroxypurine was maximized (to 3-4% for 1, and ~2% for the respective 7-methyl derivative from 2). Under these conditions a new photoproduct, 8-trifluoromethylhypoxanthine was formed in ~2% yield. We believe these reactions at the 8-position may be radical mediated and plan further studies (see "Proposal"). Preliminary studies on the irradiation of 1-hydroxyxanthine in 3 N CF_3CO_2H show that there is much reduction and that some 8-trifluoromethylxanthine may be formed. Since this compound has not been previously reported, a synthesis of it is underway to confirm the identification of the photoproduct.

B. Another example of 8-substitution was provided by the irradiation of 2-oxo-3-hydroxypurine[24] (4), which gave the reduction products of 2-hydroxypurine (21%), and 2,8-dihydroxypurine (1%) when irradiated (300 nm) at pH 3. Irradiation of the anion of 4 (pH 9) also gave 2-hydroxypurine (7%) and a trace of 2,8-dihydroxypurine, but the major product, as yet unidentified, seems to be an imidazole formed by ring opening of an intermediate. We hope to identify this product by mass spectral analysis.



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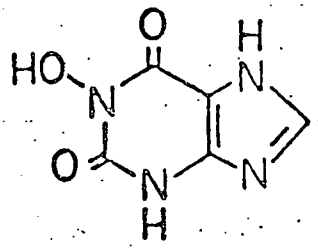

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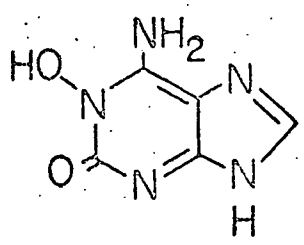
C. The irradiation of 1-hydroxyxanthine[25] (5) causes extensive photoreduction, but concomitantly, there is a small amount of photoisomerization to 3-hydroxyxanthine. The latter is also subject to photoreduction under the irradiation conditions. The rearrangement occurs from both the neutral species of 1-hydroxyxanthine (pH 3.0) and the anion (pH 9.3).

1-Hydroxyisoguanine[26] (6) is structurally related to 5 and it was hoped that it might provide another example of the

unusual 1- to 3-hydroxyl rearrangement. Irradiation of the monoanion of 6 (pH 8.5), however, showed no evidence of a product with the properties that might be anticipated for 3-hydroxyisoguanine, but instead yielded two imidazoles, 7 and 8, as the major photoproducts and isoguanine (8%).



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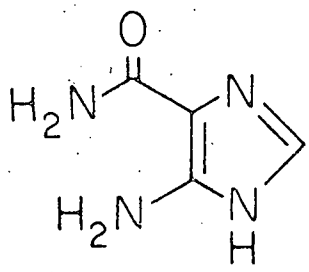

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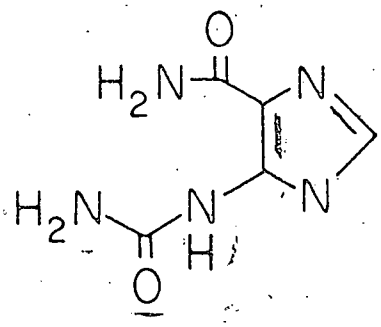

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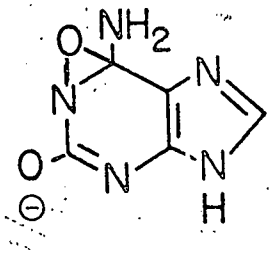
The formation of the imidazoles 7 and 8 suggests that an oxazirane, 9, formed between N-1 and C-6, undergoes ring expansion to imidazolo-oxadiazepine, 10, and that this undergoes subsequent ring opening to 7 and 8.



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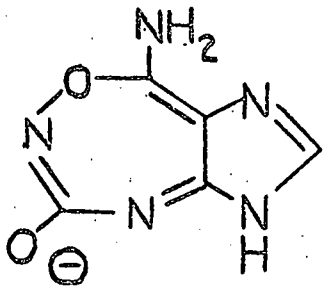

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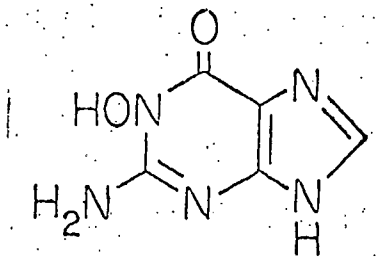

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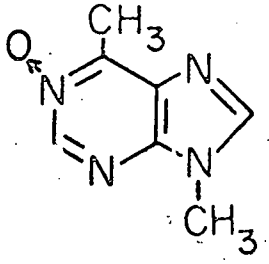
Thus in 6 oxygen migration occurs in a direction unfavorable for 1- to 3-hydroxyl migration. A synthesis of 8 is currently in progress to confirm the structure (presently based on NMR and mass spectral data) and to determine its ϵ_{max} for a quantitative evaluation of the photoproducts.

A second compound that offered the possibility of 1- to 3-migration was 1-hydroxyguanine, 11.[16] However, no 3-hydroxyguanine could be detected from the irradiation of 1-hydroxyguanine at pH's 2, 5.5, or 10.5. Instead, photoreduction was the predominant reaction at all pH's.

II. UV Irradiation of Purine N-Oxides in Solution

6,9-Dimethylpurine-1-oxide, 12, which is constrained in a single tautomeric form, proved much less susceptible to changes in the yield of photoproduct with changes in the solvent (Table II) than did 6-methylpurine-1-oxide. There was less photoreduction of 12 in organic solvents than in aqueous solution, but the yields for the rearrangement product, 6,9-dimethyl-2-hydroxypurine, 14, were nearly the same in all solvents. The major photoproduct under most conditions is as yet unidentified. It can be converted to 14 by treatment with base and may be the intermediate 13, or a 4-acetyl-5-ureidoimidazole, comparable to 8. A preparation of sufficient quantities of 12 to permit a structure determination of 13 is in progress. A sample of 14, a previously



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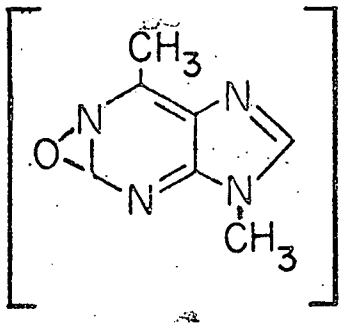

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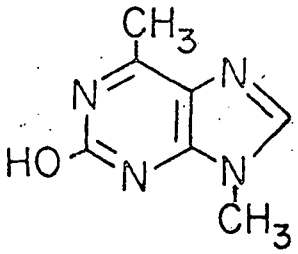

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unreported compound, has been synthesized for structure proof of the photoproduct and for ϵ_{max} values. An improved synthesis of 6,9-dimethylpurine resulted from these studies and this will facilitate preparation of the quantities of 12 required to isolate 13. When ϵ_{max} values of 13 and 14 are available, a quantitative evaluation of the photolysis of 12 in a series of solvents, both with and without sensitizers, can be undertaken.

TABLE II

Solvent[a]	Time[b]	6,9-Dimethylpurine-1-oxide	6,9-Dimethylpurine	6,9-Dimethyl-2-hydroxypurine	4-Acetyl-5-amino-1-methylimidazole
H_2O, pH 3	60	0	9%	15	6%
H_2O, pH 9	60	0	15	11	0
CH_3OH	15	13	1	8	0
Ethanol	15	13	1	9	0
CH_3CN	15	14	1	9	1
$CHCl_3$	30	0	5	7	0

[a] High pressure Hg lamp with N_2 flushing, pyrex filter.

[b] Minutes.

III. UV Irradiation of 3-Hydroxyxanthines in the Solid State.-

This study (discussed in previous Progress Reports) has been completed and submitted for publication.[207] Additional studies on the 3-nitroxyl derivatives of 3-hydroxyxanthine and its 8-methylderivative were included in this paper. The nitroxyls were generated in solution by ceric sulfate oxidation. The chemically generated nitroxyls and the photo-induced solid-state radicals showed differences in ESR spectra, in the extent of interaction of the impaired electron with the 8-methyl groups, and in their production of xanthine upon reaction with water. These data provided additional evidence in support of the characterization of the photo-induced radical as an amidogen radical. It was also shown that the photo-induced radical from solid 3-acetoxyxanthine reacts to give xanthine under conditions (1 N HCl solution) where no xanthine is normally formed from 3-acetoxyxanthine. 3-Actoxyxanthine was also irradiated in dioxane solution and was found to yield xanthine as the major product, and also an unidentified product.

IV. Actions of Ionizing Radiation on Purine Derivatives

With the use of the ion exchange columns mentioned in the 1970 Report, we have reinvestigated the ^{60}Co-γ irradiation of adenine. All of the products reported by others have been separated on the A-6 resin column with pH 4.7 buffer.[27] We have repeated the experiments of Van Hemmen[28] with his Sephadex G-10 column and pH 7 0.05 M phosphate. The compound which he claims is 7-hydroxy-7,8-dihydroadenine is present in freshly irradiated samples. It reverts to adenine at pH 7.0, with a t 1/2 of less than a day. In acid it reverts to adenine with a t 1/2 of minutes. It is not seen in the pH 4.7 or 0.05 N or 1 N Dowex 50 columns. Its possible identity with the 7-hydroxyadenine claimed by Rhaese was investigated. Repettion of Rhaese's experiments yielded mixtures in all cases. His "7-hydroxyadenine" fraction proved to be a complex mixture, possibly impure adenine 1-oxide. No compound comparable to Van Hemmen's was observed at pH 7.0 on Sephedex.

As previously mentioned we have repeated A. Wacker's observation, that 5'-AMP-1-oxide is obtained from 5'-AMP when it is irradiated in the presence of organic material which can form peroxides (~.1 N acetic acid). The trace of 2-hydroxyadenine produced from adenine (Conlay[30]) is confirmed. It could arise via a small amount of adenine 1-oxide, since adenine 1-oxide is more sensitive than is adenine to ionizing radiation.

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We have continued and expanded our studies of embryonal antigens and components which appear in the tumors of adult mice (1-5). Such antigens have been detected in each of 99 different tumors in at least 12 strains of mice and, in consequence, we regard the occurrence of embryo-specific tumor-associated antigens as an important characteristic of cancer. We have been using neutral aqueous extracts of whole mouse embryos with which to produce diagnostic antisera in the rabbit. After exhaustive absorption with extracts of normal adult internal organs, the resulting sera reacted in Ouchterlony double diffusion assays with aqueous extracts of all murine tumors tested. The carcino-embryonic antigen (CEA), widely studied by others, contrasts with our material in that CEA is derived from a perchloric acid extract of tumor tissue which produces antisera which react with embryonal tissue.

Micro-Ouchterlony assays failed to reveal embryonal antigens in normal adult tissues. However, cross-reacting substances were found in skin and blood. We have distinguished the antigens in embryos and tumors, referred to as ET-proteins, from the cross-reacting components in skin, referred to as S-proteins. The ET-proteins, with molecular weights of approximately 65,000 are completely separated from the S-proteins by chromatography on Sephadex G-200; the latter group has molecular weights in the range from 10,000 to 20,000.

Although approximately one-fifth the molecular weights of the ET-proteins, the S-proteins nevertheless completely absorb tumor-associated anti-ET activity from the rabbit antisera, as measured by the double diffusion assay. We do not yet know if the S antigens in the skin are precursors or subunits of the ET antigens. All skin extracts tested from 12 different strains of mice were positive. We refer to the entire group of fetal components under investigation as embryo-specific tumor-associated antigens.

Embryo-derived embryo-specific tumor-associated antigens of the ET group yield seven antigenically active peaks upon fractionation on DEAE-Sephadex. Each of these, in turn, produces multiple bands on polyacrylamide gel electrophoresis, and so must still be considered heterogeneous. Three of the seven DEAE peaks contain normal adult serum components, one appears to be identical to serum albumin, and one peak contains murine α-fetoglobulin, identified serologically.

The three remaining peaks appear to contain unique embryo-specific tumor-associated proteins. Gel fractionations of two of these have yielded sufficient material for amino acid analyses. The analyses indicate proteins containing the usual twenty amino acids, and little or no sugar components.

By means of chromatography on Sephadex G-100 and re-fractionation of the antigenically active fractions on Sephadex G-200, tumor extracts yield immunologically active ET-proteins only and adult skin extracts yield only S-proteins. No S-proteins were detected in extracts of skinned embryos. ET-proteins of tumors and embryos were indistinguishable by Ouchterlony double diffusion and immuno-electrophoresis.

The embryo-specific tumor-associated proteins of the ET group are soluble in water and insoluble in 9% trichloroacetic acid. The immunological reactivity is stable at 80°C for 10 minutes. The distribution of embryo-specific tumor-associated antigens in embryos has been examined by indirect immunofluorescence with absorbed rabbit antisera and fluorescein-conjugated goat anti-rabbit sera. The antigens appear to be present in all visceral tissues examined between the 16th day of gestation and up to one week after birth, with the notable exception of the spleen. Highest levels appear to be in kidney, heart, lung, and liver, and in all cases antigen is undetectable in the nuclei of the cells. Though fairly evenly distributed throughout the cytoplasmic regions in most tissues, some preparations showed higher concentrations of embryo-specific tumor-associated antigens near or at the cell membranes.

We have developed a radial immunodiffusion assay for the quantitative estimation of embryo-specific tumor-associated antigens, expressed as units of antigen per mg. of protein in our preparations. Amounts as low as 0.01 µg are detectable, an increase in sensitivity of approximately 100-fold over the double diffusion method previously employed. This assay has revealed low levels of embryo-specific tumor-associated antigens in normal adult tissues that originally appeared negative in the Ouchterlony assays. Levels of antigen (units per mg. protein) in fetal and cancer tissues range between 5 and 100 times the concentrations detected in various normal adult mouse tissues.

Quantitative analysis of whole embryo extracts has indicated that fetal antigen is high (>100 units/mg.) up to the 17th day of gestation after which time there is a sharp drop to about one-fifth of the maximum by the 2nd day after birth. When examined separately,

the concentration of the embryo-specific tumor-associated antigens extracted from the skin and remaining carcass showed an interesting reciprocal relationship and periodicity. Antigen was high (50 to 70 units/mg. protein) in skin at 2, 6, and 10 to 12 weeks after birth, and undetectable, at these times, elsewhere in the skinned animals. However, between these peak periods, the concentration in skin dropped by about half, while significant values (ca. 30 units/mg.) appeared in the carcasses.

To determine possible protective effects with fetal or skin cell components, adult C57 black mice were immunized with x-irradiated isogenic adult skin or embryo cells and then challenged by implantation with QUA tumor cells (a highly malignant methylcholanthrene-induced C57 black tumor line, obtained from Dr. J.L. Biedler, which routinely gives 100% tumor takes). Half of the mice immunized with fetal cells failed to develop tumors; those tumors in the remainder were small and appeared much later than in controls. Thirty percent of the mice immunized with adult skin cells had no tumors; development of tumors in the remainder was delayed. Immunoprotection was not afforded by immunization with irradiated isogenic adult visceral cells (6). We have begun studies to isolate and identify the protection-conferring component(s) present in embryo and adult skin cells and obviously absent from adult visceral cells in significant quantities. We also plan to see whether any of the embryo-specific tumor-associated fractions are immunoprotective.

The characteristic appearance of embryonal antigens in many tumors prompted study of the interaction of sperm with non-malignant diploid cells to learn, for example, whether synthesis of such antigens could be turned on thereby (as occurs after fertilization) and whether the cells would then become malignant. For orienting experiments, living sperm, collected aseptically from mouse epididymus and vas deferens, were added to cultures of various non-tumor and tumor cell lines maintained in our laboratory. Apparent penetration occurred with cells of all the lines as judged by light microscopy. When sperm were mixed in equal numbers with suspended Chinese hamster cells, penetration of many of the cells could be seen within the first few hours by light microscopy, and by scanning and transmission electron microscopy. When sperm were used which had been labeled in vivo with ^{3}H-thymidine, autoradiography revealed DNA uptake in nuclei of Chinese hamster cells after three days of co-culture suggesting release of DNA after penetration (7). Descendent cells are under study to see whether functional information transfer had resulted, if murine gene products, especially embryonal antigens,

can be detected, and if malignant potential had been acquired. Preliminary results suggest the appearance of murine-reactive antigen in descendent cells. This was revealed by immuno-fluoroscopy using anti-mouse embryo antisera.

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