Relation of Structure to Activity of Purine 3′-Deoxynucleosides in KB Cell and Chick Embryo Fibroblast Cell Cultures[1]

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A number of 3′-deoxyribonucleosides were compared with 3′-deoxyadenosine for cytotoxic activity and effects on uridine-^{3}H incorporation in KB cells and chick fibroblasts. In KB cells 6-methylaminopurine 3′-deoxyriboside exhibited delayed cytotoxicity greater than 3′-deoxyadenosine but was about as potent an inhibitor of uridine incorporation as was 3′-deoxyadenosine. In chick cells, it was neither as cytotoxic as 3′-deoxyadenosine, nor was it as potent an inhibitor of uridine incorporation. The N-ethyl- and N-dimethylaminopurine derivatives were significantly less active than 3′-deoxyadenosine. Very striking differences in the response of KB cells and chick cells to the 3′-deoxyribosides of guanine and 2,6-diaminopurine were observed. In chick cells, these two compounds were nearly as cytotoxic and as effective as inhibitors of uridine incorporation as was 3′-deoxyadenosine. In KB cells, however, the compounds were only slightly cytotoxic and actually stimulated uridine incorporation.

3′-Deoxyadenosine has been isolated from the fermented broth of *Aspergillus nidulans*, using cytotoxic activity against KB cells to help guide the fractionation of the broth.[2] Cordycepin, a metabolic product previously isolated from *Cordyceps militaris*,[3] has been shown to be identical[4] with 3′-deoxyadenosine. Other 3′-deoxyadenosine nucleosides were synthesized in our laboratories[5] and were tested for cytotoxic activities against KB cells and chick embryo fibroblast cells. Because 3′-deoxyadenosine was known to be an inhibitor of RNA synthesis,[6] each compound was also compared with 3′-deoxyadenosine for its effect on the incorporation of uridine-^{3}H into the acid-insoluble fraction of KB cells and chick embryo fibroblast cells. The results of these studies are reported here.

Materials and Methods

Eagle's medium,[7] with Earle's balanced salts solution, supplemented with calf serum was used throughout. Stock medium contained 10% calf serum; test medium contained 5% calf serum. All media contained, per milliliter, 100 units of penicillin and 50 γ of streptomycin.

Primary cultures of chick embryo fibroblast cells were prepared from 11-day-old embryos by a method similar to that described by Temin and Rubin.[8] The suspension of cells obtained from treatment of the embryos with trypsin was diluted in stock medium to a concentration of 7.5×10^5 cells/ml. The diluted suspension (20 ml.) was seeded into each of several milk dilution bottles. These were gassed with a CO_2–air mixture (5:95), closed with rubber stoppers, and incubated at 37°. The medium was renewed on the third and fifth day. On the sixth day, the cells were harvested with the aid of trypsin, centrifuged, and suspended in test medium for the preparation of secondary cultures of chick embryo fibroblast cells.

For the determination of the cytotoxic activities of the compounds, 3×10^5 cells in 0.9 ml. of test medium were added to each of a series of 16×125 mm. test tubes to which the compounds under test in volumes of 0.1 ml. of water had been added. The tubes were gassed with the CO_2–air mixture, closed with rubber stoppers, and incubated at a 5° angle at 37°. The cell sheets which formed along the surface of the tubes were examined for evidence of cytotoxicity after 2 and 5 days' incubation. Cytotoxic end points in chick cells were determined as described previously for KB cells.[9]

Cytotoxic activities of compounds against KB cells were determined as described previously.[9] Stock medium was used for the propagation of KB cells in continuous passage; test medium was used for growing cells when the cytotoxic activities of compounds were being determined.

In some experiments, the ID_{50} (the concentration resulting in 50% inhibition of growth) for test compounds was determined from a comparison of the protein content of treated and control cultures. Cellular protein was determined by the phenol method.[10]

For studies on the effects of the compounds on uridine-^{3}H incorporation into cellular RNA, secondary cultures of chick embryo fibroblast cells were prepared as for cytotoxicity tests, except that each tube received 1 ml. of medium containing 2.75×10^5 cells; the cultures were incubated for 3 days, the medium was changed, and test compounds in 0.10 ml. of water were added. After 2 hr. incubation at 37°, uridine-^{3}H[11] was added using a 0.5-ml. syringe fitted with a Hamilton repeating dispenser which delivered 0.01 ml./tube. The cultures were incubated for 4 hr. at 37°. The tubes were chilled, the medium was removed, and the tubes were stored at −20° until the cells were processed.

Studies on uridine-^{3}H incorporation into RNA of KB cells were performed as for chick embryo fibroblast cells except that the tubes were incubated in an upright position and each tube was inoculated with 3×10^4 cells.

Uridine-^{3}H incorporation into the total acid-insoluble fraction of the cells was determined as follows. The tubes containing the cells were thawed and 1 mg. of bovine serum albumin was added as carrier at 4°. Acid-insoluble material was precipitated with 5 ml. of cold 1% $HClO_4$ and the precipitate was washed twice by suspending in 5 ml. of cold 1% $HClO_4$ and centrifuging. The residue was then washed once with 5 ml. of ethanol–ether (1:1) and dissolved in 1.0 ml. of 1 *M* methanolic solution of hydroxide of Hyamine® 10-X (Packard Instrument Co.).

These solutions were transferred to vials and counted in a Packard Tri-Carb liquid scintillation spectrometer using 20 ml. of

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(2) E. A. Kaczka, E. L. Dulaney, C. O. Gitterman, H. B. Woodruff, and K. Folkers, *Biochem. Biophys. Res. Commun.*, **14**, 452 (1964).

(3) H. R. Bentley, K. G. Cunningham, and F. S. Spring, *J. Chem. Soc.*, 2301 (1951).

(4) E. A. Kaczka, N. R. Trenner, B. Arison, R. W. Walker, and K. Folkers, *Biochem. Biophys. Res. Commun.*, **14**, 456 (1964).

(5) (a) E. Walton, F. W. Holly, G. E. Boxer, R. F. Nutt, and S. R. Jenkins, *J. Med. Chem.*, **8**, 659 (1965); (b) S. R. Jenkins, F. W. Holly, and E. Walton, *J. Org. Chem.*, **30**, 2851 (1965).

(6) (a) H. Klenow and S. Frederickson, Abstracts, the VIth International Congress of Biochemistry, New York, N. Y., Aug. 1964, Vol. 8, p. 66; (b) H. T. Shigeura and C. N. Gordon, *J. Biol. Chem.*, **240**, 806 (1965).

(7) H. Eagle, *J. Exptl. Med.*, **102**, 596 (1955).

(8) H. M. Temin and H. Rubin, *Virology*, **6**, 669 (1958).

(9) C. O. Gitterman, E. L. Dulaney, E. A. Kaczka, G. W. Campbell, D. Hendlin, and H. B. Woodruff, *Cancer Res.*, **24**, 440 (1964).

(10) (a) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951); (b) V. I. Oyama and H. Eagle, *Proc. Soc. Exptl. Biol. Med.*, **91**, 305 (1956).

(11) Uridine labeled with tritium (New England Nuclear Corp.) was diluted with cold uridine (Pabst Labs) so that 0.01 ml. contained 46 mμmoles of uridine with a specific activity of 0.98×10^4 c.p.m./mμmole.

0.3% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene in toluene as the phosphor solution.

Results

Table I shows the cytotoxic activities found for each of the purine 3′-deoxyribosides in KB cells and in chick embryo fibroblast cells. The cytotoxic end point for

TABLE I

CYTOTOXIC ACTIVITIES OF PURINE 3-DEOXYNUCLEOSIDES AGAINST KB CELLS AND CHICK EMBRYO FIBROBLAST CELLS

9-(3-Deoxy-β-D-ribofuranosyl)-	Cytotoxic activity, CE, γ/ml.[a] KB cells 2 days	KB cells 5 days	Chick embryo fibroblast cells 2 days	Chick embryo fibroblast cells 5 days
Adenine (1)	10–25	10–25	Sl. <10	10–25
2,6-Diaminopurine (2)	Sl. >1000	300	10	<10
Guanine (3)	>300	100	...	...
6-Methylaminopurine (4)	10–25	6	50	50
6-Dimethylaminopurine (5)	100	30	100–300	100–300
6-Ethylaminopurine (6)	Sl. >100	30	...	...
Purine-6-thiol (7)	300	100	1000	100–1000
Purine (8)	>500	500	>500	>500

[a] Cytotoxic end point determined from visual observations.

3′-deoxyadenosine (**1**) in both cell systems was about 10–25 γ/ml. The inhibitory effect of **1** at 2 days was equal to or slightly greater than the effect at 5 days.

The diamine **2** was as cytotoxic as 3′-deoxyadenosine to chick embryo fibroblast cells. The cytotoxic end point (CE) at 2 days was about 10 γ/ml. Unexpectedly its cytotoxic activity against KB cells was much lower (CE about 1000 γ/ml. at 2 days, 300 γ/ml. at 5 days).

In KB cells, 3′-deoxyguanosine (**3**) was much less cytotoxic (CE 300 γ/ml. at 2 days, 100 γ/ml. at 5 days) than 3′-deoxyadenosine. The cytotoxic end point in chick embryo cells was not determined.

The 6-methylamino compound (**4**) in KB cells appeared to be clearly more cytotoxic (CE 6 γ/ml.) than 3′-deoxyadenosine at 5 days. However, at 2 days, the cytotoxic effect of **4** was found to be less than at 5 days. Unlike the diamine, **4** was more cytotoxic to KB cells than to chick embryo cells. The 6-dimethylamino compound (**5**) also was more cytotoxic to KB cells than to chick embryo fibroblast cells, but the order of activity was much lower than that for the monomethyl derivative (**4**). The 6-ethylamino compound (**6**) inhibited KB cells to about the same extent, but the cytotoxic end point was not determined in chick embryo fibroblast cells. Substitution at position 6 of purine with a mercapto group (**7**) or with hydrogen (**8**) resulted in a marked reduction in cytotoxic activity in both cell systems.

ID_{50} values obtained from measurements of the protein content of KB cells exposed, for 1, 2, and 5 days, to compounds **1**, **2**, and **4** are shown in Table II. These agree with and extend the cytotoxic end points derived from visual observation. 3′-Deoxyadenosine exerted a maximum cytotoxic effect on KB cells within 1 day while the other two compounds exerted maximum cytotoxic effects only at 5 days. The ID_{50} values for 3′-

TABLE II

CYTOTOXIC ACTIVITIES OF PURINE 3′-DEOXYNUCLEOSIDES AGAINST KB CELLS

9-(3-Deoxy-β-D-ribofuranosyl)-	Cytotoxic activity, ID_{50}, γ/ml.[a] Day 1	Day 2	Day 5
Adenine	20	23	17
2,6-Diaminopurine	...[b]	250–1000[c]	130
6-Methylaminopurine	50	15	2

[a] Dose which inhibited protein synthesis 50%. [b] 500 and 1000 γ/ml inhibited cell growth 30%. [c] Cytotoxic effect remained constant with concentrations of drug from 250–1000 γ/ml.

deoxyadenosine, 6-methylaminopurine 3′-deoxyriboside, and 2,6-diaminopurine 3′-deoxyriboside were 17, 2, and 130 γ/ml., respectively.

Dose–response effects of the substituted purine 3′-deoxyribosides on incorporation of uridine-^{3}H into the acid-insoluble fraction of KB cells and chick embryo fibroblast cells are shown in Figures 1 and 2 and in Table III. These are pooled results from two separate

TABLE III

EFFECT OF THE 3′-DEOXYRIBOSIDES OF 2,6-DIAMINOPURINE, GUANINE, AND PURINE ON INCORPORATION OF URIDINE-^{3}H INTO THE RNA OF KB CELLS AND CHICK EMBRYO FIBROBLAST CELLS

9-(3-Deoxy-β-D-ribofuranosyl)-	Concn., γ/ml.	Uridine-^{3}H incorporation, % of control KB	Chick embryo fibroblast
2,6-Diaminopurine	300	153	24
	100	148	33
	30	118	40
	10	109	46
	3	100	48
Guanine	750	140	30
	250	142	34
	75	156	36
	25	145	43
	300	142	16
	100	168	31
	30	146	31
	10	127	36
	3	125	48
Purine	600	79	101
	200	85	122
	60	98	118
	20	108	100

experiments. The data in Figure 1A and B indicate that chick fibroblasts were inhibited more effectively than KB cells by 3′-deoxyadenosine, the approximate ID_{50} values being 3 and 15 γ/ml., respectively. 6-Methylaminopurine 3′-deoxyriboside was about as effective as 3′-deoxyadenosine in inhibiting the incorporation of uridine into KB cells. In chick fibroblasts, the 6-methylamino compound was significantly less active than 3′-deoxyadenosine, the approximate ID_{50} values being 30 and 3 γ/ml., respectively. The 6-methylamino compound was, however, equally effective in KB and chick fibroblast cells, the respective ID_{50} values being about 25 and 30 γ/ml. Two points for inhibition of uridine incorporation into chick fibroblasts by the 6-methylamino compound are shown in Figure 1B but were not used for drawing the dose–response line. They are from a separate experiment and are unexplainably low and are included only for the sake of completeness.

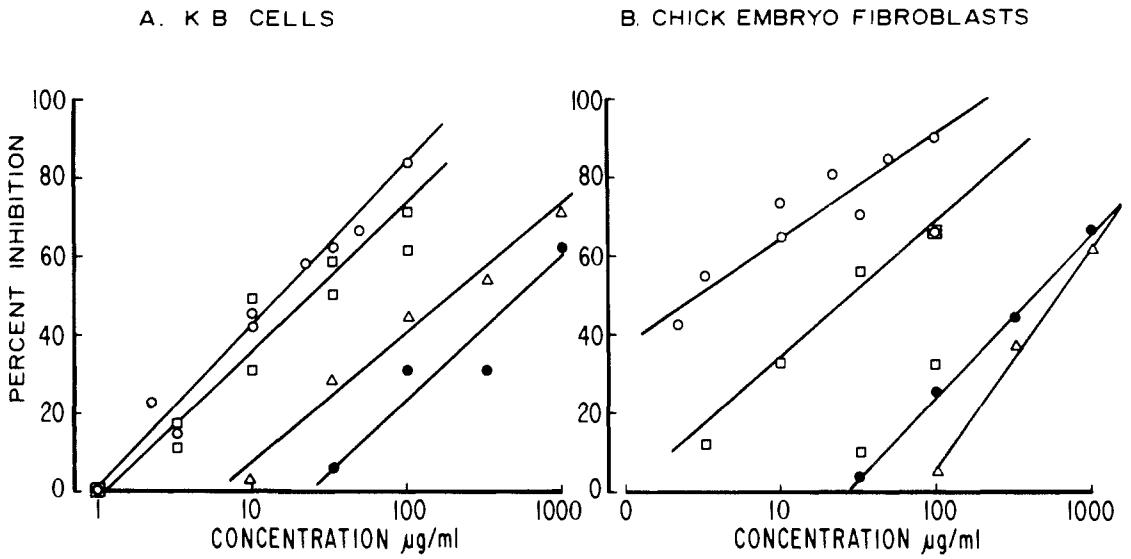

Figure 1.—Effect of purine 3′-deoxynucleosides on uridine-^{3}H incorporation into the acid-insoluble fraction of KB cells and chick embryo fibroblast cells: O—O, adenine; □—□, 6-methylaminopurine; △—△, 6-dimethylaminopurine; ●—●, 6-ethylaminopurine.

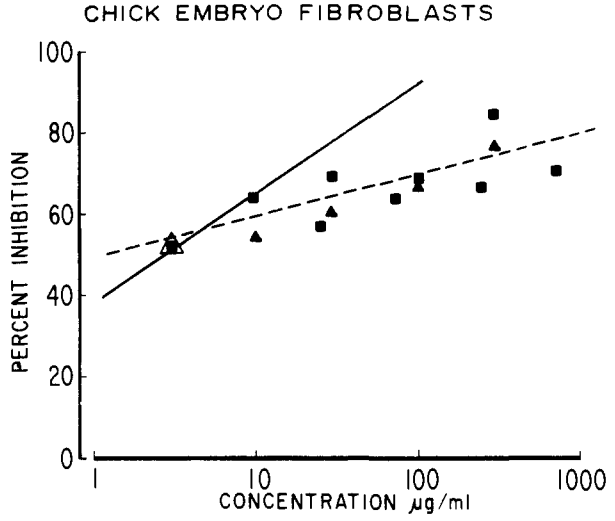

Figure 2.—Effect of purine 3′-deoxynucleosides on uridine-^{3}H incorporation into the acid-insoluble fraction of chick embryo fibroblast cells: ■---■, guanine, ▲---▲, 2,6-diaminopurine; —, adenine (line derived from Fig. 1B).

The 6-dimethylamino and the 6-ethylamino compounds were decidedly less effective in inhibiting uridine-^{3}H incorporation into both KB cells and chick embryo fibroblast cells. The apparent differences in the ID_{50} values for the two cell lines are not considered significant within the precision of the experiment. In general, the slopes of the dose–response lines for the N^6-alkyl-substituted derivatives are about the same as that for 3′-deoxyadenosine in both cell lines.

The 2,6-diaminopurine and the guanine 3′-deoxyribosides inhibited uridine-^{3}H incorporation only in chick embryo fibroblasts. The slopes of the dose–response lines for these two derivatives are indistinguishable but are decidedly flatter than that for 3′-deoxyadenosine which is included for comparison in Figure 2. The slope of the dose–response lines is so flat for these two compounds that the ID_{50} value can be given only by extrapolation but must be lower than that of 3′-deoxyadenosine.

The 2,6-diaminopurine and guanine compounds not only did not inhibit uridine-^{3}H incorporation into KB cells but actually stimulated it (Table III). For comparison, inhibition values for chick embryo fibroblasts plotted in Figure 2 are also given in Table III. In addition, data on the unsubstituted purine 3′-deoxyriboside are also included. The latter compound showed minimal inhibition of uridine incorporation into KB cells and had no significant effect in chick fibroblasts. This compound also had no significant cytotoxic activity in the two cell lines (Table I).

Discussion

Based on their effects on growth of KB cells and of chick embryo fibroblasts and on incorporation of uridine-^{3}H into the acid-insoluble fraction of the cells, the purine 3′-deoxynucleosides studied here can be grouped in distinctive categories. 3′-Deoxyadenosine exerted its maximum cytotoxic effect within 1–2 days, and it was an effective inhibitor of uridine-^{3}H incorporation in both cell systems. The early appearance of its cytotoxic effect was observed also in H.Ep. No. 1 cells.[12]

In Ehrlich ascites cells, 3′-deoxyadenosine is phosphorylated to the 5′-triphosphate (3′-deoxy-ATP).[6] *In vitro* experiments with a preparation of RNA polymerase from *Micrococcus lysodeikticus* have shown that 3′-deoxy-ATP is a potent inhibitor of RNA synthesis, probably by being incorporated in competition with ATP and thereby terminating further growth of the polynucleotide chain.[13] The inhibitory action of 3′-deoxyadenosine in KB cells and chick embryo cells is probably due to this mechanism.

At 5 days, the cytotoxic effect of the 6-methylaminopurine 3′-deoxyriboside was greater than that of 3′-deoxyadenosine in KB cells, but this was a delayed effect since 3′-deoxyadenosine was clearly more effec-

(12) M. A. Rich, P. Meyers, C. Weinbaum, and R. J. Suhadolnik, Abstracts of Papers, 145th National Meeting of the American Chemical Society, New York, N. Y., Sept. 1963, p. 35-C.

(13) H. T. Shigeura and G. E. Boxer, *Biochem. Biophys. Res. Commun.*, **17**, 758, 1964.

tive after 1 day and about equally effective after 2 days (Table II). The mechanism of this delayed toxicity is not yet understood but would indicate that the compound is metabolized differently from 3′-deoxyadenosine. It should be noted, however, that uridine-^{3}H incorporation was inhibited effectively during only 6 hr. of contact of the 6-methylamino compound with either KB cells or chick embryo fibroblasts.

The inhibitory mechanisms for the 6-dimethylamino- and 6-ethylaminopurine 3′-deoxyribosides probably resemble that of the 6-methylamino compound except for degree since they exhibited delayed cytotoxicity in KB cells, and also, the slopes of inhibition of uridine incorporation for all three compounds in each cell system were similar.

The results obtained with 2,6-diaminopurine 3′-deoxyriboside and 3′-deoxyguanosine presented a markedly different picture. The 2,6-diaminopurine derivative showed low toxicity in KB cells but was, if anything, more cytotoxic than 3′-deoxyadenosine in chick fibroblasts. Unfortunately, the supply of 3′-deoxyguanosine was insufficient to obtain similar comparisons in chick embryo fibroblasts. Both compounds effectively inhibited the incorporation of uridine-^{3}H into the acid-insoluble fraction of chick fibroblasts, but a meaningful comparison with the effect of 3′-deoxyadenosine could not be made since the slopes of the dose–response lines were different (Figure 2).

The effect of these two compounds on uridine-^{3}H incorporation was strikingly different in KB cells where actually stimulation rather than inhibition was observed (Table III). Until these two compounds are tested against a wider variety of cells in culture, one cannot speculate whether the observed qualitative differences between KB cells and chick fibroblasts with regard to uridine-^{3}H incorporation and cytotoxic effects reflect species differences, differences between a malignant and a normal cell, or differences between an established cell line and a primary explant of an embryonic cell.

The Synthesis and Properties of 6-Mercaptomethylpurine and Derivatives[1a]

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Reaction of 6-chloromethylpurine with either thioacetic acid or ammonium dithiocarbamate led to 6-acetylthiomethylpurine and 6-dithiocarbamylmethylpurine, respectively, which gave after ammonolysis, 6-mercaptomethylpurine (6-homomercaptopurine). This compound was reduced to 6-methylpurine with Raney nickel or by prolonged refluxing with thioacetic acid. Chlorination of 6-mercaptomethylpurine produced 6-trichloromethylpurine, which in turn, was reduced to 6-dichloromethylpurine with thioacetic acid. Reaction of equivalent amounts of 6-bromomethylpurine with potassium thiocyanate or with thiourea resulted in the synthesis of 6-purinylmethyl thiocyanate and 2-(6-purinylmethyl)pseudothiourea hydrobromide, respectively. The corresponding alkylthiomethylpurine derivatives were prepared from 6-chloromethylpurine and methyl, ethyl, and benzyl mercaptan, benzenethiol, and 6-mercaptopurine. Some physical and chemical properties of the new compounds are reported and results of animal screening tests are included. The tumor-inhibitory activity of 6-acetylthiomethylpurine on mouse Sarcoma 180 (ascites) was marked and its effect on mouse Glioma 26 was moderate; the other derivatives proved toxic in mice.

The inhibitory activity of 6-mercaptopurine and other thiopurines and their nucleosides on neoplastic growth[2] stimulated our interest in the synthesis of new mercaptopurine derivatives as potential chemotherapeutic agents. The outstanding toxic effects shown by 6-methylpurine[3] led also to a search for alterations in its structure that might result in derivatives of lower toxicity which still possessed carcinostatic properties. Previous studies on the fluorination,[4] oxidation,[5] chlorination, and bromination[6] of 6-methylpurine afforded derivatives with insignificant biological activity. In studies of the synthesis of thiated purines carried out recently in this laboratory, novel routes of the thiation of purine and 6-methylpurine[7] were developed. The 2-mercapto- and 8-mercapto-6-methylpurine and purine-6-thiocarboxaldehyde which were synthesized did not show any activity in tumor screening tests.

It has been established that the introduction of a methylene group into certain pharmacologically active compounds leads to an alteration or increase in their

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(2) (a) G. B. Elion and G. H. Hitchings, *J. Am. Chem. Soc.*, **69**, 2138 (1947); (b) G. H. Hitchings and C. P. Rhoads, *Ann. N. Y. Acad. Sci.*, **60**, 185 (1954); (c) G. B. Elion and G. H. Hitchings, *J. Am. Chem. Soc.*, **77**, 1676 (1955); (d) D. A. Clarke, G. B. Elion, G. H. Hitchings, and C. C. Stock, *Cancer Res.*, **18**, 445 (1958); (e) J. A. Johnson, Jr., and H. J. Thomas, *J. Am. Chem. Soc.*, **78**, 3863 (1956); (f) J. J. Fox, I. Wempen, A. Hampton, and I. L. Doerr, *ibid.*, **80**, 1669 (1958); (g) I. L. Doerr, I. Wempen, D. A. Clarke, and J. J. Fox, *J. Org. Chem.*, **26**, 3401 (1961).

(3) (a) F. S. Philips, S. S. Sternberg, L. Hamilton, and D. A. Clarke, *Ann. N. Y. Acad. Sci.*, **60**, 283 (1954); (b) J. J. Biesele, Proceedings of the 3rd National Cancer Conference, Detroit, Mich., 1956, p. 405; (c) O. Bodansky, F. S. Philips, J. Scholler, and S. S. Sternberg, *Cancer Res.*, **18**, 687 (1958).

(4) A. Giner-Sorolla and A. Bendich, *J. Am. Chem. Soc.*, **80**, 5744 (1958).

(5) (a) A. Giner-Sorolla, I. Zimmerman, and A. Bendich, *ibid.*, **81**, 2515 (1959); (b) M. A. Stevens, A. Giner-Sorolla, H. W. Smith, and G. B. Brown, *J. Org. Chem.*, **27**, 567 (1962).

(6) S. Cohen, E. Thom, and A. Bendich, *ibid.*, **27**, 3545 (1962).

(7) A. Giner-Sorolla, E. Thom, and A. Bendich, *ibid.*, **29**, 3209 (1964).