Title

Flavonol inhibition of cancer growth in vivo, cellular nucleic acid synthesis, and polynucleotide polymerases in vitro

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FLAVONOL INHIBITION OF CANCER GROWTH *IN VIVO*, CELLULAR NUCLEIC ACID SYNTHESIS, AND POLYNUCLEOTIDE POLYMERASES *IN VITRO*

by

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B.S. University of Denver, 1971

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GRADUATE DIVISION

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of the

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ABSTRACT

FLAVONOL INHIBITION OF CANCER GROWTH *IN VIVO*, CELLULAR NUCLEIC ACID SYNTHESIS, AND POLYNUCLEOTIDE POLYMERASES *IN VITRO*

Epidemiological evidence suggests that populations whose diets are high in plant foods and low in animal foods have lower cancer incidence rates than people who consume a larger proportion of animal foods. Several studies have implicated meat and animal fat as inciting factors in the development of various human cancers. It is also possible that certain constituents of plant foodstuffs act as cancer suppressing factors and, thus, contribute to the decreased occurrence of cancer in some populations. Flavonoids, 2-phenylchromones, are naturally occurring plant substances often found in plant foodstuffs. These compounds might be considered as possible cancer suppressing influences because, in addition to their wide distribution in food plants, they appear to have antibacterial, antiviral, and antitumor properties.

This research was designed to determine whether the flavonoids possess pharmacological actions which are consistent with a direct or indirect anticancer action and to investigate the biochemical basis of such an effect. The first experiments established that certain flavonols reproducibly inhibited the growth of P_{388} mouse leukemia *in vivo*. Acitivity was dependent on the presence of both the 3-hydroxyl and 4-keto functions. The most potent drugs, which inhibited growth by as much as 99%, were flavonols with 3', 4', 5'-trihydroxylated phenyl rings.

Inhibition of cellular macromolecular synthesis was studied as a possible mode of action of the flavonoids. The best inhibitors of tumor

growth, such as the flavonol myricetin, were found to dramatically decrease nucleic acid synthesis in P_{388} leukemia cells. DNA synthesis was particularly sensitive to the flavonols; inhibition of protein synthesis appeared to be a secondary effect. l-Epicatechin, a flavonol which was inactive against cancer growth in vivo, did not significantly alter the synthesis of cell macromolecules.

The next question was whether inhibition of polynucleotide polymerases could account for the effects on cellular nucleic acid synthesis. Two enzymes, representing forward transcription, E. coli DNA-dependent RNA polymerase (DRP), and reverse transcription, Rous sarcoma virus RNA-dependent DNA polymerase (RDP) were studied. Although both reactions were blocked by some of the flavonoids, RDP was considerably more sensitive. The active compounds were characterized: all had an unsaturated heterocyclic ring and hydroxyl groups at both the 3 and 4' positions. Potency is a function of the number and the pattern of B-ring hydroxylation. Again, as was seen for the effects on tumor growth and cellular nucleic acid synthesis, the best inhibitors were flavonols with a trihydroxylated phenyl ring. They are capable of inhibiting reverse transcription by 50% at 9×10^{-6} M.

The active flavonols were found to be unstable under the conditions of the in vitro enzyme assays. They undergo an alkaline pH and oxygen dependent chemical transformation to form intermediates which are responsible for the enzyme inhibition. The flavonols that are most susceptible to this reaction are the best inhibitors. The proximate transcription inhibitors appear to be polyhydroxylated 1, 3-diphenyl-1, 2, 3-propanetriones, a new class of polynucleotide polymerase inhibitors.

This study shows that the observed inhibition of nucleic acid transcription, a new mode of action of flavonols, may be sufficient to account for their anticancer and antiviral effects. The most potent inhibitors of *in vivo* P_{388} leukemia growth strongly inhibit cellular nucleic acid synthesis and are also the best DNA polymerase inhibitors *in vitro*. Inhibition of certain polymerases may explain some of the selective toxicity of the flavonols. The active intermediates formed by oxidative alkaline degradation of flavonols might provide prototypes for new chemotherapeutic agents.

INTRODUCTION

Cancer Epidemiology

"Cancer is not an inevitable consequence of life. This conclusion, which holds equally true for other diseases prevalent in developed societies, such as atherosclerosis, is clearly borne out by epidemiological evidence. The major differences in the incidence of cancers among different countries, between the sexes, among population groups within each country, and between migrant and native populations, as well as time trends, point to environmental factors as explaining such variances." (1)

Ernst Wynder related the importance of environmental factors in the etiology of human cancer with these remarks in his introduction of a recent symposium, Nutrition in the Causation of Cancer (2). The diet is one such variable and, although the intake levels of specific constituents have been correlated with the incidence rates of certain cancers, the mechanisms underlying these influences are unknown. A discussion of the epidemiology of colon cancer will illustrate several different approaches which have been used to identify possible etiological factors.

Colon cancer incidence rates vary throughout the world (3) and differences in dietary patterns rather than genetic constitution have been implicated as the important etiological variables. Haenszel et al. studied Japanese populations which migrated first to Hawaii and then to California and found that the patterns of cancer occurrence changed and began to resemble those of the new environment. Colon cancer, normally rare in Japan, is increasingly prevalent in Hawaiian and especially Californian Japanese, and is approaching the high rates found in North America. Furthermore, a positive association between meat intake and the incidence of colon cancer was noted. A world-wide correlation between animal, but not vegetable fat and colon cancer has been shown (5),

suggesting that the fat in meat may be the important variable.

Epidemiological studies can identify correlations but a supporting biochemical mechanism helps to establish etiological importance. In 1971 Hill et al. (6) showed that the feces from people in Britain and the U.S.A., where the incidence of colon cancer is high, have increased concentrations of anaerobic bacteria, steroids and degraded steroids, as compared to the feces from people in Uganda, South India, or Japan, where the incidence of the disease is low. They postulated that intestinal micro-organisms might be able to produce carcinogens from dietary fats or from bile steroids, and that dietary differences may alter the bacterial flora and thus influence colon cancer incidence. Although data supporting this hypothesis have accumulated (7,8), two major investigations have not been able to confirm that significant differences in the composition of intestinal flora exist between different risk groups (9). Burkitt (10) has offered an alternative hypothesis, suggesting that dietary plant fiber accounts for the low incidence of colon cancer seen in populations whose diet is high in plant foods and low in fat and meat. He proposed that fiber, which decreases the transit time of feces in the colon, would shorten the available time for carcinogen production and subsequent contact with the bowel. A world-wide correlation between the dietary intake of fiber and colon cancer does not, however, appear to exist (11).

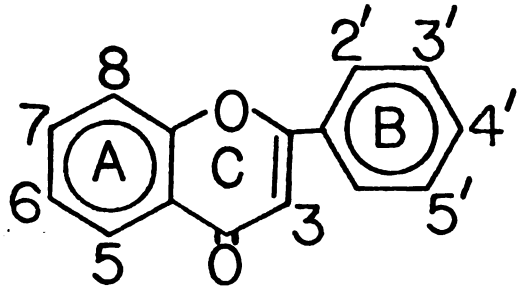
Dietary intake of animal fat is important, but unlikely to be the only factor involved in the development of colon cancer. In a careful, county by county, comparison of gastrointestinal cancer incidence rates in the U.S., Jansson (12) pointed out that since the consumption of beef and animal fat is relatively homogeneous, it is difficult to explain the distinct geographic variations in colon cancer rates without assuming

other etiological variables. The presence of carcinogens other than those found in the diet or of factors protecting against carcinogenesis may be responsible. For example, the concentration of selenium, an antioxidant, in the soil appears to be inversely correlated with cancer incidence and may be such a protecting factor (13,14).

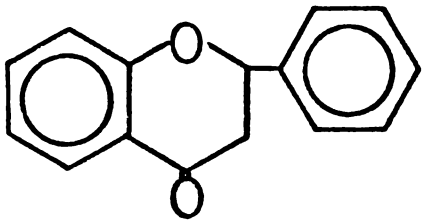
The Flavonoids: Possible Cancer Suppressing Influences

The present investigation tests the hypothesis that certain plant constituents, which are commonly found in many foods, may act as cancer suppressing influences. This suggestion is not inconsistent with the results of the epidemiological studies which, as have been discussed, suggest complex etiological patterns. Thus, the risk of developing cancer may depend on the relationship between inciting factors, such as components in animal fat, and protecting factors. The food plant flavonoids might be considered as possible cancer suppressing influences, explaining the epidemiological association of plant fiber diets and low cancer incidence found by Burkitt (10).

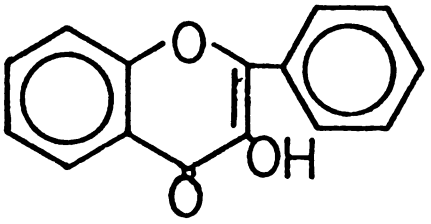
The flavonoids, widely distributed in the plant kingdom, are found in many foods, including fruits, vegetables, cereals, and teas (15,16). They are characterized by a C_6-C_3-C_6 carbon skeleton consisting of two aromatic rings joined by a three carbon, oxygen containing heterocyclic ring. Structural classification is based on the pattern of substitution in the aromatic rings and the nature of the oxidation of the carbon fragment. Figure 1 shows the basic structures and numbering of several classes of flavonoids. Flavones are 2-phenylchromones and flavonols are the 3-hydroxylated subclass; flavonones and flavanonols, or dihydroflavonols, are the 2-3 saturated derivatives, respectively. The flavyliums, or anthocyanidins, are permanently charged 2-phenyl-benzopyrylium cations, and the 4-deoxyflavanols, or catechins, are



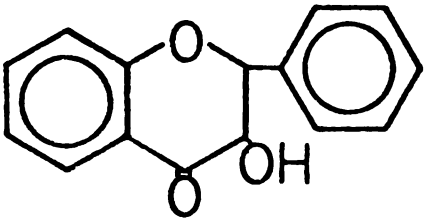
FLAVONES



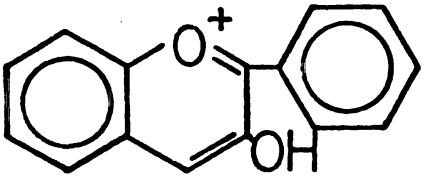
FLAVANONES



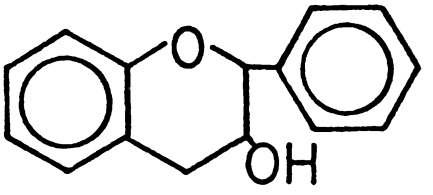
FLAVONOLS



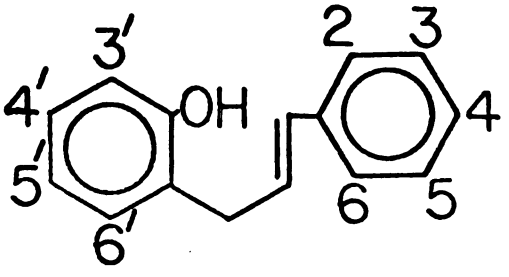
DIHYDROFLAVONOLS



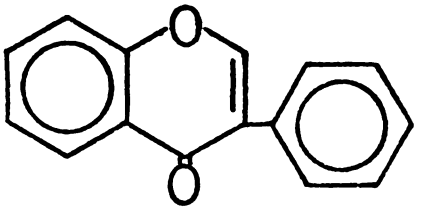
FLAVYLIUMS



FLAVANOLS



CHALCONES



ISOFLAVONES

FIGURE I

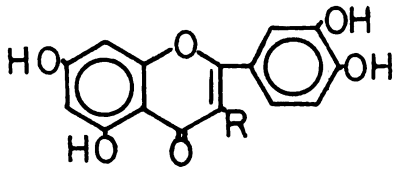
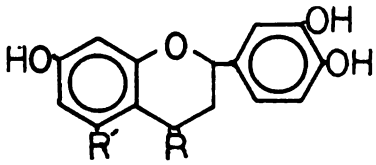
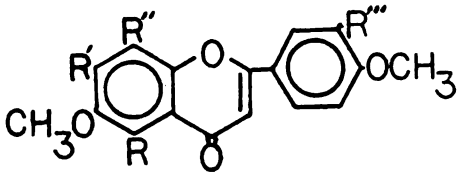
saturated in the C-ring. Chalcones are open chain compounds and the isoflavones are 3-phenylchromones. The aromatic rings of the naturally occurring flavonoids are usually hydroxylated or methoxylated at one or more positions and may normally exist in plants as glycosides in which certain of the phenolic hydroxyls are combined with sugar residues, often glucose or rhamnose. The colors of many flowers and berries, ranging from red through purple to deep blue, are provided by flavyliums, whereas the favones are yellow pigments in many species. Despite their abundance, no specific role in plant life has been ascribed to these compounds.

Flavonoids might be considered as possible cancer suppressing influences because, in addition to their wide distribution in food plants, they have a number of interesting pharmacological properties. Antifungal, antibacterial, antiviral, and anticancer activities have been attributed to both naturally occurring and synthetic flavonoids. The most active compounds, their structures, and their actions are summarized in Table 1. While the effects are not dramatic or consistently reproducible, the observation that diverse organisms may be affected is intriguing.

"Mal secco", a fungal disease of citrus varieties, is inhibited by nobiletin, a polymethoxylated flavone. This compound has been identified as the main fungistat present in tangerines that are resistant to the disease (17). Taxifolin, a polyhydroxylated dihydroflavonol, is toxic to several decay fungi (18,19).

Inhibition of Clostridium botulinum and Staphlococcus aureus growth by quercetin, a flavonol, but not by its glycosides, quercitrin or rutin, has been reported (20,21). Some gram negative organisms, particularly Shigella sonnei, are also sensitive to the effects of several

TABLE 1

COMPOUND	EFFECT	REFERENCE
		
Quercetin R = OH	Antibacterial Antiviral Cytotoxicity(Mammalian) Antitumor	20,21,22 23,24,25 41 31
Quercitrin R = glucose	Antiviral	24,25
		
Taxifolin R = O; R'= OH	Antibacterial Antifungal Antiviral	22 18,19 23
Fustin R = O; R'= H	Antibacterial Antiviral	22 23
Catechin R = H; R'= OH	Cytotoxicity(Mammalian)	34,35
		
Eupatorin R,R'''= OH R'= OCH_3; R''= H	Cytotoxicity(Mammalian)	29,30
Tangeretin R,R',R''= OCH_3 R'''= H	Zebra-Fish Toxicity Rat Neonatal Toxicity	27 28
Nobiletin R,R',R'',R'''= OCH_3	Antifungal	17

polyhydroxylated flavonols and dihydroflavonols (22). However, the data are insufficient to determine the structural features required for, or the spectra of, the antifungal and antibacterial effects of the flavonoids.

An *in vitro* virucidal effect of quercetin and, to a lesser extent, of two dihydroflavonols on Herpes simplex virus, a DNA virus, has been reported (23). Two RNA viruses, parainfluenza and polio, have also been studied. Quercetin inhibited replication of the former, but the latter was unaffected by any of the flavonoids tested. A series of reports on the *in vivo* antiviral effects of some flavonoids have appeared (24,25). Inhibition by quercetin and quercitrin of rabies virus induced mouse death was, at times, marked but not readily reproducible. Similar effects on ectromelia virus were noted. Infection by neurovaccinia, parainfluenza, and Columbia-SK viruses was not significantly altered by any of the flavonoids. A synthetic compound, 4'-hydroxy-5,6,7,8-tetramethoxyflavone, inhibits the replication of Rhinovirus types 13 and 56 grown in human KB cells (26). No clear pattern of activity is evident, neither DNA or RNA viruses were consistently affected.

The polymethoxylated flavones, in addition to their antifungal and antiviral properties, are cytotoxic. Two of the compounds, both commonly found in citrus fruit peels, are highly toxic to zebra fish embryos (27) and rat neonates (28). Although they are not toxic to adult mammals, some closely related flavones, possessing both methoxy and hydroxy groups, inhibit mammalian cell growth *in vitro* (29,30).

Several investigators have reported that flavonoids affect *in vivo* tumor growth. Subcutaneous injection of quercetin consistently induced damage in mouse sarcomas (31). In other studies crude plant extracts

were tested, making interpretation of the results difficult. A flavonoid preparation injected daily for 19 days inhibited growth of the Crocker rat carcinoma by 80% at 40 days after tumor implantation (32). Inhibition of rat carcinoma 175-G and mouse sarcoma 180 growth was also demonstrated. Oral administration, but not injection, of a "flavonoid complex" to mice bearing mammary tumor decreased the formation of metastases (33). These findings are suggestive but inadequate.

The action of catechin on the growth of mammalian cells in culture is biphasic (34). At low concentrations (10^{-6}M) cellular proliferation is stimulated whereas it is inhibited at higher concentrations (10^{-3}M). The authors postulated that quinones, resulting from the auto-oxidation of catechin, are responsible for growth inhibition (35). Previously Swayne (36) had found several flavonoids to be spermicidal *in vitro* and suggested that orthoquinone formation is necessary for the effect. Examples of such oxidations are shown in Figure 2. In this connection it was Bartlett (37) who, after studying the rather non-specific inhibition of succinate dehydrogenase by certain flavonoids, first suggested that quinone formation may be important in the biochemical actions of these compounds.

Some flavonols are, however, specific enzyme inhibitors. Mitochondrial and Na^+, K^+ stimulated ATPase activities are blocked at concentrations which do not alter oxidative phosphorylation (38,39), an effect which mediates quercetin induced inhibition of aerobic glycolysis in Erhlich ascites cells *in vitro* (40). The relationship of this action to the marked inhibition of P_{388} leukemia cell growth *in vitro* by quercetin is under investigation (41).

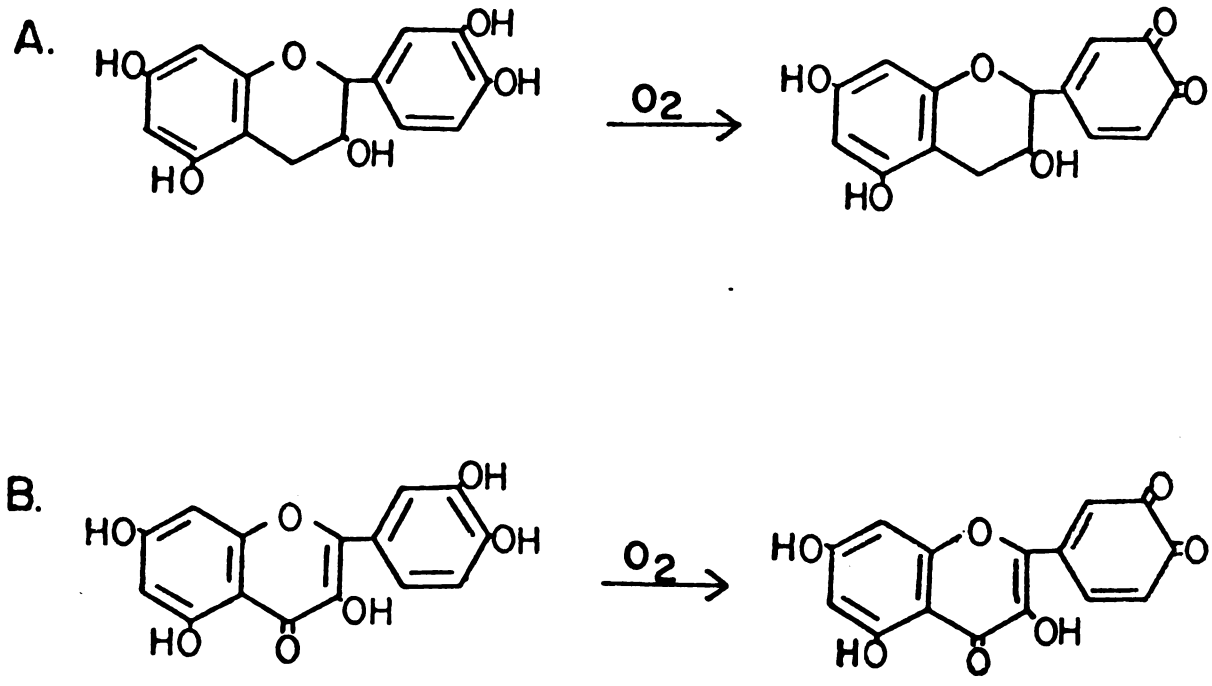


Figure 2: O-Quinone Formation from the Oxidation of 3',4'-dihydroxy Flavonoids: A) Catechin, B) Quercetin.

This brief review indicates that the growth and/or some basic replicative processes(es) of a wide variety of organisms are affected by a number of flavonoids. However, these effects are often limited to a particular class or subclass of flavonoids, suggesting the need to consider specific compounds and specific classes of flavonoids with reference to any pharmacological actions.

Possible Modes of Action

On the basis of their wide distribution in food plants and known pharmacological properties, flavonoids are reasonable candidates as possible cancer suppressing influences. Since precise data on the dietary intake levels of these compounds in different populations around the world do not exist, epidemiological verification is not presently feasible. However, specific questions relating to possible modes of action can be generated, and using *in vivo* and *in vitro* models, tested. In general, anticancer factors could act directly or indirectly and affect either carcinogenesis, tumor growth, or both.

A direct antitumor effect must be considered as a potential mechanism of action. The very low mammalian toxicity of the flavonoids suggests that the compounds are not highly cytotoxic to normal tissues. There is, however, some evidence to indicate that they are selective inhibitors of cancer growth. Soulinna *et al.* (41) found that transformed cells were more sensitive than normal cells to the antiproliferative effects of quercetin. In any case, small, rapidly growing tumors are more sensitive than large, slowly growing tumors to cytotoxic agents. Thus, any effects of the flavonoids would probably be exerted on early, preclinical neoplasias. This would be consistent with the idea of a dietary constituent displaying a weak, but chronic,

anticancer effect.

Many human cancers are caused, mediated, or modified by environmental factors (42). These factors, synthetic and naturally occurring, often require metabolic activation, a process which is effected by both human and bacterial enzymes (43). Chemical carcinogens are also subject to metabolic detoxification and the ultimate potency of a compound may depend on the balance between activating and detoxifying pathways. Wattenberg (44) has shown that the potent carcinogenic action of benzo(a)-pyrene can be inhibited by the administration of certain flavones. He had previously reported that the naturally occurring polymethoxylated flavones, nobiletin and tangeretin, induce benzpyrene hydroxylase activity (45). Induction of this enzyme decreases the carcinogenicity of many compounds (43). The importance of bacterial transformation of dietary and metabolic products, particularly fats, in the development of colon cancer has been discussed. Other types of microbial enzymes can alter the fate of carcinogens as well. A glucosidase, which converts cycasin into a carcinogenic aglycone (46), and reductases, which detoxify azo dyes (47), are examples. Flavonoids could influence the metabolism of carcinogens in a number of ways. Host or bacterial activating or inactivating enzymes could be induced or inhibited. Selective depletion of the bacterial flora that are responsible for these metabolic reactions would likely alter neoplastic development. In this regard, the previously cited antimicrobial effects of the flavonoids are of interest.

The role of viruses in the etiology of cancer is a matter of great interest (48). The cause of many animal cancers is certainly viral and it is likely that at least some human tumors, such as nasopharyngeal carcinoma and Burkitt's lymphoma, are also virally induced (49-52).

It is reasonable to anticipate that disruption of tumor virus infection or genome expression might lower cancer incidence rates. A specific, antiviral effect of the flavonoids is an attractive possibility. As natural dietary compounds, the prophylactic nature and low host toxicity of these compounds are consistent with such an action.

The precise relationship between neoplasia and the immune response are not defined. Confusion regarding the importance of both cellular and humoral immunity in controlling the appearance and growth of tumors exists (53). Nonetheless, immunotherapy, based on non-specific stimulation of the immune system, has been of some value in the treatment of malignant melanoma (54). It is possible that the flavonoids could, in some poorly defined way, influence the immunological response and thus suppress cancer development. It may be of interest that Kilburn et al. (55) have found that quercetin is capable of recruiting leukocytes in lung tissue.

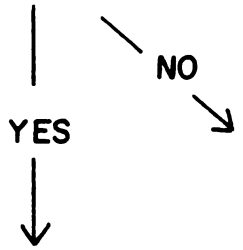
Experimental Approach

The purpose of this research was to determine whether the flavonoids possess pharmacological actions which are consistent with a direct or indirect anticancer action and to investigate the biochemical basis of such an effect. An effort was made to ask specific, experimentally testable questions in such a way that the results would either rule out or generate new hypotheses. The basic questions asked are outlined in Figure 3.

The first experiments were designed to establish if any of the flavonoids, particularly the commonly occurring flavonols, reproducibly inhibit the growth of a murine leukemia in vivo. Effects on tumor cells numbers rather than on increases in life span were determined. The greater sensitivity of this procedure was needed to compensate for the

FIGURE 3: EXPERIMENTAL APPROACH

I. A. Do any of the Flavonoids Exhibit Reproducible Inhibition of <u>in vivo</u> Tumor Growth?

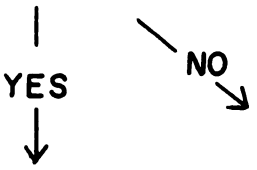


1) Do they Inhibit Virally Induced Carcinogenesis?

2) Do they Alter Bacterial Flora and the Metabolism of Carcinogens?

3) Do they Alter the Immune Response?

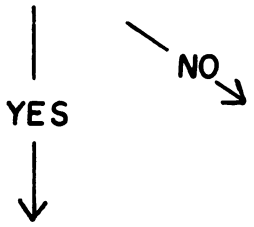
B. Do they Inhibit Nucleic Acid or Protein Synthesis?



1) Are they Mitotic Inhibitors?

2) Do they Disrupt Energy Metabolism?

C. Are they Inhibitors of Polunucleotide Polymerases <u>in vitro</u>?



1) Is Purine or Pyrimidine Nucleotide Synthesis Blocked?

2) Is Nucleotide Phosphorylation Inhibited?

II. A. What is the Nature of the Enzyme Inhibition?

B. What Chemical Species Accounts for the Effect?

1) Does the Inhibitor Result from a pH Dependent Reaction?

2) Does the Inhibitor Result from an Oxygen Dependent Reaction?

C. Are Compounds Structurally Related to the Reaction Products Inhibitors of Polynucleotide Polymerases?

expected low toxicity of the flavonoids. Since some of the flavonols exhibited surprisingly strong antileukemic activity, the mechanism of action underlying this cytotoxicity was investigated. The synthesis of cell macromolecules, a process which is common to the variety of organisms including viruses inhibited by different flavonoids, was considered a possible site of action. Changes in DNA, RNA, and protein synthesis were studied in leukemia cells of the same type used in the *in vivo* experiments. The best inhibitors of tumor growth, the flavonols, were found to dramatically decrease cellular nucleic acid synthesis. Several different metabolic lesions can lead to such an effect. Polynucleotide polymerases are immediately and directly involved, however, and so the actions of flavonoids on two representative transcribing enzymes were investigated.

E. coli DNA dependent RNA polymerases (DRP) and Rous sarcoma virus RNA dependent DNA polymerase (RDP) were studied for several reasons. Both enzymes are relatively stable (56,57), facilitating reproducibility and the meaningful comparison of a large number of compounds. They catalyze the two classes of transcription, forward (DRP) and reverse (RDP). Forward transcription is required for cell growth (58,59) and its blockade by drugs can produce an anticancer effect (60). Furthermore, reverse transcription is required for the carcinogenic action of Rous sarcoma virus (61-63). The structure/activity relationships were similar for both enzymes, although RDP was generally more sensitive to inhibition. The best enzyme inhibitors most effectively blocked cellular nucleic acid synthesis and *in vivo* tumor growth as well.

During investigations designed to understand why certain aspects of the flavonoid molecule are required for the *in vitro* inhibition of polynucleotide polymerases, the lability of the active compounds in mild

alkali was detected. Experiments were performed to determine if the flavonols or the products resulting from this chemical reaction were the proximate enzyme inhibitors. It was learned that an alkaline and oxygen dependent reaction is required for the formation of the active compounds. Several compounds, simplified analogs of the proposed flavonol reaction products, were synthesized in an effort to clarify the nature of the enzyme inhibitors. Attempts to isolate the active compound from the breakdown reaction and to synthesize the inhibitor directly were also made.

MATERIALS AND METHODS

General Procedures

Ultraviolet spectra were determined with a Cary, model 14PM, recording spectrophotometer in ethanol, distilled water (pH 5.2), or 50 mM tris buffered solutions (pH 7.6, pH 8.0, pH 8.5). For analyses done in nitrogen or oxygen enriched atmospheres the buffer and test compound solutions were first gassed under positive pressure in rubber stoppered test tubes for 10 minutes. A 0.1 ml sample of the compound was then added to 2.9 ml of the buffer using a 1.0 ml syringe. Gassing was continued for a specified length of time, the mixture transferred to a quartz cuvette, and the absorbance determined immediately with a Beckman DU spectrophotometer.

A Perkin-Elmer, model 457, grating infrared spectrophotometer was used for the determination of IR spectra (halocarbon mulls). Melting points, uncorrected, were determined with a Thomas-Hoover, unimelt, in open capillaries. Samples were prepared for mass spectrophotometric analysis for an AEIMS-9 using chemical ionization by acidification of the alkaline flavonol reaction mixtures to pH 6, extraction with ether, and concentration in vacuo.

The following firms supplied the flavonoids used in this study: The Aldrich Chemical Co. Inc., Milwaukee, Wisc., 2'-hydroxychalcone, chrysin, fisetin, morin, and myricetin; Eastman Organic Chemicals, Rochester, N.Y., 3-hydroxyflavone; ICN K and K Laboratories Inc., Hollywood, Ca., delphinidin chloride, fustin, naringenin, pelargonidin chloride, and taxifolin; Pfaltz and Bauer, Inc., Flushing, N.Y., apigenin; and Transworld Chemicals, Inc., Washington, D.C., kaempferol, quercetin, and robinetin.

In Vivo Antitumor Assay

The methodology of Apple (64) was used to determine *in vivo* anticancer activity. This assay is based on a comparison of the number of cancer cells present in drug treated and control animals after 7 days of tumor growth. A transplantable tumor with reproducible growth characteristics was used so that results from independent experiments can be compared. The P_{388} lymphocytic leukemias used in these studies was chosen because of the correlation of its drug sensitivity to that of human cancer *in vivo*; it is now the primary *in vivo* screen for potential anticancer drugs used by the National Cancer Institute.

Male, $BDF_1(C_{57}$ BL/6 x DBA/2) mice, 5-7 weeks old, were purchased from Simonsen Laboratories, Gilroy, California. The animals were housed 6 to a cage and given food and water *ad libitum*. The National Cancer Institute provided the P_{388} leukemia, which was maintained as an ascitic tumor by passage every seven days. On day 0 of each experiment the animals were injected intraperitoneally with 1×10^5 cells diluted in 0.1 ml of ice-cold sterile saline and then divided into a control group of 10-12 animals and experimental groups of 5-6 animals each. Cold saline was used to increase cell viability (65). Intraperitoneal injections of saline or a flavonoid (150 mg/kg) were administered daily for the next 6 days. The tumor burden of each animal was estimated by counting the leukemic cells in the peritoneal cavity on day 7 (64). The mice were killed by cervical dislocation, and the abdominal walls cut, and the peritoneal cavity thoroughly washed with 100 ml of sterile saline was collected, diluted, red blood cells were lysed, and then the P_{388} cells were counted with a model-A coulter counter. Three counts were made and averaged. Background levels were established by determining the number of cells present in animals not injected with

tumors but treated with saline for days 1-6. Each flavonoid was tested twice, in independent experiments, and the results are expressed as the percent inhibition of growth in drug treated compared to saline treated animals. The mice were weighed throughout the experiment to determine if the drugs induced host toxicity.

Cellular DNA, RNA, and Protein Synthesis

The effects of different flavonoids on cellular macromolecular synthesis were determined by pulse labeling P_{388} leukemia cells with radioactive precursors (66). The rates of DNA, RNA, and protein synthesis were estimated by the incorporation of [methyl-^{3}H]-thymidine (specific activity = 6.7 Ci/mMole), [5,6-^{3}H]-uridine (specific activity = 37.6 Ci/mMole), and 1-[4,5-^{3}H]-leucine (specific activity = 5 Ci/mMole), respectively, into acid precipitable material. The labelled compounds were obtained from New England Nuclear. P_{388} cells were removed from a BDF_1 tumor bearing animals and diluted with Fischer's leukemic cell media (67) supplemented with 10% horse serum (Gibco), and buffered with 25 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) at pH 7.3 to approximately 3 X 10^6 cells/ml. The flavonoids, dissolved in ethanol, or vehicle control solutions and the tritiated precursors were added in 15 µl volumes to 3 ml of the cell suspension and incubated at 37° C. At 10, 30, and 50 minute intervals 0.8 ml samples were removed and added to ice-cold normal saline, centrifuged for 4 minutes at 500 g at 4° C, and the supernatants removed. The pellets were resuspended in 0.25 ml of 50 mM EDTA and sonicated for 5 seconds at a setting of 30 with a Bronwil Biosonik, Rochester, N.Y.

Replicate 75 µl portions were applied to glass fiber filters (Schleicher and Schuell, No. 25, Keene, N.H.). The samples were soaked in 10% and 5% trichloroacetic acid solutions and 95% ethanol for 20

minutes, 2 X 10 minutes, and 15 minutes, respectively (68). The filters were air dried and radioactivity determined with a Packard-Tri Carb liquid scintillation spectrophotometer using an Omnifluor (New England Nuclear, Boston, Mass.)-toluene cocktail. Counting efficiencies, approximately 33%, were estimated using ^{3}H-toluene standards and the automatic external standardization as a measure of sample quenching. The results are expressed as disintegrations per minutes per 10^{6} cells and are plotted as a function of incubation time.

In Vitro Enzyme Assays

The DRP and RDP assays are basically the same. Enzyme activity is determined by the amount of radioactive substrate that is incorporated into acid precipitable polynucleotides. The buffered reaction mixtures contain a template, four nucleoside triphosphate substrates, the necessary ions, vehicle or drug, and the enzyme. The reactions are initiated by the addition of the enzyme or substrate and a shift from 4^{o} C to the incubation temperature. Ice-cold acid is added to quench the reactions and precipitate the ^{3} H polymers which are trapped by filtration and washed to eliminate excess unincorporated label. (Figure 4)

I_{50} values, determined graphically, represent the drug concentration required to inhibit the control enzyme reaction by 50% and were used to compare the potencies of different compounds. Three assays were done for each concentration tested and the enzyme inhibition curves were compiled from at least two independent experiments.

DNA Directed RNA Polymerase Assay (DRP)

E. coli DRP (Sigma Chem. Co., St. Louis, Mo.) was assayed in a manner similar to that of Burgess (56). Modifications included the use of glass fiber filters (Schleicher and Schuell, No. 25) and [5,6-^{3}H]-UTP (Swartz/Mann, Orangeburg, N.Y.; specific activity = 30 Ci/mMole) as

FIGURE 4: Flow Diagram of

Polynucleotide Polymerase Assays In Vitro

	Enzyme	Buffer	Substrates	Template	Ions
DRP	E. coli	pH 7.6	NTP's	ds DNA	Mg^{++}
RDP	RSV Virus	pH 8.0	dNTP's	ss RNA	Mg^{++}

↓ (+/- Drug)

Incubation

DRP 37°C

RDP 40°C

↓

Quench Reaction

↓

Filtration

DRP Glass Fiber

RDP Millipore

↓ (Dry Filters)

Liquid Scintillation Counting

the labelled substrate. A standard incubation mixture (0.225 ml) contained 40 mM tris buffer (pH 7.9 at 25° C), 10 mM Mg Cl_2, 0.10 mM EDTA, 0.10 mM dithiothreitol, 150 mM KCl, 50 mg/ml bovine serum albumin, 0.15 mM ATP, GTP, CTP, 0.11 μM ^{3}H-UTP, 0.15 mg/ml calf thymus DNA, and 0.12 units of enzyme. One unit is the amount of enzyme required to incorporate 1 nmole of ATP into an acid insoluble product in 10 minutes at pH 7.9 at 37° C and, in this case, was approximately 2.5 μg protein.

After 14 minutes the reaction tubes were put on ice, two 0.10 ml samples were removed and mixed with 2 ml 5% trichloroacetic acid. The acid precipitates were collected on glass fiber filters, washed 3 times with 2 ml of 2% trichloroacetic acid, once with ethanol and dried. The samples were counted in a Packard-Tri Carb liquid scintillation spectrophotometer using an Omnifluor (New England Nuclear)-toluene cocktail. Since some of the compounds tested are poorly water soluble, an accurate determination of activity was difficult. In certain indicated experiments, the flavonoids were dissolved in dimethylsulfoxide (DMSO) and diluted 1:1 with glass-distilled water before adding to the incubation mixture, yielding a final DMSO concentration of 5%. Under these conditions the compounds readily dissolved.

In some experiments the enzyme was assayed under a nitrogen enriched oxygen-deficient atmosphere. This was done by using rubber stoppered, glass test tubes which were connected to a nitrogen source with polyethylene tubing. Nitrogen was slowly bubbled through the incubation mixture, drug, and substrate solutions prior to initiation of the reaction. The test tubes remained sealed until they were transferred to an ice bath.

RNA-Directed DNA Polymerase Assay (RDP)

Conditions for the assay of detergent disrupted Rous sarcoma virus RDP activity were as follows. A typical reaction mixture contained

88 mM tris buffer (pH 8.0 at 25° C), 5.3 mM $MgCl_2$, 70 uM each dATP, dCTP, dTTP, 1.5 uM 8-^{3}H GTP (ICN, Irvine, Ca.; specific activity = 25 Ci/mMole), 11 mM dithiothreitol, 11 mM NaCl, and 0.011% Nonidet P-40, a nonionic detergent. Drug or control solutions and appropriately diluted virus were added in volumes of 25 µl and 75 µl, respectively. Following a 60 minute incubation at 40° C the assay mixtures were put on ice and replicate samples withdrawn and added to an ice cold 4.5% sodium pyrophosphate solution. The addition of perchloric acid (7%) and hydrochloric acid (9%) solutions precipitated the nucleic acids. The labelled DNA was collected on filters by vacuum filtration (Millipore Corp., HAWP02500, Bedford, Mass.), washed with 9% HCl and 4.5% sodium pyrophosphate solutions, air dried, and counted in a liquid scintilation spectrometer. These conditions are drived from the Temin-Baltimore reverse transcriptase assay (69,70).

Synthesis

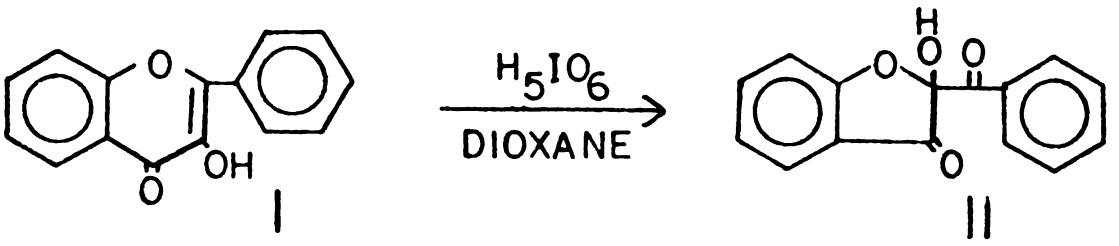
Materials: The Aldrich Chemical Company supplied the following reagents: O-hydroxyacetophenone, benzoylchloride, formic acid, veratric acid, thionyl chloride, 2-hydroxy-4,6-dimethoxyacetophenone. Hydrogen peroxide (30%) and periodic acid were obtained from Fisher Scientific, Fair Lawn, N.J., and Sigma Chemical Co., St. Louis, Mo., respectively. Silica gel, no. 6061, and cellulose, no. 6064, thin layer chromatographic plates were manufactured by Eastman Organic Chemicals. The preparation silica gel plates, F-254, were purchased from EM Reagents, Westbury, N.Y. Thin layer chromatography was used routinely to assess the progress of the organic reactions and in the evaluation of chemical purity.

2-Aroyl-2-hydroxy-3(2H)-benzoylfuranone (Figure 5 -A)

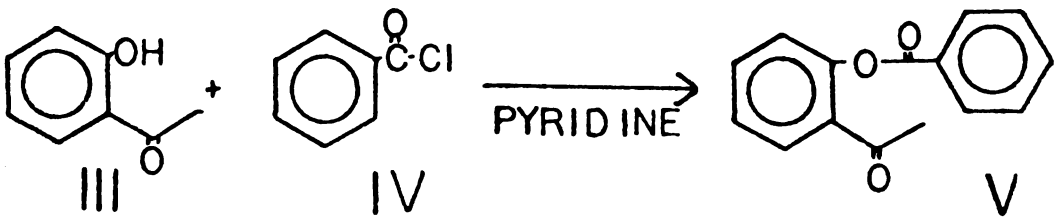
This compound was prepared by the periodic acid oxidation of 3-hydroxyflavone as described by Smith (71). 50 ml of 0.01 M periodic

FIGURE 5: Synthesis of A) 2-aroyl-2-hydroxy-3(2H)-benzofuranone(II) B) O-benzoyloxyacetophenone(V), C) O-hydroxydibenzoylmethane(VI), and D) O-hydroxydibenzoylcarbinol(VII).

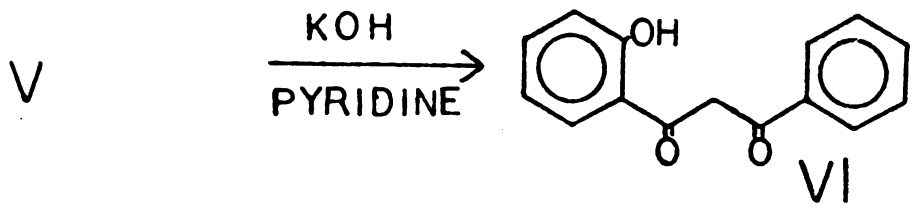
A


O
OH
O
I
H_5IO_6
DIOXANE
H
O
O
O
O
II

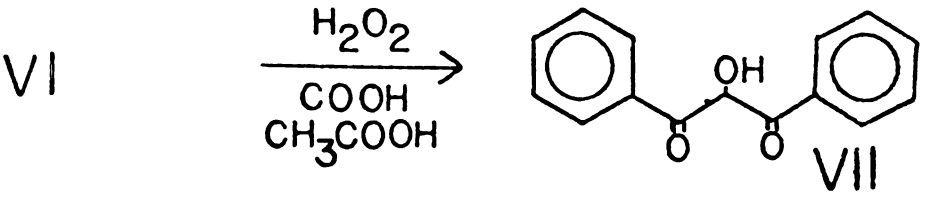
B


OH
+
O
III
O
C·Cl
IV
PYRIDINE
O
O
O
V

C


V
KOH
PYRIDINE
OH
O
O
VI

D


VI
H_2O_2
COOH
CH_3COOH
OH
O
O
VII

acid were slowly added to 1 gram of flavonol dissolved in 50 ml of purified dioxane (72). After stirring for 20 minutes the reaction mixture was added to 10 ml of 3 M sodium bisulfite and 0.1 M sulfuric acid. Most of the dioxane was removed in vacuo and the remaining oil and aqueous mixture was extracted with ether (3 X 200 ml) and then washed with saturated sodium bicarbonate. The ether was evaporated and the residue was crystallized from methylene chloride-hexane to furnish a colorless solid. The yield was 74%, mp 93° C; UV(Etoh): λ max 252(log ε 4.22), 325(log ε 3.58) nm; IR:ν max 3310, 1770i, 1720, 1690, and 1605 cm^{-1}.

O-Benzoyloxyacetophenone (Figure 5 -B)

The procedure of Wheeler (73) was used for this preparation. O-Hydroxyacetophenone (13.6 g) and benzoylchloride (21.1 g) were mixed in 20 ml of pyridine. After 15 minutes the mixture was poured into 600 ml of ice-cold 3% hydrochloric acid. The product was filtered, washed with methanol and water, and then dried. The yield was 73%. Following recrystallization from methanol the melting point was 82-84° C.

O-Hydroxydibenzoylmethane (Figure 5-C)

This Baker-Venkataraman rearrangement has been outlined (73). A solution of O-benzoyloxyacetophenone (10 g) in pyridine (37.5 ml) was warmed to 50° C and hot pulverized potassium hydroxide (3.5 g) was added. The mixture was stirred for 15 minutes and the resulting yellow precipitate was cooled and acidified with 50 ml of 10% acetic acid. The mixture was filtered, dried, and recrystallized from methanol to give a product melting at 115-116° C in a yield of 73%.

O-Hydroxydibenzoyl carbinol (Figure 5-D)

The method of Winicki (74), a hydrogen peroxide oxidation of the

propandedione, was used for this synthesis. To 167 ml of acetic acid, 2 g of O-hydroxydibenzoylmethane, 9.4 ml of formic acid, and 6.8 ml of hydrogen peroxide were added. The mixture was maintained at 50° C for 5 hours and then diluted 1:10 with distilled water and extracted with ether. Following neutralization, the ether was evaporated and the resulting oily residue was washed with a 95:5 mixture of petroleum ether: chloroform and concentrated to 50 ml. Cooling and the addition of petroleum ether furnished a white solid which, upon recrystallization from petroleum ether:chloroform, had a melting point of 97-99° C. Yield 23%; UV(EtOH):λ max 251(log ε 4.02), 316(log ε 3.56) nm; IR:ν max 1680, 1638, and 1610 cm^{-1}.

1-(2,4,6-Trihydroxy)-phenyl-3-(3',4'-dihydroxy)-phenyl-1,3-dione-2-propanol

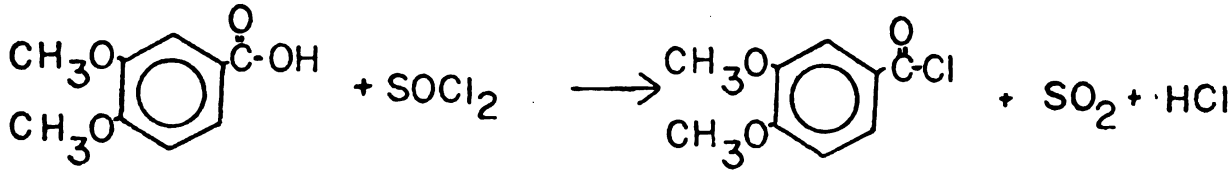
The synthesis of this compound, a polyhydroxylated analog of the carbinol(VII), was attempted according to the scheme outlined in figure 6. Veratroyl chloride(IX) was prepared by the procedure of Fieser and Fieser (75). Veratric acid(15 g)(VIII) and thionyl chloride(20 ml) were refluxed for one hour. The excess thionyl chloride was removed in vacuo, the mixture allowed to solidify, and then the crude acid chloride was purified by vacuum distillation. Yield 80%; mp 75-78° C.

The polymethoxylated propanedione(XI) was obtained from a condensation and rearrangement of (IX) and 2-hydroxy-4,6-dimethoxyacetophenone (X) (Figure 6-B). The conditions of Bayer and Krämer (76) were used. The product was recrystallized from boiling ethanol to furnish yellow needles, melting at 171-172° C, in a 90% yield.

All efforts to oxidize the propanedione(XI) by the methods successful in the oxidation of (VI) failed. The reaction was also run at lower temperatures, with less hydrogen peroxide, and under less acidic conditions. However, mixtures of compounds in yields of less than 10%

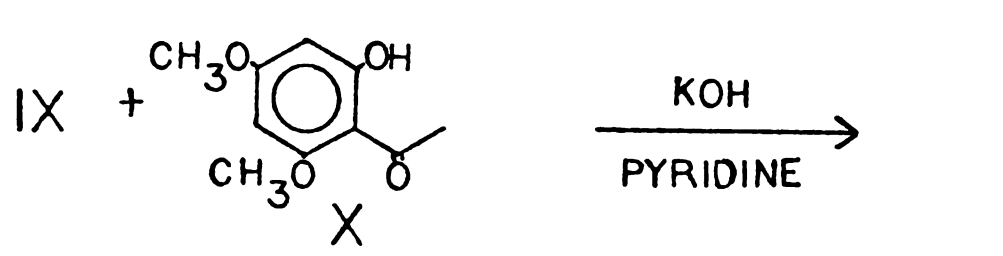
FIGURE 6: Synthesis of A) veratroyl chloride(IX), B) 1-(2-hydroxy-4, 6-dimethoxy)-phenyl-3-(3',4'-dimethoxy)-phenyl-1,3-propanedione (XI), C) 1-(2-hydroxy-4,6-dimethoxy)-phenyl-3-(3',4'-dimethoxy)-phenyl-1,3-dione-2-propanol(XII), D) 1-(2,4,6-trihydroxy)-phenyl-3-(3',4'-dihydroxy)-phenyl-1,3-dione-2-propanol(XIII).

A.



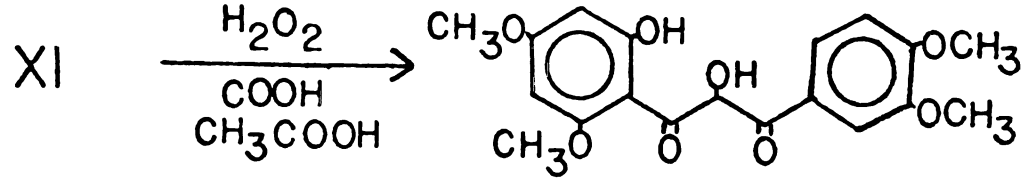
VIII IX

B.



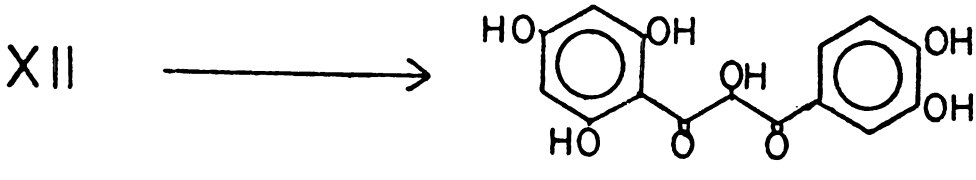
X XI

C.



XII

D.



XII

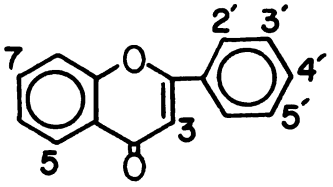
RESULTS

In Vivo Antitumor Activity

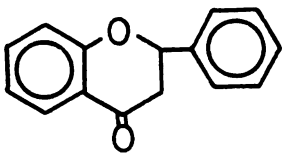
In vivo P_{388} leukemia growth is inhibited by a number of flavonoids. The data are summarized in Table 2 and the actual cell counts are given in Appendix I. The results show that all of the flavonols (XV-XX) were active at the dosage administered (150 mg/hg); but that apigenin, which lacks the 3-hydroxyl group, was inactive. The flavonols with trihydroxylated B-rings, such as myricetin and robinetin, are the best inhibitors, capable of decreasing tumor growth by 99%. The mono and dihydroxylated flavonols (XV-XVII) were less active, inhibiting growth by about 75%. The 5-hydroxyl function is not required, as evidenced by the effects of fisetin and robinetin. Taxifolin, saturated at the 2-3 position and possessing 3-hydroxyl and 4-keto groups, was moderately active; l-epicatechin, which lacks the keto function, did not inhibit tumor growth. Drug induced weight losses were less than 10% for all of the experimental groups.

Cellular Macromolecular Synthesis

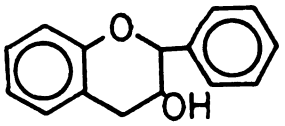
To determine whether inhibition of DNA, RNA, or protein synthesis could account for the *in vivo* anticancer properties of the flavonoids, the effects of several of these compounds on cellular macromolecular synthesis were investigated. Figure 7 shows substantially decreased ^{3}H-thymidine incorporation in P_{388} cells treated with myricetin (A) or quercetin (B). The inhibition, readily apparent by 10 minutes, increased to almost 75% by 50 minutes in the presence of 5×10^{-5} M myricetin. The response was dose-related, increasing the flavonol concentration to 1×10^{-4} M decreased ^{3}M-thymidine incorporation by



FLAVONES



FLAVONONES



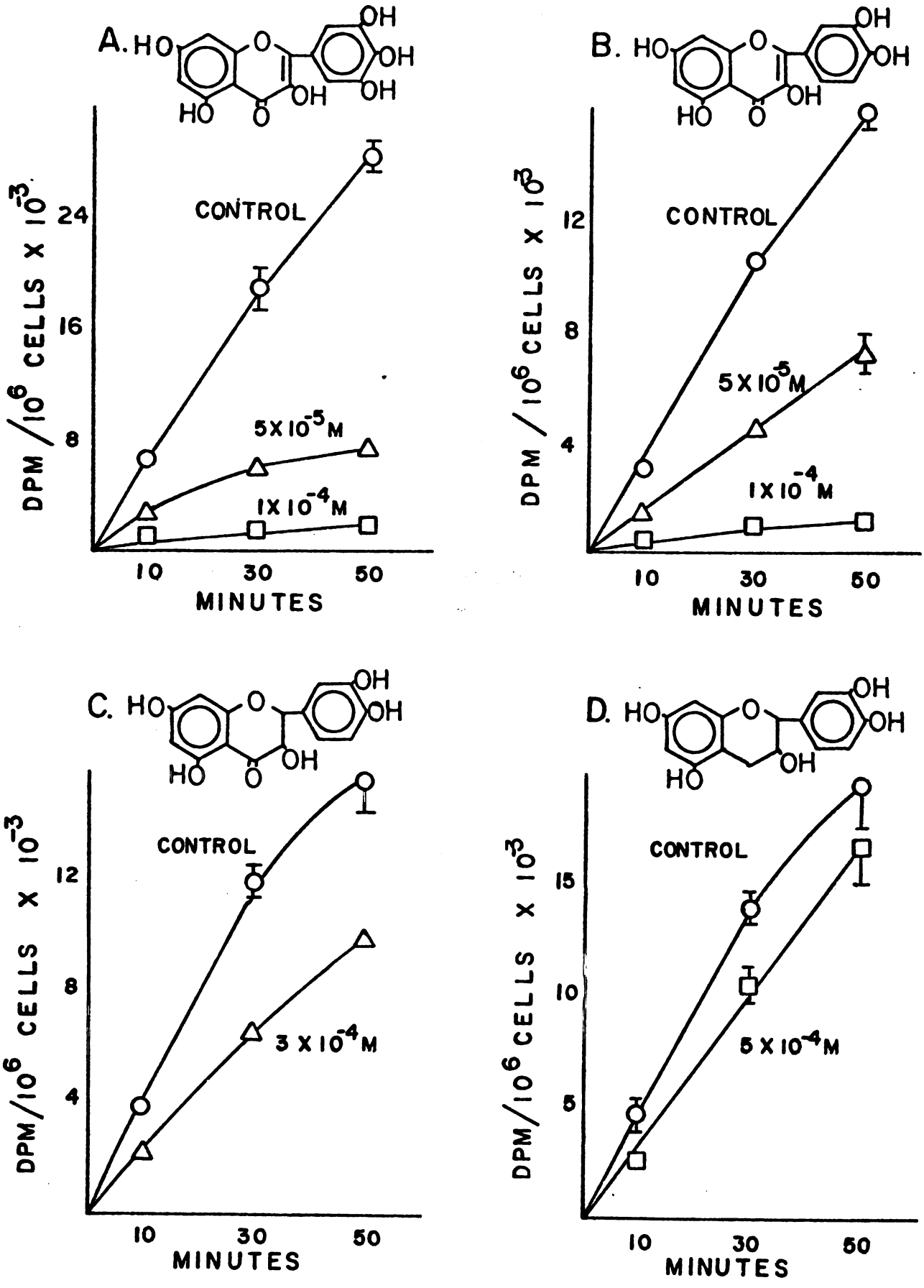
FLAVANOLS

COMPOUND NAME	NUMBER	HYDROXYLATION PATTERN	PERCENT INHIBITION OF P_{388} GROWTH		
			A	B	AVERAGE
FLAVONES					
Apigenin	(XIV)	5,7,4'	0	0	0
Kaempferol	(XV)	3,5,7,4'	79	75	77
Fisetin	(XVI)	3,5,3',4'	76	79	78
Morin	(XVII)	3,5,7,2',4'	71	45	58
Quercetin	(XVIII)	3,5,7,3',4'	58	87	70
Robinetin	(XIX)	3,7,3',4',5'	79	91	85
Myricetin	(XX)	3,5,7,3',4',5'	98	99	99
FLAVANONES					
Taxifolin	(XXI)	3,5,7,3',4'	82	72	78
FLAVANOLS					
l-Epicatechin	(XXII)	3,5,7,3',4'	12	6	9

TABLE 2: The effects of various flavonoids on the in vivo growth of P_{388} leukemia. BDF_1 mice were inoculated with 10^5 cells on day 0 and injected with a flavonoid(150 mg/kg) or saline on days 1 through 6. The cancer cells were collected and quantified on day 7. A and B are the results of the first and second assays of each compound.

FIGURE 7: The effects of myricetin(A), quercetin(B), taxifolin(C), and l-epicatechin(D) on ^{3}H-thymidine incorporation in P_{388} leukemia cells *in vitro*. The flavonoid and the thymidine(1.25 μCi/ml) were added to the cells and samples were taken 10, 30, and 50 minutes later. Each point represents the mean ± standard deviation of three independent samples (for those points without bars the standard deviation was less than the symbol size). The tritium counting efficiency was approximately 33%.

^{3}H-THYMIDINE INCORPORATION



over 90%. Quercetin inhibited DNA synthesis slightly less than myricetin. Dihydroquercetin (C), however, was clearly less effective than the flavonols, decreasing incorporation by about 40% at 3 X 10^{-4} M. 1-Epicatechin (D) was minimally active, even at 5 X 10^{-4} M.

The effects of myricetin on DNA synthesis are cumulative. As shown in Figure 8, incubation of P_{388} cells with the flavonol (1 X 10^{-4} M) for two hours prior to adding the ^{3}H-thymidine completely blocked its incorporation into acid precipitable polymers.

RNA synthesis was also altered by some of the flavonols (Figure 9). The effects are similar to, but less pronounced than, the inhibition of ^{3}H-thymidine incorporation. In particular, the higher concentrations (1 X 10^{-4} M) of myricetin (A) and quercetin (B), were less active. ^{3}H-uridine incorporation was decreased by about 30% and 20% by dihydroquercetin at 3 X 10^{-4} M (C) and 1-epicatechin at 5 X 10^{-4} M (D), respectively.

Protein synthesis was less sensitive than either DNA or RNA synthesis to inhibition by flavonoids. Figure 10 shows that both myricetin (A) and quercetin (B) at 1 X 10^{-4} M inhibit ^{3}H-leucine incorporation by less than 50%. In addition the effect is slower to appear, since at ten minutes quercetin did not inhibit protein synthesis. The presence of 3 X 10^{-4} M dihydroquercetin (C) and 5 X 10^{-4} M 1-epicatechin resulted in 23% and 35% reduction in ^{3}H-leucine incorporation, respectively.

Inhibition of Nucleic Acid Polymerases In Vitro

I_{50} values, as previously described, were used to compare the potencies of different compounds as enzyme inhibitors. Figure 11 illustrates the graphical procedure used to estimate I_{50}'s, presenting the data for inhibition of E. coli RNA polymerase and Rous sarcoma

FIGURE 8: Inhibition of ^{3}H-thymidine incorporation into DNA by myricetin. The drug was incubated with the P_{388} cells for two hours before adding the precursor(1.25 μCi/ml). Samples were removed at 20 minute intervals. Each point represents the mean ± standard deviation of three independent samples, which were counted at an efficiency of 33%.

^{3}H-THYMIDINE INCORPORATION

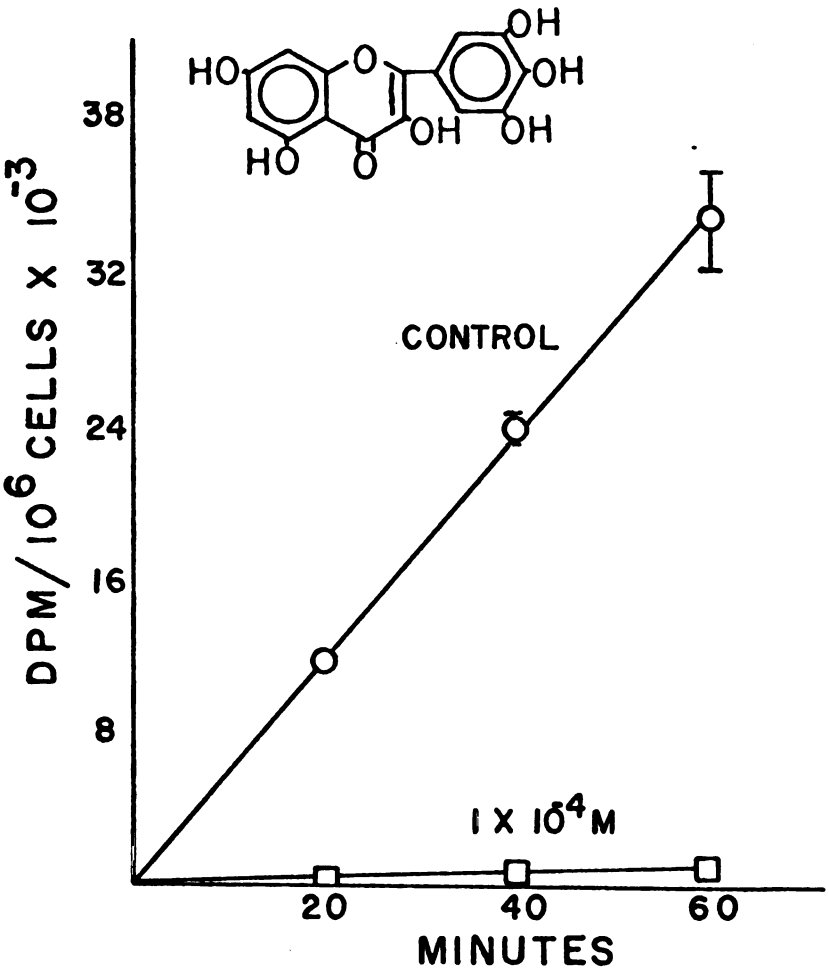


FIGURE 9: The effects of myricetin(A), quercetin(B), taxifolin(C), and l-epicatechin(D) on ^{3}H-uridine incorporation in P_{388} leukemia cells *in vitro*. The flavonoid and the uridine(1.25 μCi/ml) were added to the cells and samples were taken 10, 30, and 50 minutes later. Each point represents the mean ± standard deviation of three independent samples. The estimated counting efficiencies in these experiments were between 33 and 35%.

DPM/10^{6} CELLS X 10^{3}

^{3}H-URIDINE INCORPORATION

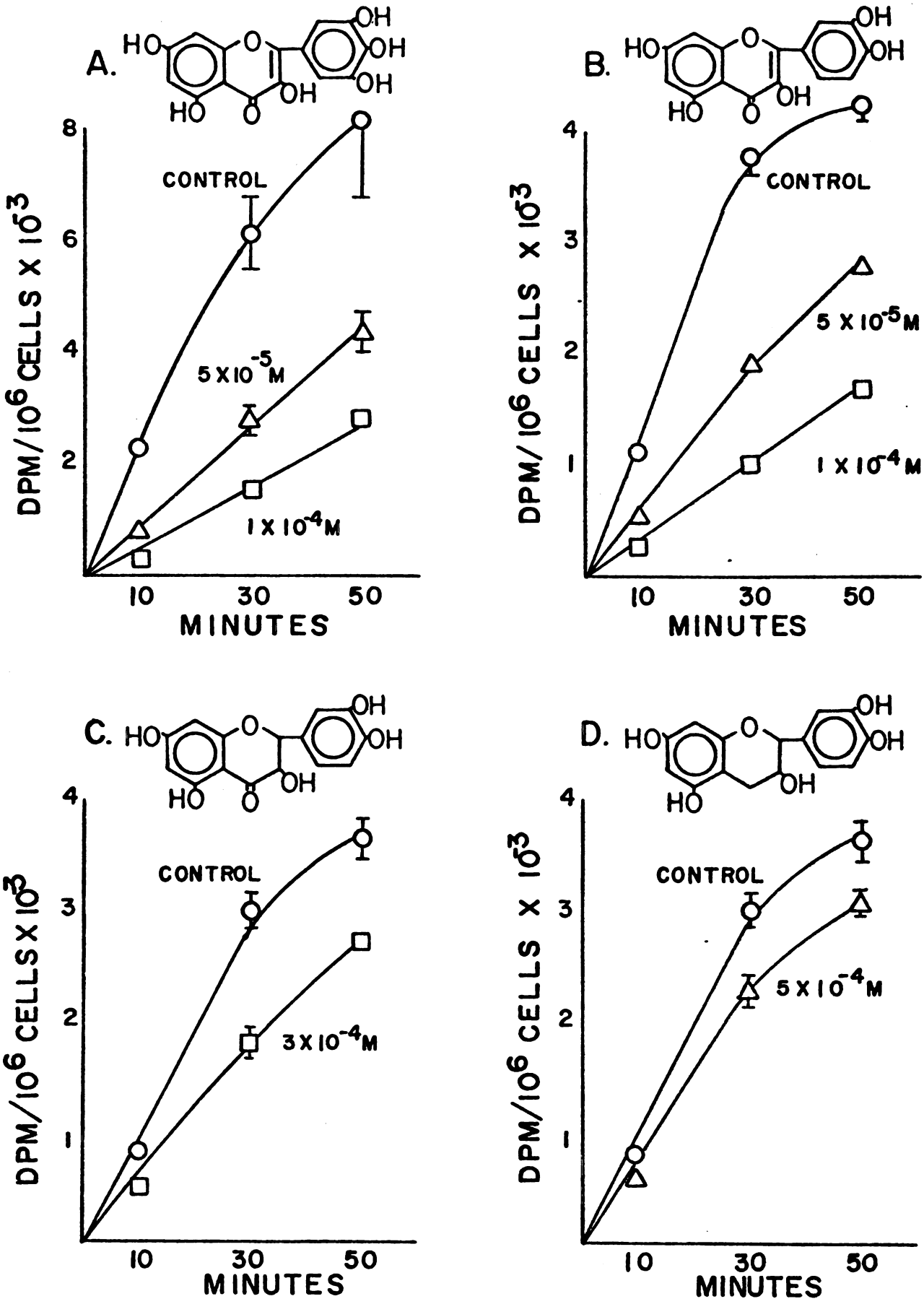


FIGURE 10: The effects of myricetin(A), quercetin(B), taxifolin(C), and l-epicatechin(D) on ^{3}H-leucine incorporation in P_{388} cells *in vitro*. The flavonoid and the leucine(1.25 μCi/ml) were added to the cells and samples were taken 10, 30, and 50 minutes later. Each point represents the mean ± standard deviation of three independent samples. The estimated counting efficiencies in these experiments were between 33% and 35%.

^{3}H- LEUCINE INCORPORATION

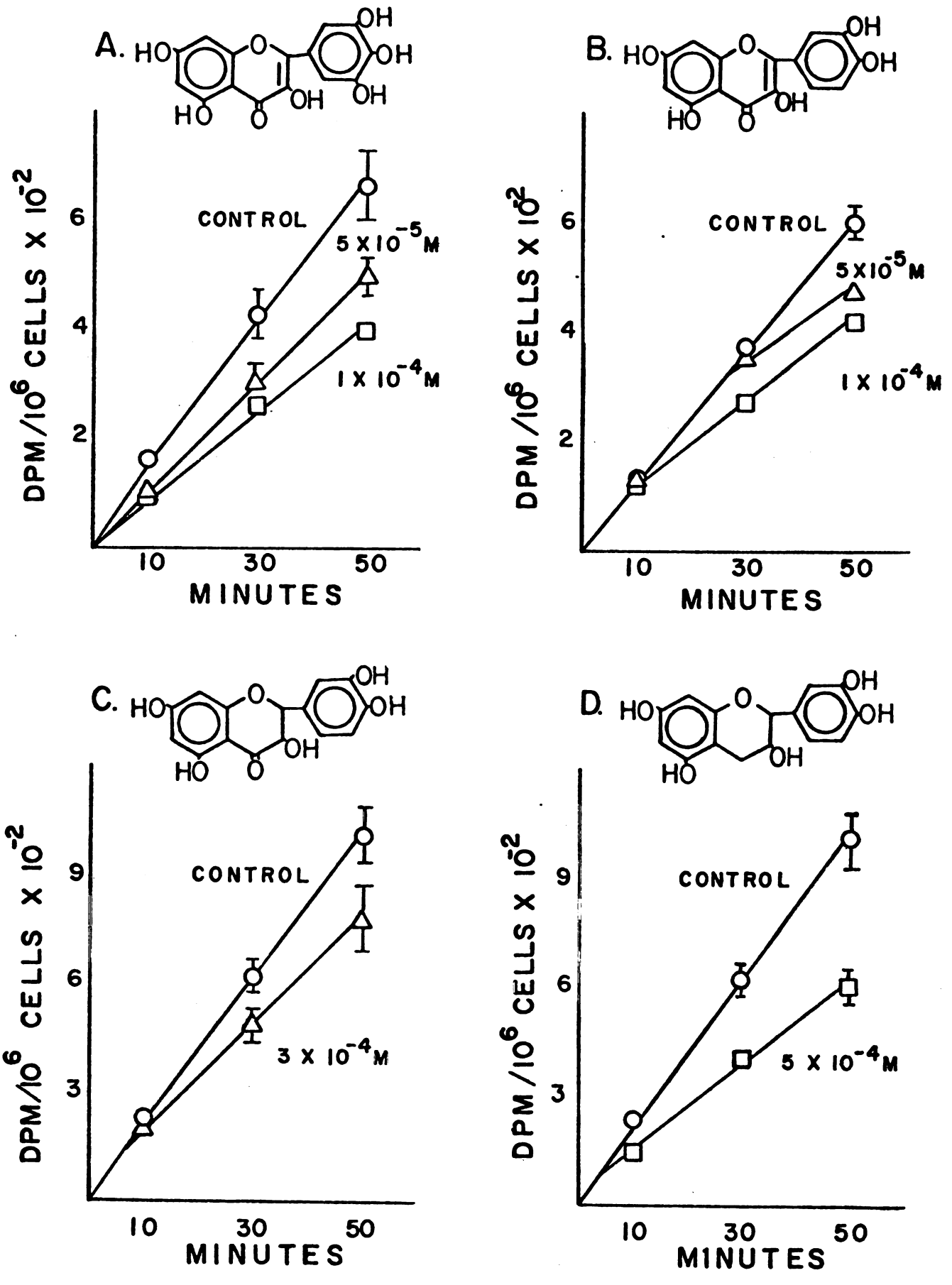
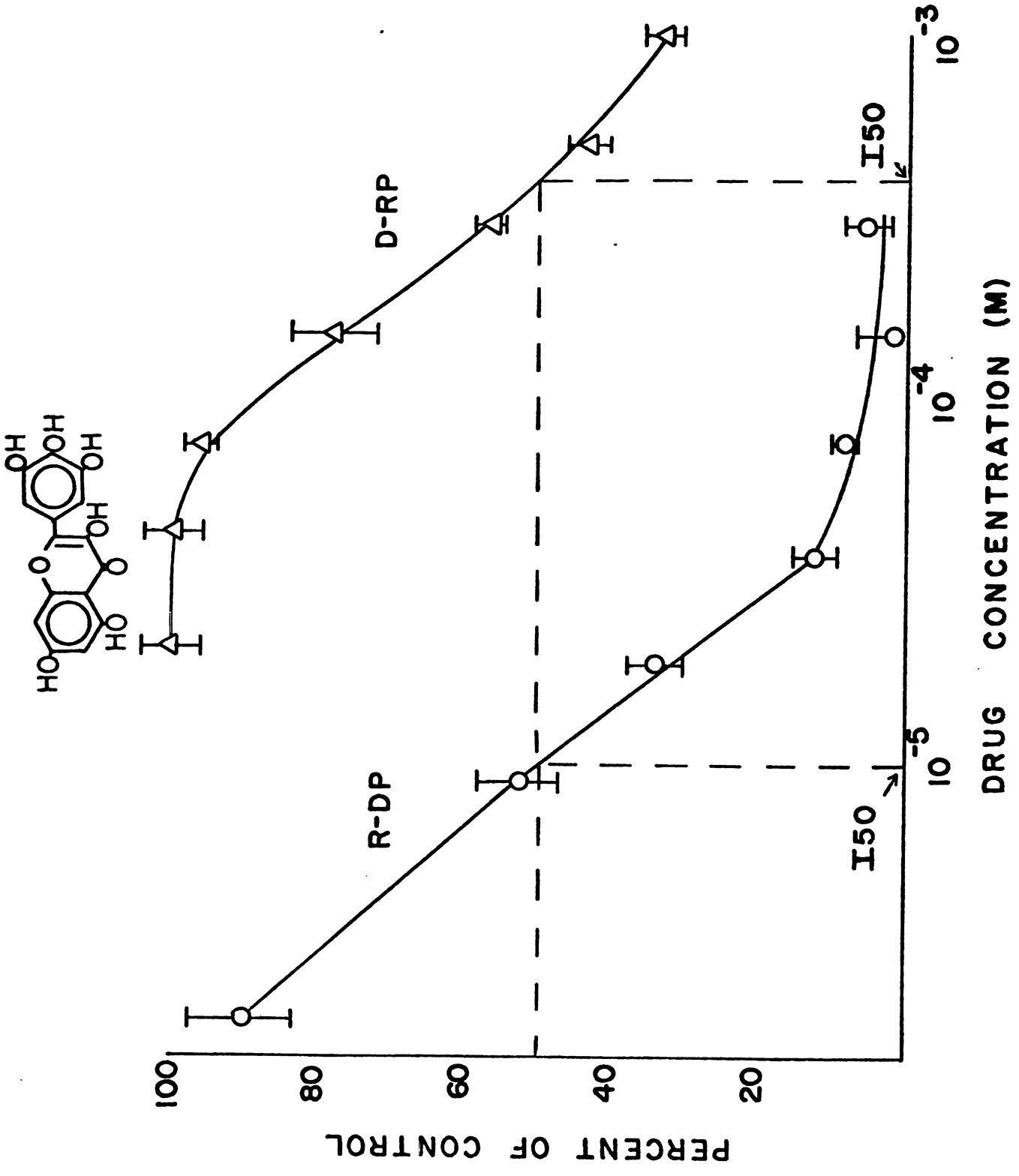


FIGURE 11: Estimation of I_{50} values for myricetin inhibition of RNA-dependent DNA polymerase(RDP) and DNA-dependent RNA polymerase(DRP). The intercept of the enzyme activity curve and the 50% inhibition point determines the I_{50} value. Each point represents the mean ± standard deviation of three independent measurements. The control values averaged about 2,000 cpm, at a tritium counting efficiency of 35%, in the RDP assay and about 2,500 cpm, at an efficiency of 32%, for the DRP reaction.


PERCENT OF CONTROL
100
80
60
40
20
DRUG CONCENTRATION (M)
10-3
10-4
10-5
I50
I50
D-RP
R-DP
OH
OH
OH
OH
OH
OH
O
O

virus DNA polymerase by myricetin as an example. Each point on the graph represents the mean ± standard deviation of three determinations.

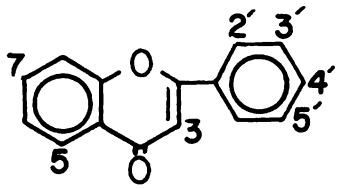
Table 3 summarizes the effect of various flavonoids on RDP and DRP enzyme activities; representative enzyme inhibition curves are presented in Appendix II (Figures 25-29). Some flavones (XV-XX), but none of the flavanones (XXI, XXIV, XXV), were effective inhibitors, indicating that unsaturation at the 2-3 position is necessary for the activity. All of the active flavones (XV-XX) possess hydroxyl groups at both the 3 and 4' positions. The most potent RDP inhibitors (XIX and XX) were hydroxylated at the 3', 4', and 5' positions of the B-ring; 3', 4' dihydroxylated flavonols (XVI-XVIII) were less active. The position of the B-ring hydroxyl groups seems to be critical as the 2', 4' flavonol (XVII) was found to be 30 times less effective than its 3', 4' isomer (XVIII) as an inhibitor of RDP.

The flavyliums (XVII-XXVII) are also good inhibitors. In these compounds the oxygen ring is unsaturated and hydroxyl groups are present at the 3 and 4' positions. DRP inhibition increases as the number of B-ring hydroxyls increases.

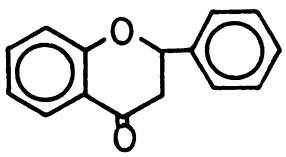
Figure 12 shows the effect of heterocyclic ring saturation on RDP inhibition by structurally related flavonoids. The activities of both the flavonol (XVIII) and flavylium (XXVII) are markedly greater than their saturated analogs, the dihydroflavonol (XXI) and flavonol (XXII), respectively.

The 3-Hydroxyl Group and Water Solubility

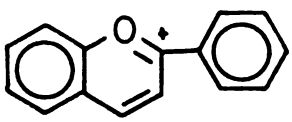
Low water solubility, limiting the effective drug concentration, may influence enzyme inhibition. To determine if the 3-hydroxyl is necessary for activity because it increased water solubility or whether it has a specific structural significance, DMSO was added to a 5% concentration in the DRP assay. Under these conditions water



FLAVONES



FLAVONONES



FLAVYLIUMS

COMPOUND NAME	NUMBER	HYDROXYLATION PATTERN	I_{50} X 10^{-5} M	
			RDP	DRP
FLAVONES				
Flavonol	(I)	3	Inactive	Inactive
Chrysin	(XXIII)	5,7	Inactive	Inactive
Apigenin	(XIV)	5,7,4'	Inactive	Inactive
Kaempferol	(XV)	3,5,7,4'	100+	100+
Fisetin	(XVI)	3,7,3',4'	5	30
Morin	(XVII)	3,5,7,2',4'	60	40
Quercetin	(XVIII)	3,5,7,3',4'	2	55
Robinetin	(XIX)	3,7,3',4',5'	0.9	50
Myricetin	(XX)	3,5,7,3',4',5'	1	40
FLAVONONES				
Naringenin	(XXIV)	5,7,4'	Inactive	Inactive
Fustin	(XXV)	3,7,3',4'	Inactive	Inactive
Taxifolin	(XXI)	3,5,7,3',4'	Inactive	Inactive
FLAVYLIUMS				
Pelargonidin	(XXVI)	3,5,7,4'	0.9	15
Cyanidin	(XXVII)	3,5,7,3',4'	2	5.5
Delphinidin	(XXVIII)	3,5,7,3',4',5'	-	3.5

TABLE 3: In vitro inhibition of RDP and DRP by some flavonoids. I_{50} values represent the drug concentration required to inhibit the control reaction by 50%. Compounds were considered inactive if inhibition was less than 50% at 1.5 mM.

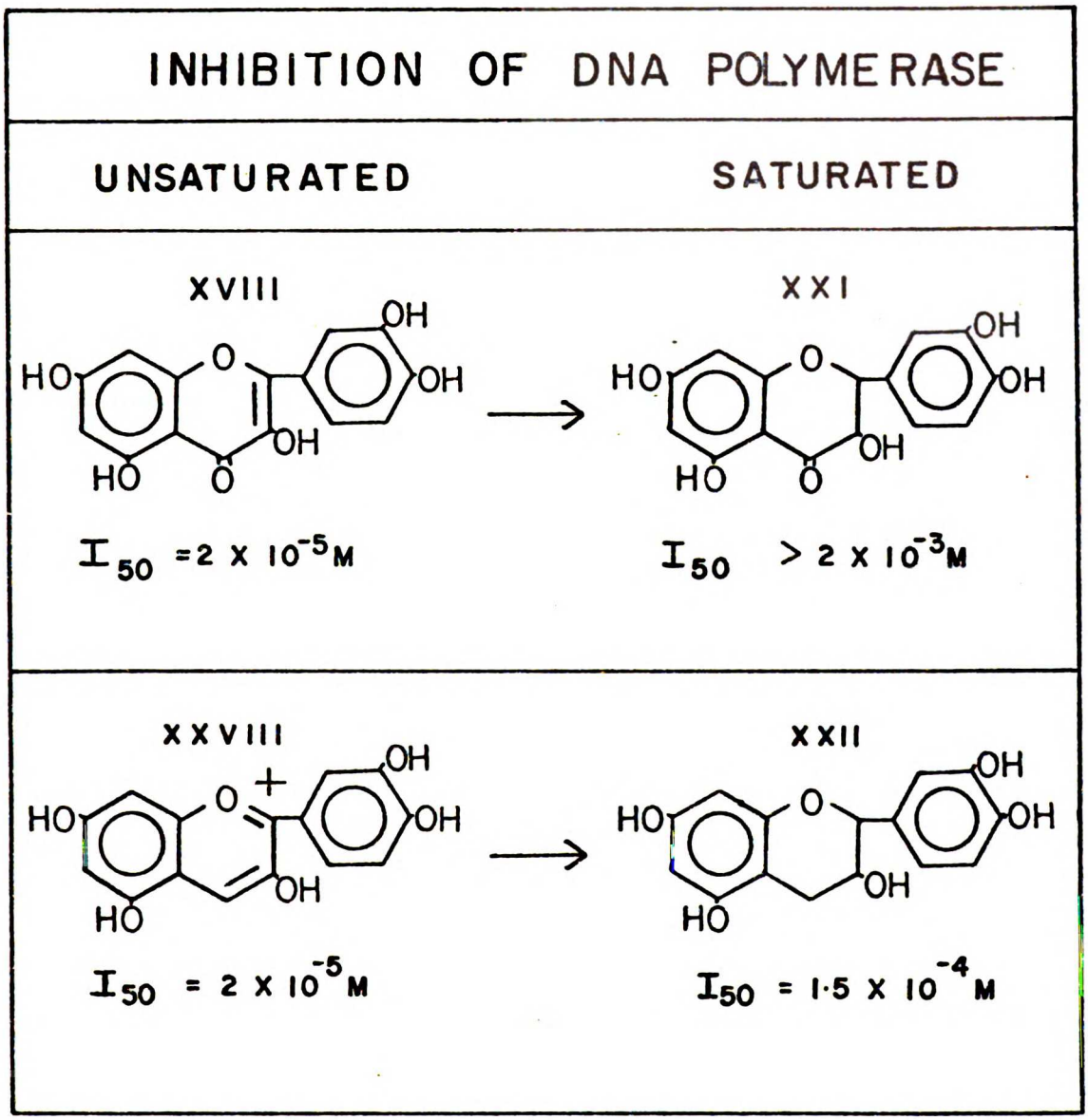


FIGURE 12: A comparison of flavonoids saturated and unsaturated in the C-ring as inhibitors of RNA-dependent DNA polymerase *in vitro*. I_{50} values are the drug concentration required to inhibit the control reaction by 50%.

insolubility was not a limiting variable and, as shown in Table 4, apigenin was inactive whereas its 3-hydroxylated analog (XV) was inhibitory. Thus, in addition to any effects on water solubility, this functional group appears to have a special importance. Quercetin and myricetin are more water soluble than kaempferol and the enhancement of polymerase inhibition by solubilization in DMSO was less dramatic. None of the otherwise inactive flavonoids were found to be inhibitory at the higher DMSO concentrations.

Hydroxyl Group Ionization

Why are the 3 and 4' hydroxyls and unsaturation of the middle ring so important for flavonoid inhibition of the polynucleotide polymerases? Ionization of hydroxyl groups, which might influence the interaction of flavones with the polynucleotide-enzyme complex, was studied since RDP, which was assayed under more alkaline conditions than DRP, was considerably more sensitive to flavonol inhibition. The bathochromatic shift in the UV spectra of many flavonoids, which accompanies an increase in and is associated with hydroxyl group ionization (77,78), could be utilized in this regard. The UV spectra of flavonols at pH 5.2 or in neutral ethanol are characterized by two major λ maxima, band I at about 320-380 nm and band II in the 240-290 nm region. In dihydroflavanols the B-ring is not conjugated with the carbonyl group and, consequently, absorbance is greatest from 270-290 nm (band II) with an inflection of low intensity at about 320-330 nm (band I). Figures 13 and 14 show the UV spectra for two inactive compounds, a dihydroflavonol and a flavone, respectively; the UV spectra for two active flavonols are presented in Figures 15 and 16. The spectra were recorded at pH 5.2 and pH 7.6 in order to compare the patterns of hydroxyl group ionization.

COMPOUND		I_{50} X 10^{-5} M	
NAME	NUMBER	1% DMSO	5% DMSO
FLAVONES			
Flavonol	(I)	Inactive	Inactive
Chrysin	(XXIII)	Inactive	Inactive
Apigenin	(XIV)	Inactive	Inactive
Kaempferol	(XV)	100	26
Quercetin	(XVIII)	55	24
Myricetin	(XX)	40	18
FLAVONONES			
Naringenin	(XXIV)	Inactive	Inactive
Fustin	(XXV)	Inactive	Inactive
Taxifolin	(XXI)	Inactive	Inactive

TABLE 4: A comparison of E. coli RNA polymerase inhibition in vitro by flavonoids in 1% or 5% dimethylsulfoxide. I_{50} values represent the drug concentration required to inhibit the control reaction by 50%. Compounds were considered inactive if inhibition was less than 50% at 1.5 mM. (See Table 3 for the structure of each compound.)

FIGURE 13: The UV spectra of taxifolin (4×10^{-5} M), a dihydroflavonol, in an aqueous solution at pH 5.2 (dashed line) and at pH 7.6 (solid line).

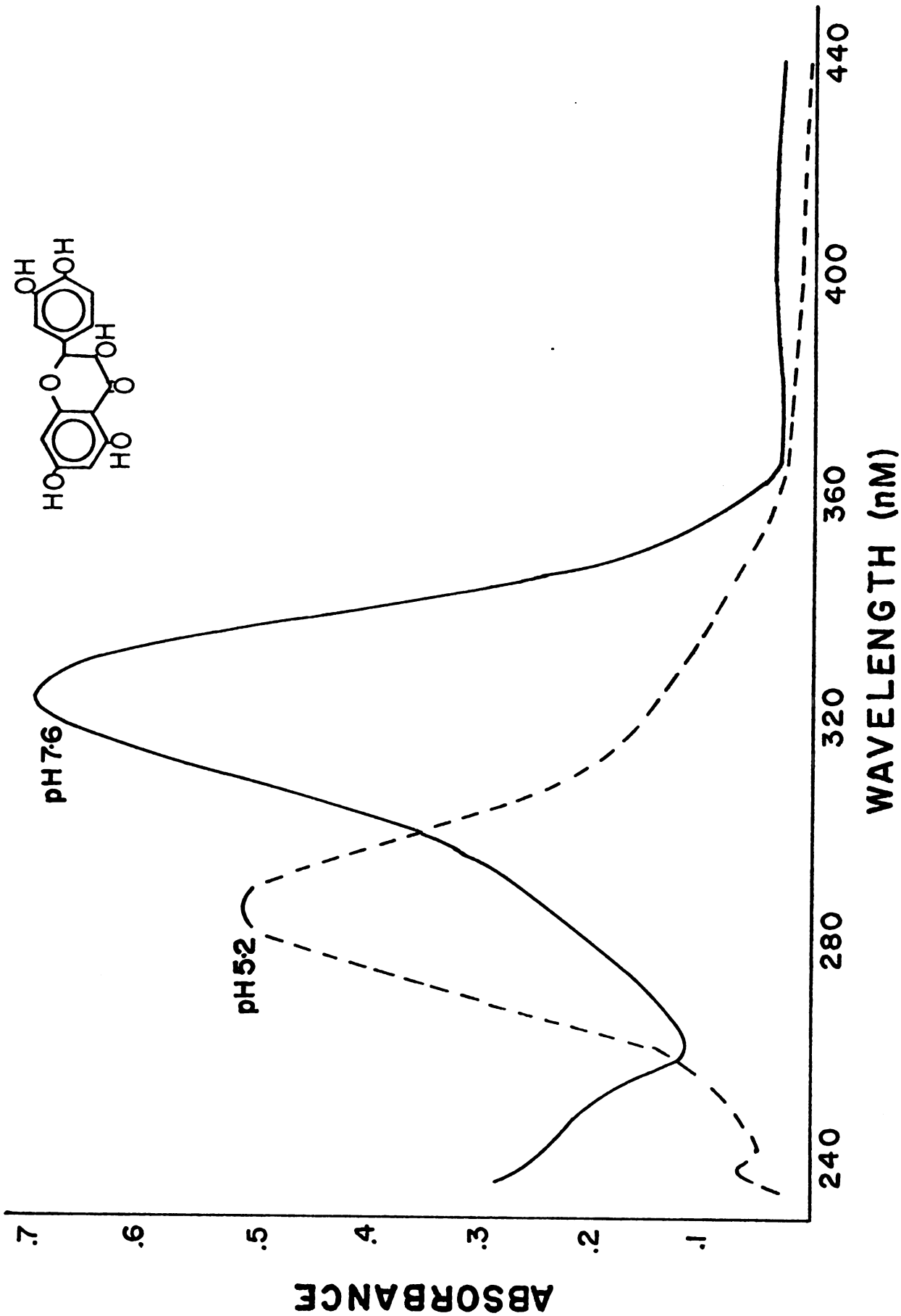

ABSORBANCE
.7
.6
.5
.4
.3
.2
.1
240
280
320
360
400
440
WAVELENGTH (nM)
pH 7.6
pH 5.2
OH
HO
O

FIGURE 14: The UV spectra of apigenin (4 X 10^{-5} M), a flavone, in an aqueous solution at pH 5.2 (dashed lined) and at pH 7.6 (solid line).

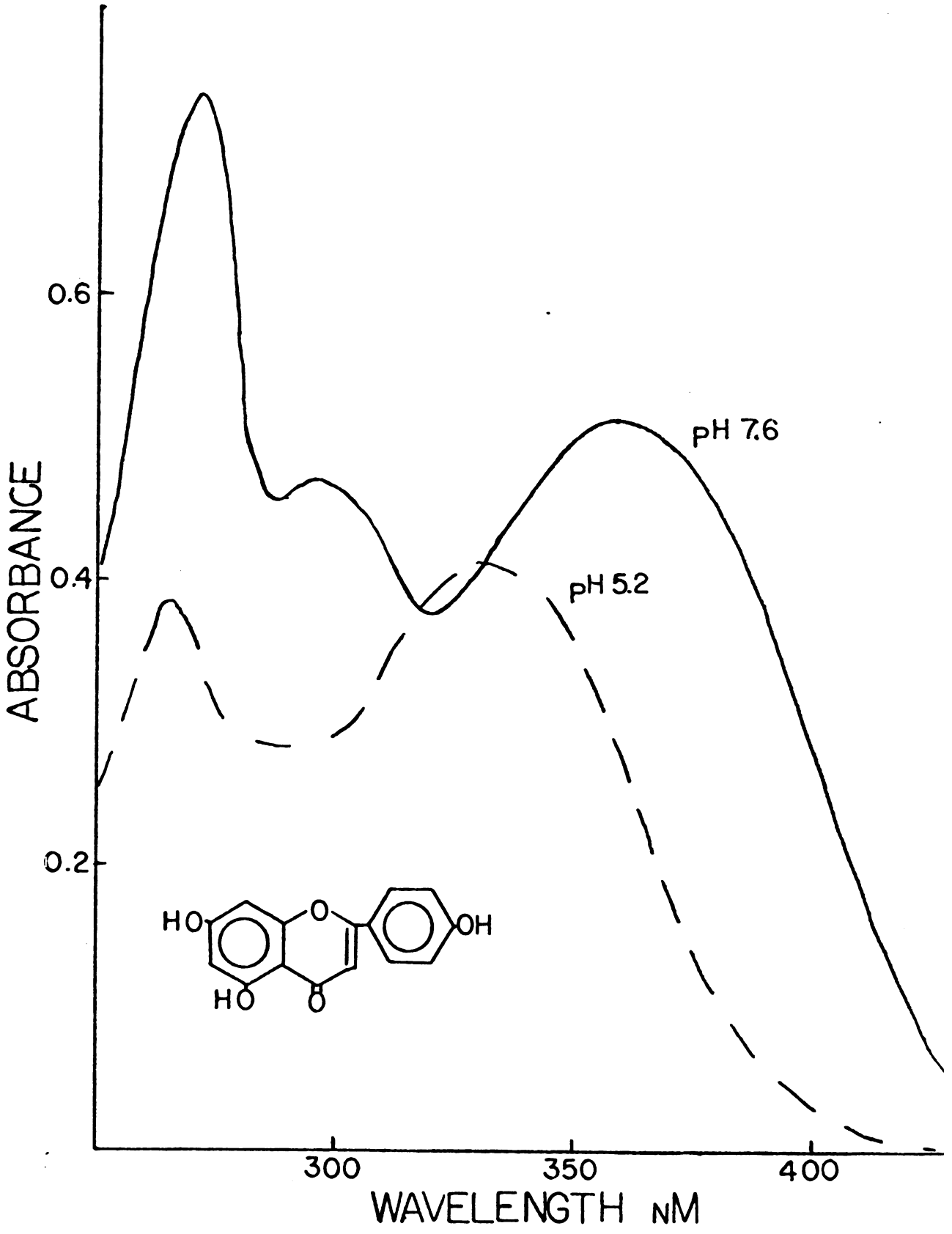

ABSORBANCE
0.6
0.4
0.2
pH 7.6
pH 5.2
HO
O
OH
HO
O
300
350
400
WAVELENGTH nM

FIGURE 15: The UV spectra of myricetin (4×10^{-5} M), a flavonol, in an aqueous solution at pH 5.2 (dashed line) and at pH 7.6 (solid line): the characteristic spectral changes of 3,4'-dihydroxy-flavones in alkali were recorded at 1, 4, 7, and 20 minutes.

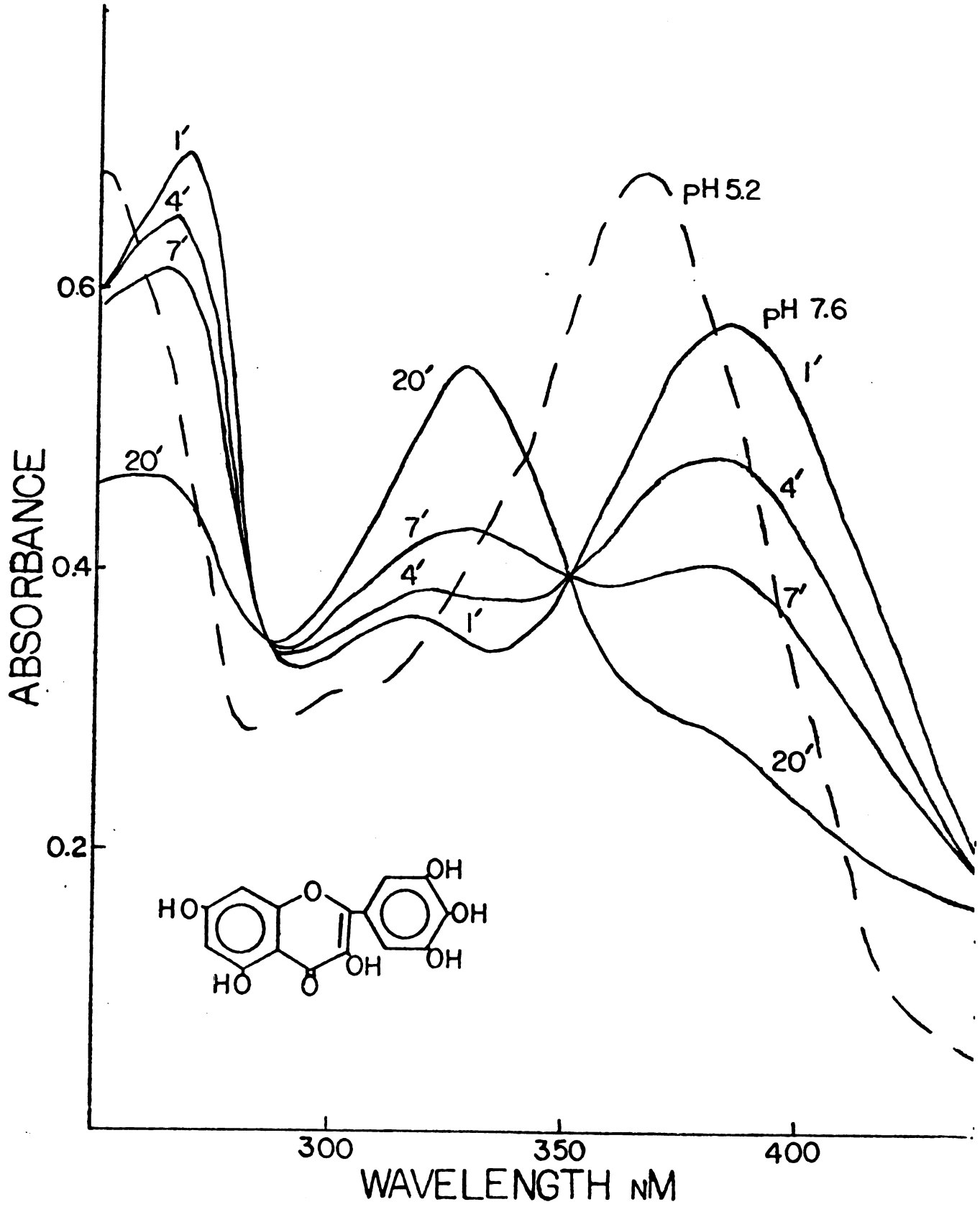
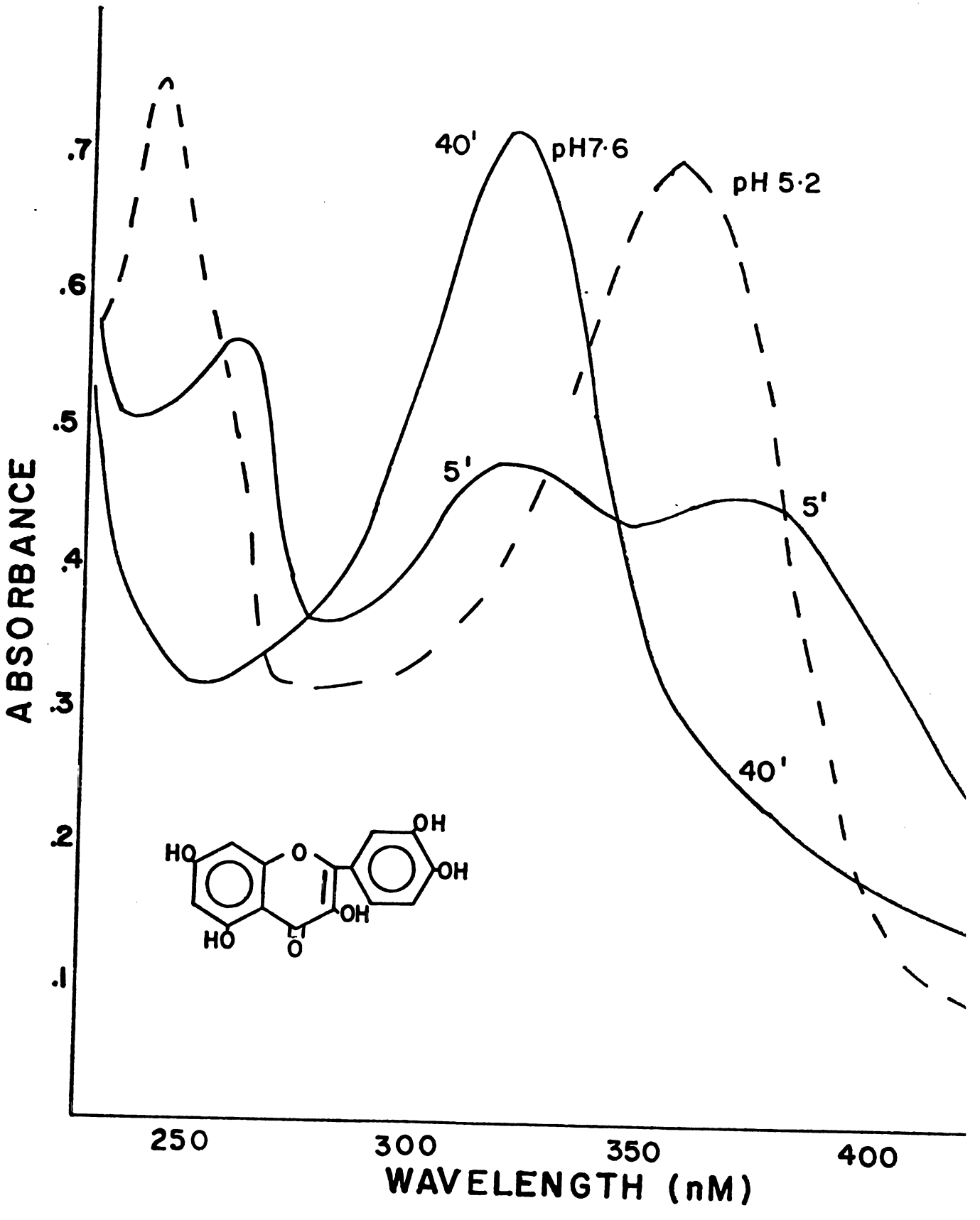

ABSORBANCE
0.6
0.4
0.2
300
350
400
WAVELENGTH NM
pH 5.2
pH 7.6
1'
4'
7'
20'
HO
O
OH
OH
OH
HO
O
OH

FIGURE 16: The UV spectra of quercetin (4×10^{-4} M), a flavonol, in an aqueous solution at pH 5.2 (dashed line) and pH 7.6 (solid lines): the spectral changes were recorded at 5 and at 40 minutes.


40'
pH7·6
pH 5·2
5'
5'
40'
ABSORBANCE
.7
.6
.5
.4
.3
.2
.1
HO
O
OH
OH
OH
HO
O
250
300
350
400
WAVELENGTH (nM)

Since bathochromatic shifts were evident in all four compounds, ionization does not appear to discriminate between active and inactive compounds.

However, the spectra of the enzyme inhibiting flavonols, i.e., those with the 3 and 4'-hydroxyl groups (XV-XX), but not those of inactive compounds, vary with time in an aqueous solution at pH 7.6. Note in Figures 15 and 16 that new λ maxima developed at 325 nm and 332 nm, respectively, as the spectra of the parent flavonols disappeared. The spectra of the inactive flavone and dihydroflavonol, Figures 13 and 14, remained stable. All of the flavonols capable of enzyme inhibition undergo similar spectral shifts with a new λ maximum characteristically appearing at about 330 nm (Table 5). Acidification of the alkaline solution with dilute hydrochloric acid did not regenerate the spectrum of the parent flavonol but resulted in the appearance of a new band at 295 nm, which, as shown in Figure 17, is consistent with protonation of the ionized hydroxyl groups of the new species. These results suggest that only those flavonols which are polymerase inhibitors undergo a chemical reaction at the pH of the enzyme assay.

Which Form Is Active in the Transcription Assay?

To determine whether the parent flavonol or the alkaline reaction product was responsible for the enzyme inhibition, the active flavonol, robinetin (XIX), was incubated at pH 7.6 for up to 200 minutes prior to assaying against DRP. If the parent compound is active, then enzyme inhibition should decrease with time, whereas the converse should hold if a reaction product is the active compound. Figure 18 indicates that polymerase inhibition increased with time. UV analysis of the flavonol solution revealed increasing amounts of an intermediate, reflected by a

λ MAXIMA (nM)							
COMPOUND	pH 5.2			pH 7.6			
	0 to 60 Min			5 Min		30 Min(New)	
Kaempferol	262		360	272	375		337
Fisetin	245	315i	362	270i	381		342
Morin	261		385	270	395		332
Quercetin	254		365	272	385		332
Robinetin	245	319i	360		375		342
Myricetin	252		365	265	385		325

TABLE 5: The λ maxima of some flavonoids capable of polynucleotide polymerase inhibition. Several UV spectral recordings (230 nm to 450 nm) were made for each compound over a 60 minute period in distilled water (pH 5.2) and 50 mM tris buffer (pH 7.6). (i = inflection point)

FIGURE 17: UV spectra of myricetin (5×10^{-5} M): 1) after 20 minutes at pH 7.6 (solid line), and 2) acidification of the pH 7.6 solution to pH 5 (dashed line).

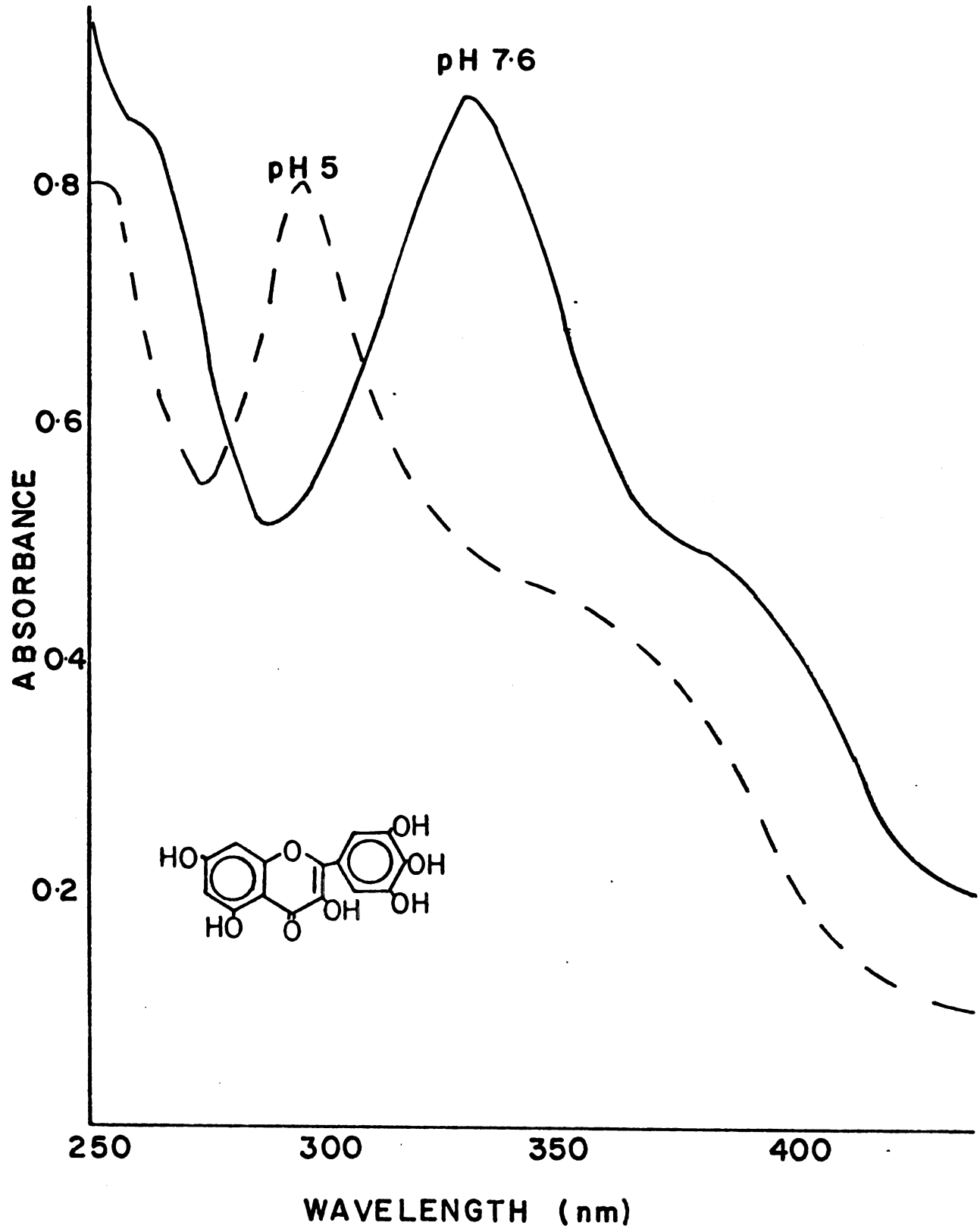
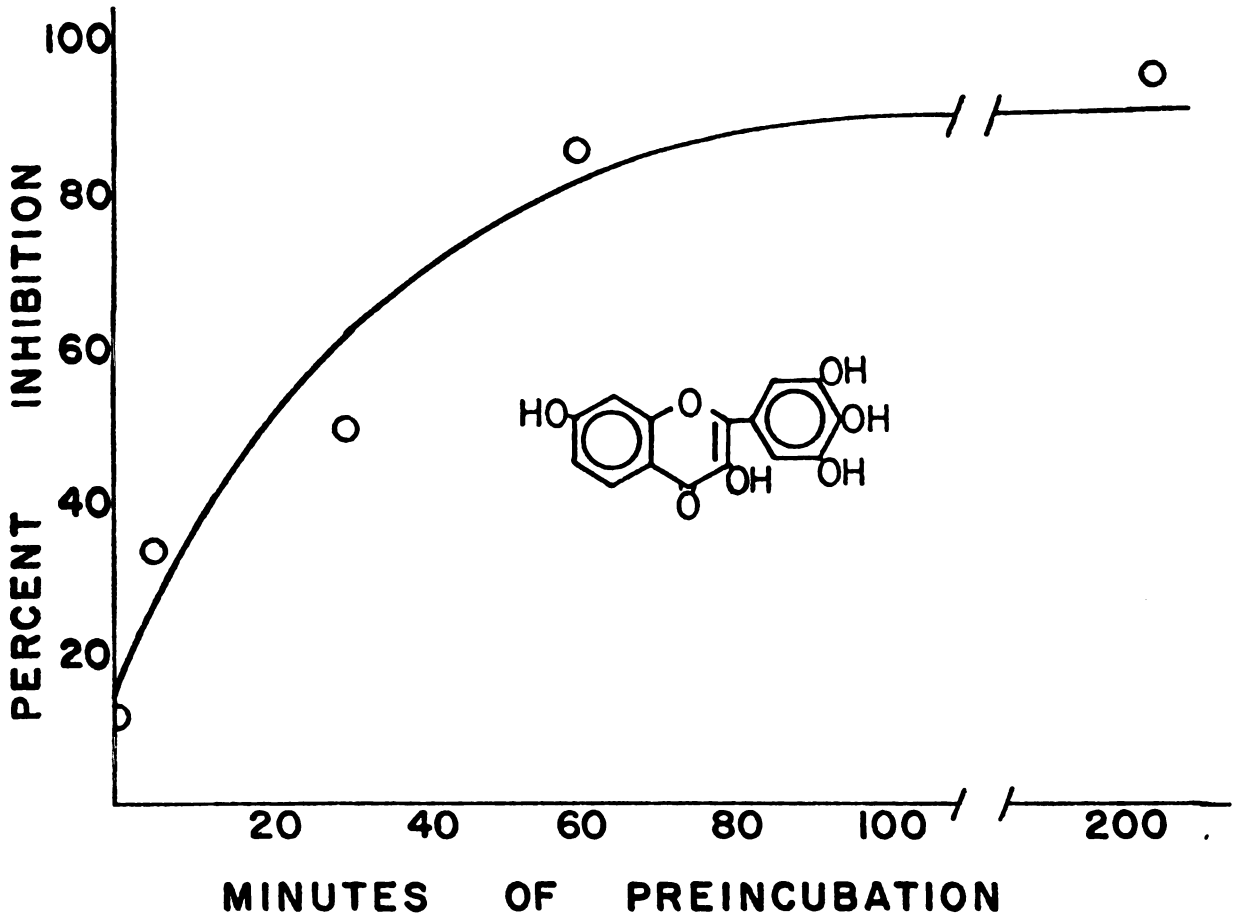

pH 7·6
pH 5
0·8
0·6
0·4
0·2
ABSORBANCE
250
300
350
400
WAVELENGTH (nm)
HO
O
OH
OH
OH
HO
O
OH

FIGURE 18: The effect of preincubating robinetin at pH 7.6 on the inhibition of DNA-dependent RNA polymerase. The drug was mixed in a tris buffered solution at 10^{-3} M and then samples were removed, acidified to pH 6, and subsequently assayed. The controls were treated similarly, but lacked the flavonol.

RNA POLYMERASE INHIBITION
DRUG PREINCUBATION AT pH 7·6



spectral shift to the new λ max (342 nm), as a function of pre-incubation time.

pH Dependence of the UV Spectral Change

To determine if the differences in pH of the enzyme assays could account for the greater sensitivity of RDP to flavonol inhibition, the pH dependence of the breakdown reaction was studied. UV spectral changes were followed by monitoring the decrease in absorbance of the band I λ max of several flavonols at pH 7.6, pH 8.0, and pH 8.5. Figure 19 illustrates that the reaction is pH dependent, being more rapid at higher pH's, but the differences in reaction rates are insufficient to explain the selective inhibition of the viral enzyme.

Oxygen Dependence of UV Spectral Changes

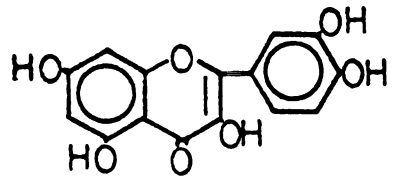
The appearance of a new λ max in the 330 nm region of the UV spectrum at pH 7.6 is characteristic of the active flavonols. This suggests that the reaction may involve saturation of the 2-3 double bond and the consequent disruption of conjugation between the carbonyl and the B-ring group. Three of the possible pathways of flavonol breakdown in alkali, which will be discussed later, are shown in Figure 20. Determination of the oxygen dependence of the reaction should help to clarify the importance of the different possibilities. The results of experiments comparing flavonol breakdown rates in the presence of sodium dithionite, an antioxidant, and in nitrogen enriched, oxygen enriched, and normal atmospheres are presented in Figure 21. Sodium dithionite and nitrogen blocked the irreversible shifts, whereas an oxygen atmosphere enhanced the rate of reaction, indicating that the reaction is highly dependent on oxygen.

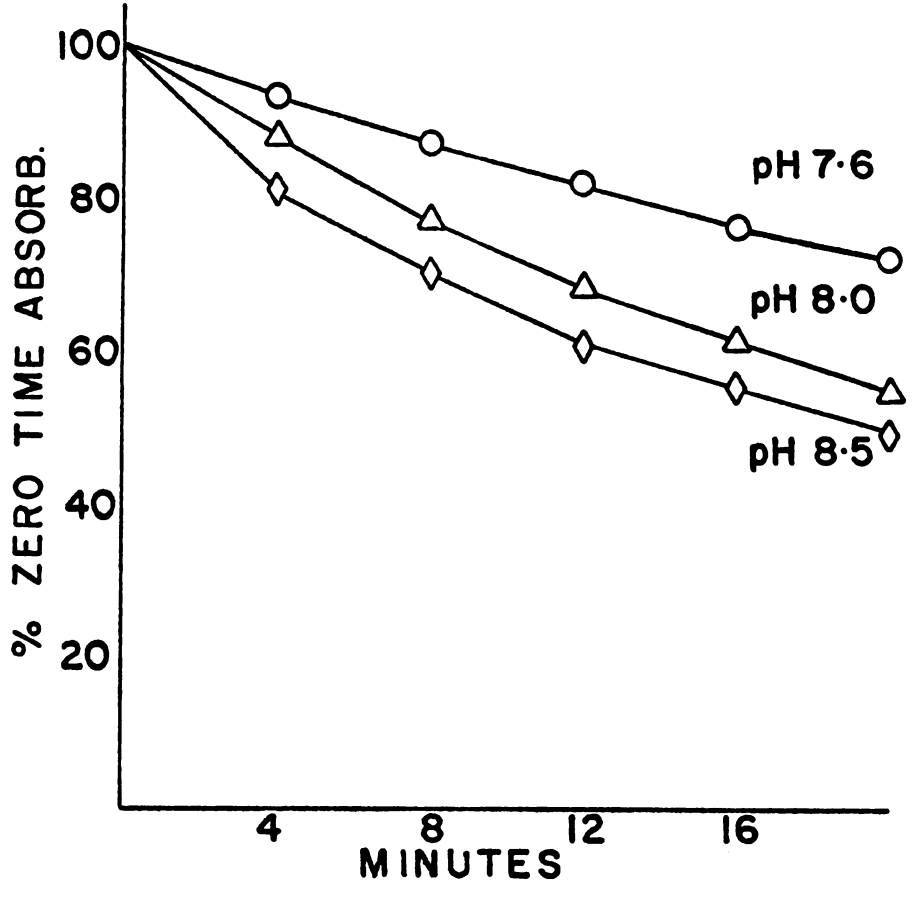
Is Flavonol Inhibition of Transcription Dependent on Oxygen?

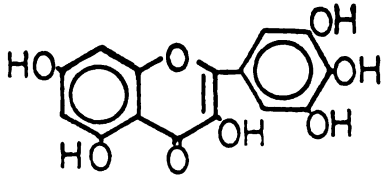
To determine if the oxygen-dependent reaction product of flavonols

FIGURE 19: The pH dependence of flavonol breakdown: Absorbance at the band I λ maxima of quercetin and myricetin at pH 7.6, 8.0, and 8.5 were monitored. The percent of zero time absorbance, a measure of breakdown, is plotted against time.

pH DEPENDENT FLAVONOL BREAKDOWN







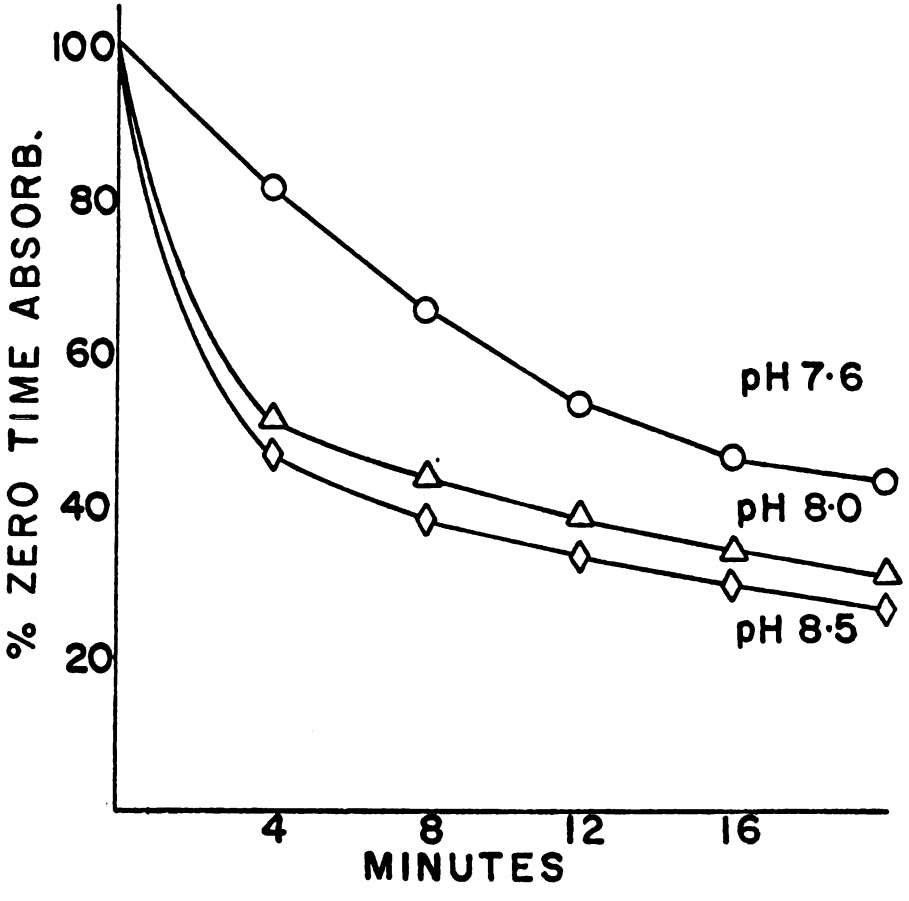


FIGURE 20: Possible pathways of flavonol breakdown (quercetin is used as an example): A) oxidation to an O-quinone, B) base catalyzed nucleophilic addition, C) oxidation of a flavandione followed by nucleophilic attack.

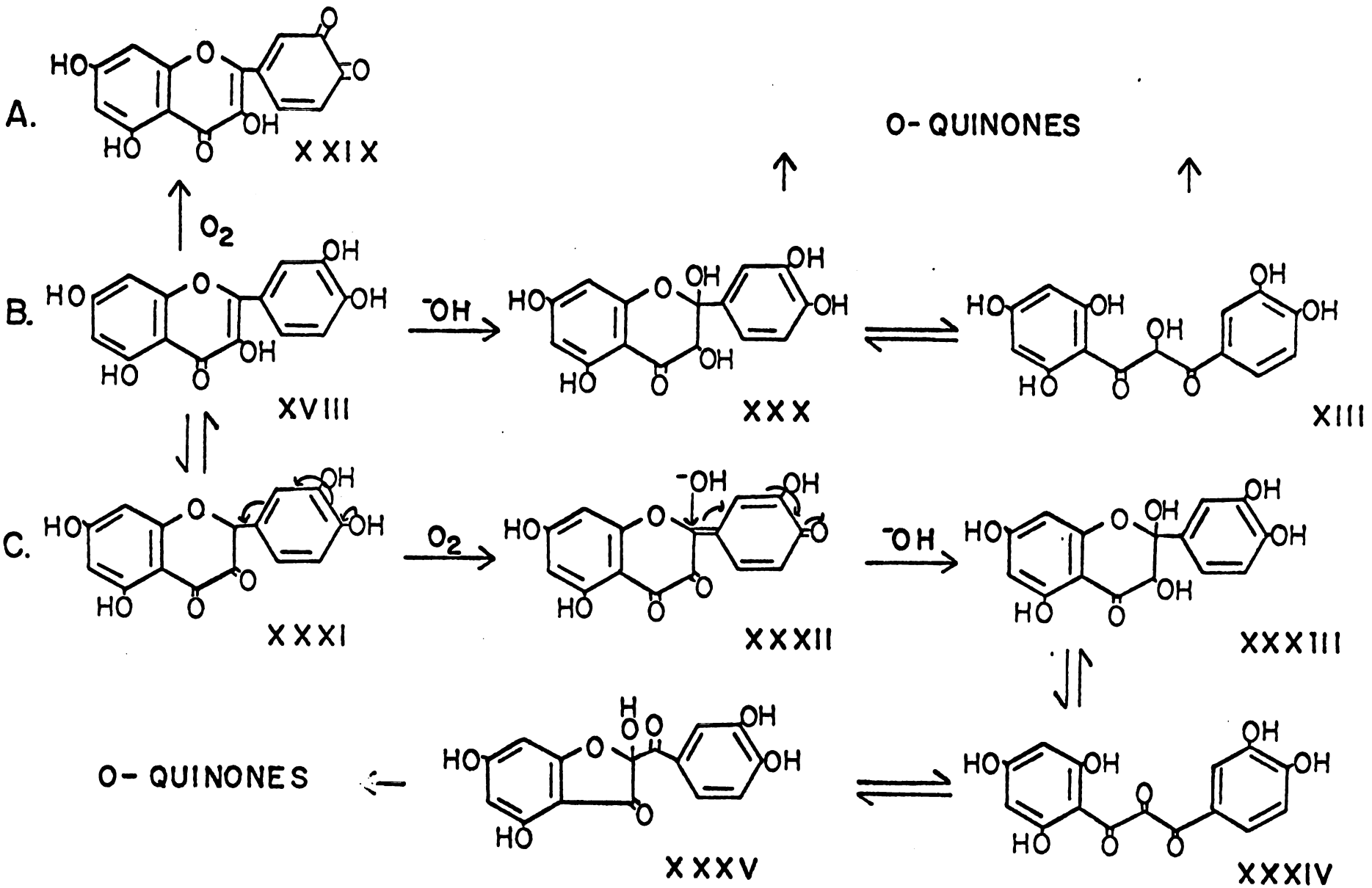
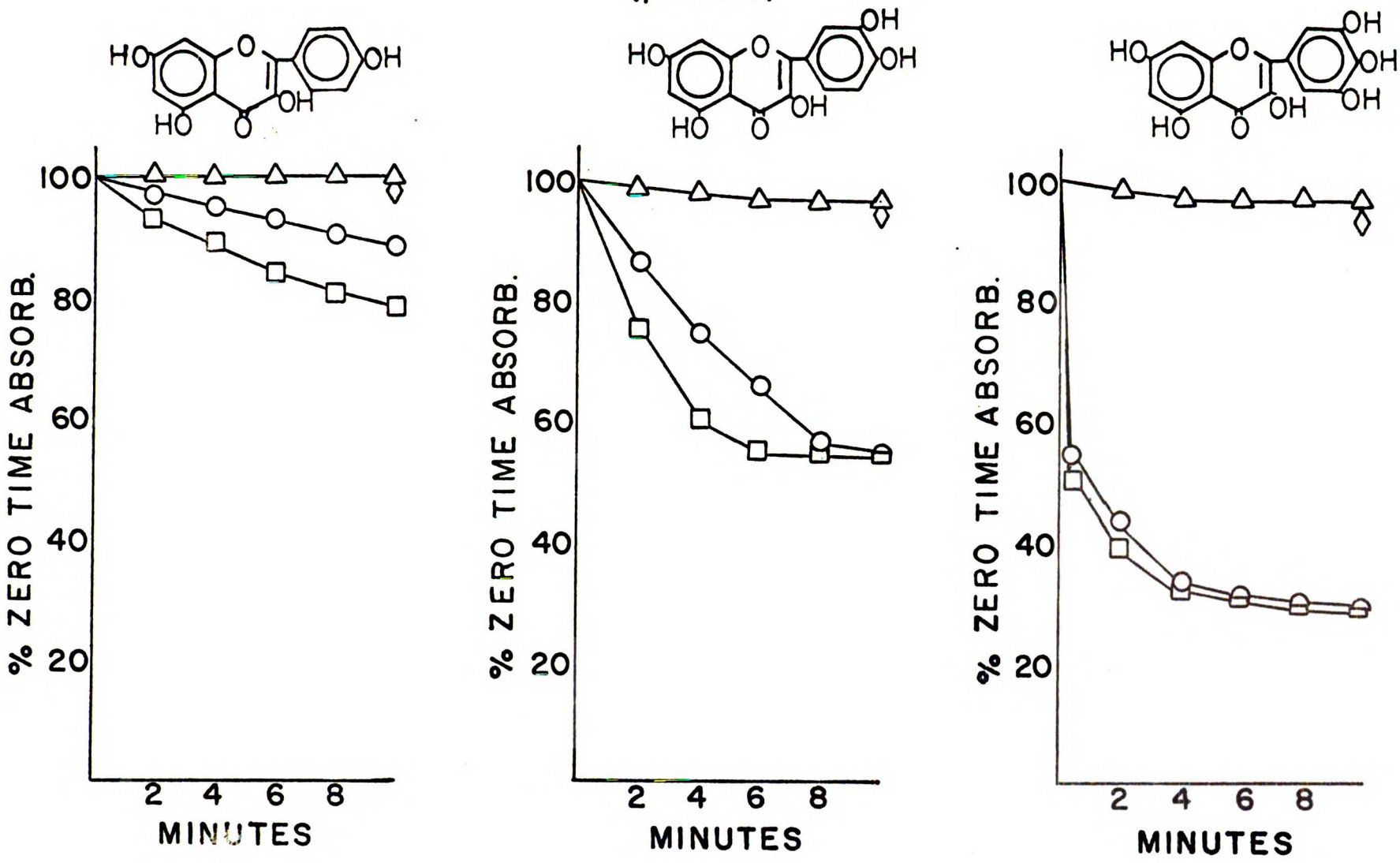

A.
B.
C.
XXIX
XVIII
XXXI
XXX
XXXII
XXXV
XIII
XXXIII
XXXIV
O- QUINONES
O- QUINONES
O2
O2
-OH
-OH

FIGURE 21: Oxygen dependent breakdown of kaempferol, quercetin, and myricetin at pH 8.5 in the presence of (△) 8 mM Na_2S_2 04, (◇) a nitrogen enriched atmosphere, (○) a normal atmosphere, and (□) an oxygen enriched atmosphere. The percent of zero time absorbance of band I maxima, a measure of flavonol breakdown, is plotted against time.

OXYGEN DEPENDENT FLAVONOL BREAKDOWN

(pH 8·5)



is required for polymerase inhibition, quercetin inhibition of DRP was compared under normal atmospheric conditions and in the presence of an oxygen-deficient, nitrogen-enriched atmosphere. Figure 22 shows that reduced oxygen tension greatly decreased enzyme inhbition, confirming the necessity of available oxygen for the reaction leading to maximal polymerase inhibition. The results document that the parent flavonol, even in high concentrations, is a poor enzyme inhibitor in the absence of O_2.

RDP Inhibition and the Rate of Flavonol Oxidation

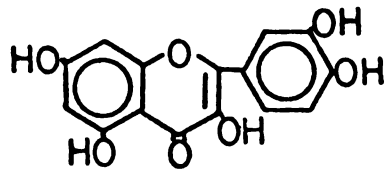
The relationship between the rate of flavonol oxidation to the active intermediate and the ability of flavonols to inhibit RDP is illustrated in Figure 23. The best inhibitors (VIII and IX) react most rapidly, moderately active drugs (V and VII) less rapidly, and the least effective inhibitor (VI) oxidizes most slowly.

Inhibition of DRP by Analogs of the Flavonol Reaction Product

Some compounds that resemble the possible breakdown products outlined in Figure 20 were synthesized and compared as DRP inhibitors (Table 6) 3-Hydroxyflavone (I), which does not undergo a change in UV spectrum at pH 7.6, is inactive. Analogs of the postulate intermediates (XIII and XXXIV), the carbinol (VII) and the propanetrione (II), contain a hydroxyl and a ketone group, respectively, at the 2-position (corresponding to the 3-position in flavonols) and are enzyme inhibitors. 2'-Hydroxychalcone (XXXVI) and O-hydroxydibenzoylmethane (VI), open chain structures lacking a substitution at the 2-position, are inactive. The ability of the intermediate analogs (VII and II) to inhibit DRP suggests that an oxygen function at the 2-position is critical. The low potency of these compounds indicates that the multiple hydroxyl groups of the active flavonols directly enhance enzyme inhibition as well as

FIGURE 22: The effect of a nitrogen enriched atmosphere on quercetin (4×10^{-4} M) inhibition of DNA-dependent RNA polymerase. Nitrogen was bubbled through the reaction mixture and drug solution (50% DMSO) before initiating the reaction with the substrates.

EFFECT OF N_2 ATMOSPHERE ON RNA POLYMERASE INHIBITION



4×10^{-4} M

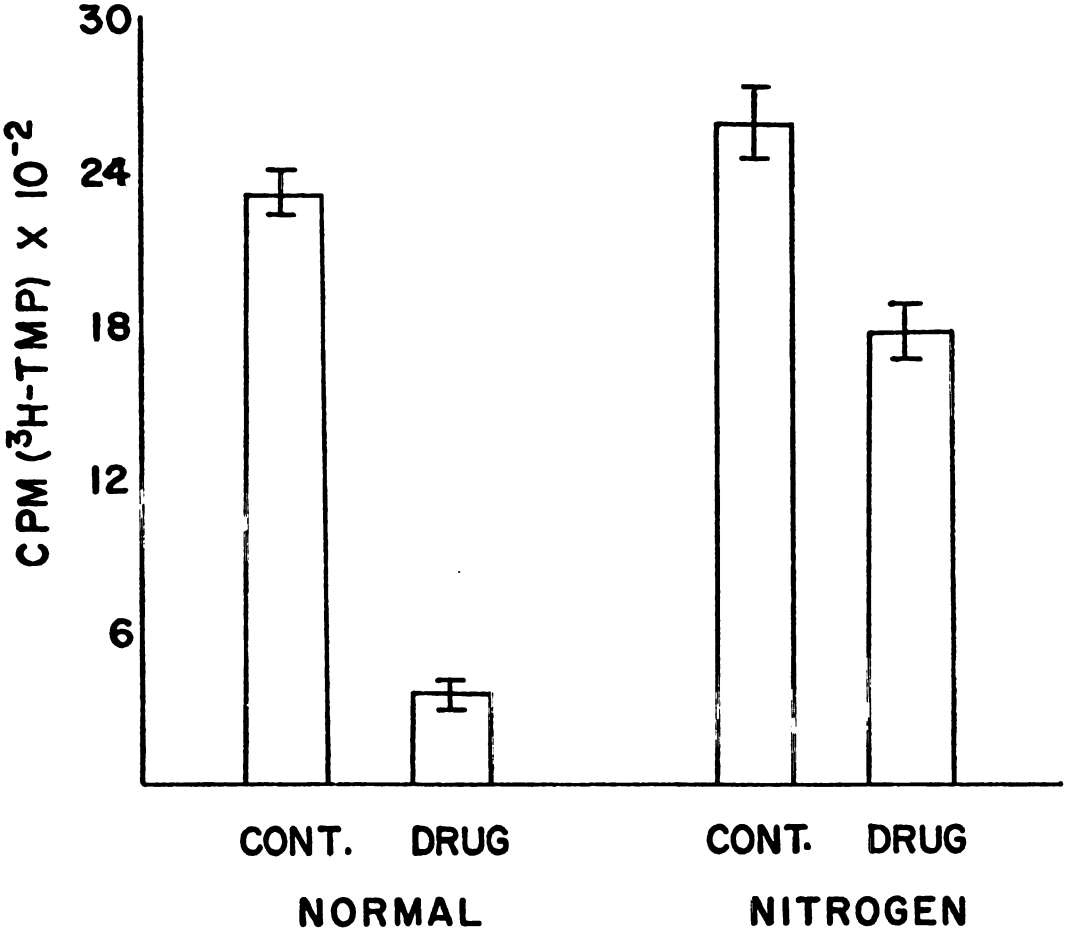


FIGURE 23: The relationship between the rate of flavonol oxidation at pH 7.6 to the intermediate and the ability of flavonols to inhibit RNA-dependent DNA polymerase. The percent decrease in band I absorbance is plotted on the ordinate; and the I_{50} values for RDP inhibition by five flavonols on the abscissa.

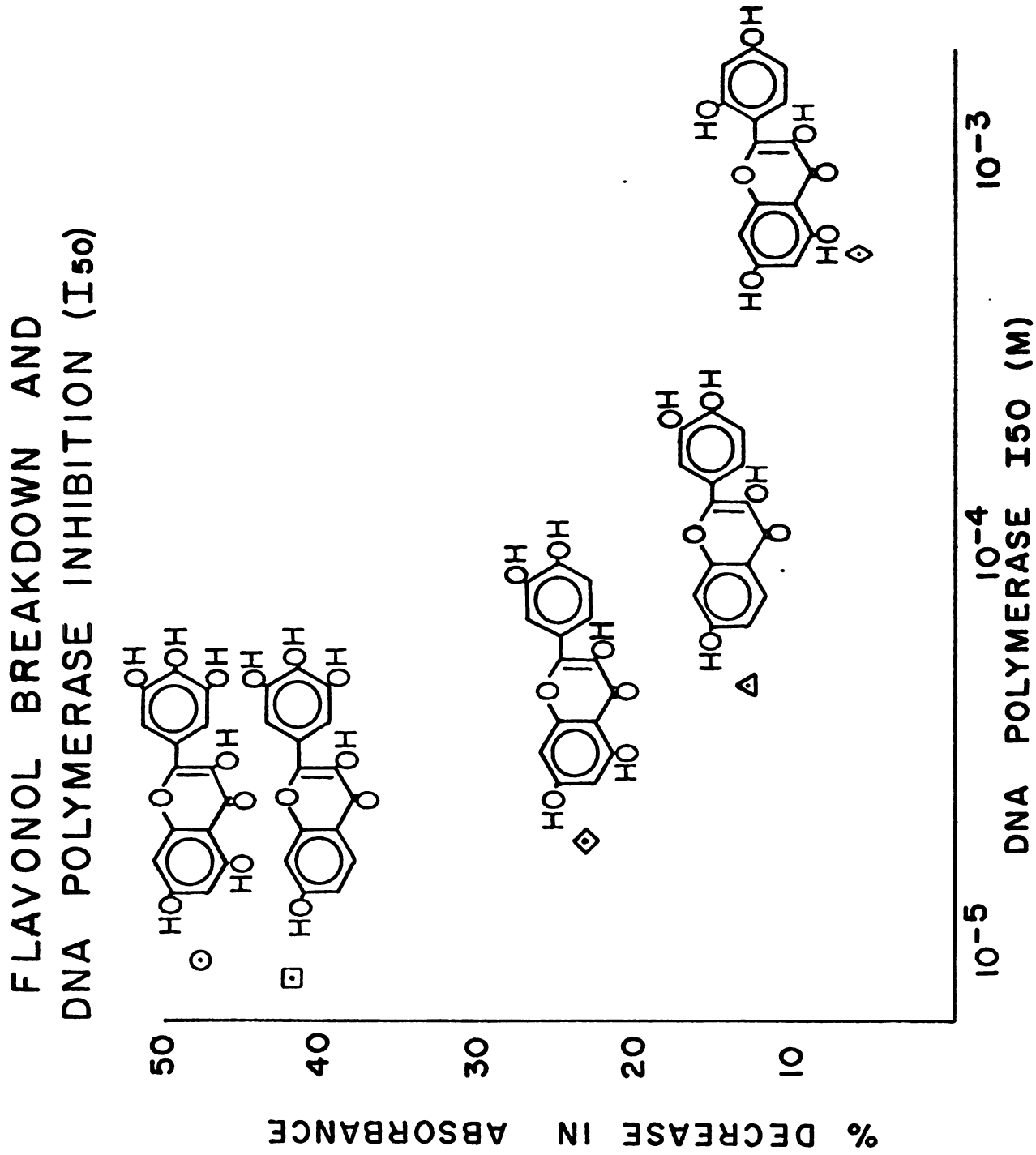
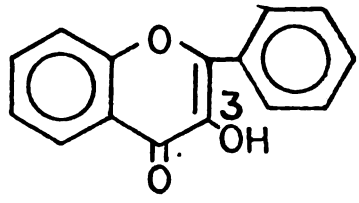
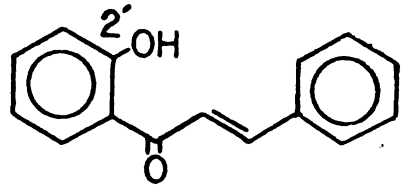
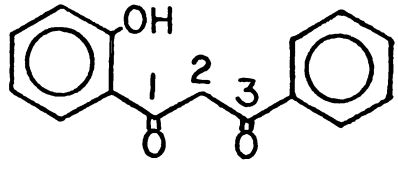
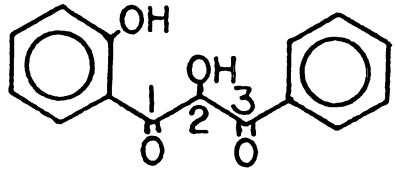
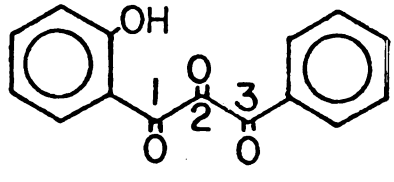

FLAVONOL BREAKDOWN AND
DNA POLYMERASE INHIBITION (I50)
% DECREASE IN ABSORBANCE
50
40
30
20
10
10-5
10-4
10-3
DNA POLYMERASE I50 (M)

TABLE 6: Inhibition of DNA-dependent RNA polymerase: The compounds were dissolved in dimethylsulfoxide (50%) to facilitate solubility in the assay mixture. Inactive compounds did not inhibit the enzyme at a concentration of 5 mM. The enzyme inhibition curves for compounds II, and VII are shown in Appendix II (Figure 30).

NUMBER	STRUCTURE	I_{50}
I		INACTIVE
XXXVI		INACTIVE
VI		INACTIVE
VII		1.2 mM
II		1.1 mM

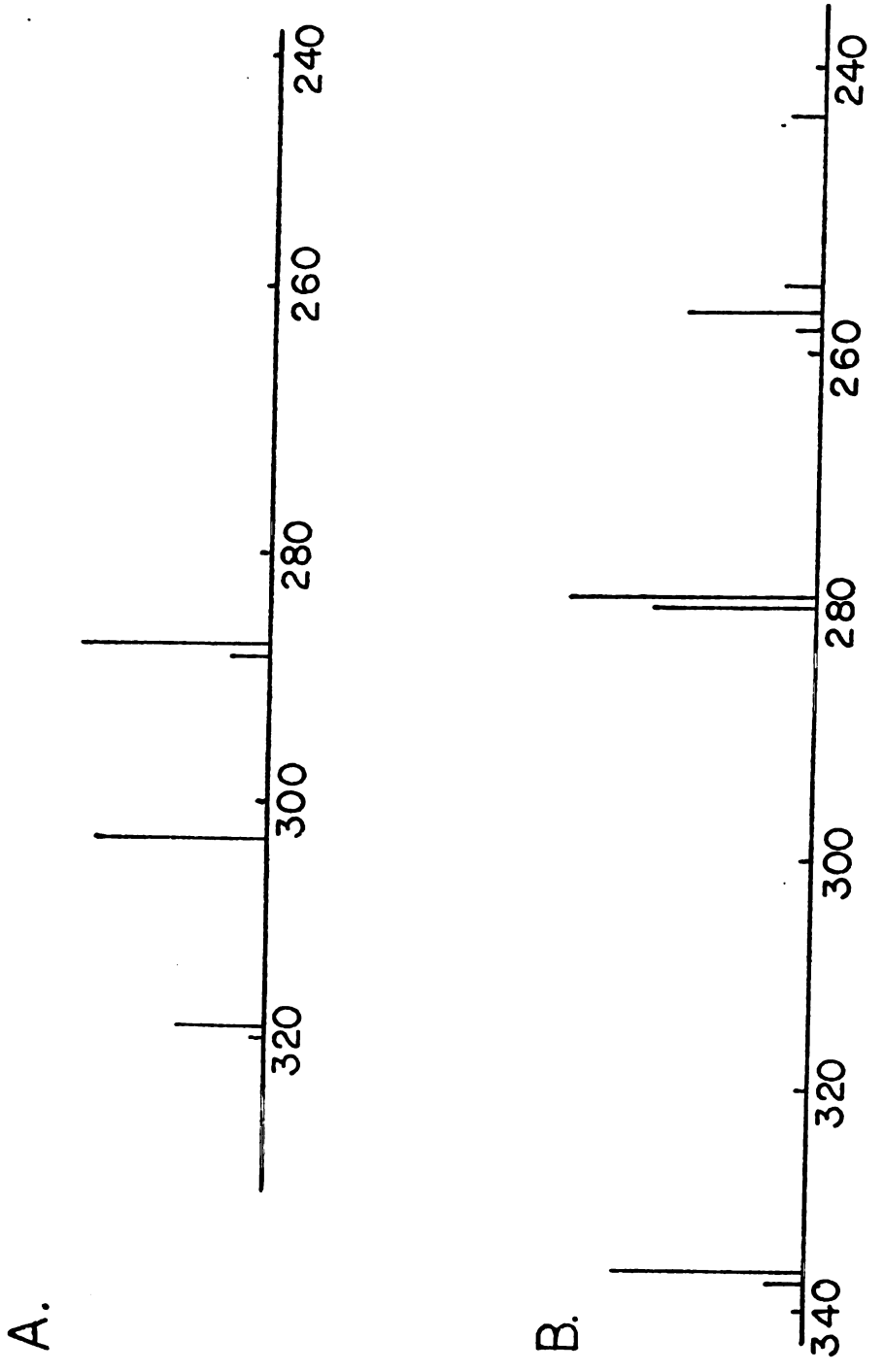
facilitating ring opening. Attempts to synthesize a stable polyhydroxylated carbinol (Figure 5, XIII) were unsuccessful and it was not possible to quantitatively determine the changes in activity induced by specific ring hydroxyl groups or the relative potency of the intermediates as enzyme inhibitors.

The products resulting from the breakdown of myricetin (5×10^{-5} M) after 30 minutes at pH 7.6 were analyzed by mass spectrophotometry. The mass spectra of myricetin and the products resulting from incubation at pH 7.6 are shown in Figure 24. No myricetin (molecular weight = 318; MH^+ = 337) and several smaller molecules were detectable. Compound XIII (Figure 20-B), resulting from the addition of water to the parent flavonol at the 2-position, would have the same molecular weight. Compound XXXIV (Figure 20-C), the oxidation product, would have a molecular weight of 334. No such species was detected. The absence of this compound is inconsistent with all the prior results confirming the importance of an oxidation reaction. Fragmentation of the product during the mass spectrometry or destruction during the work-up procedures could account for these findings.

Isolation of the Flavonol Breakdown Product

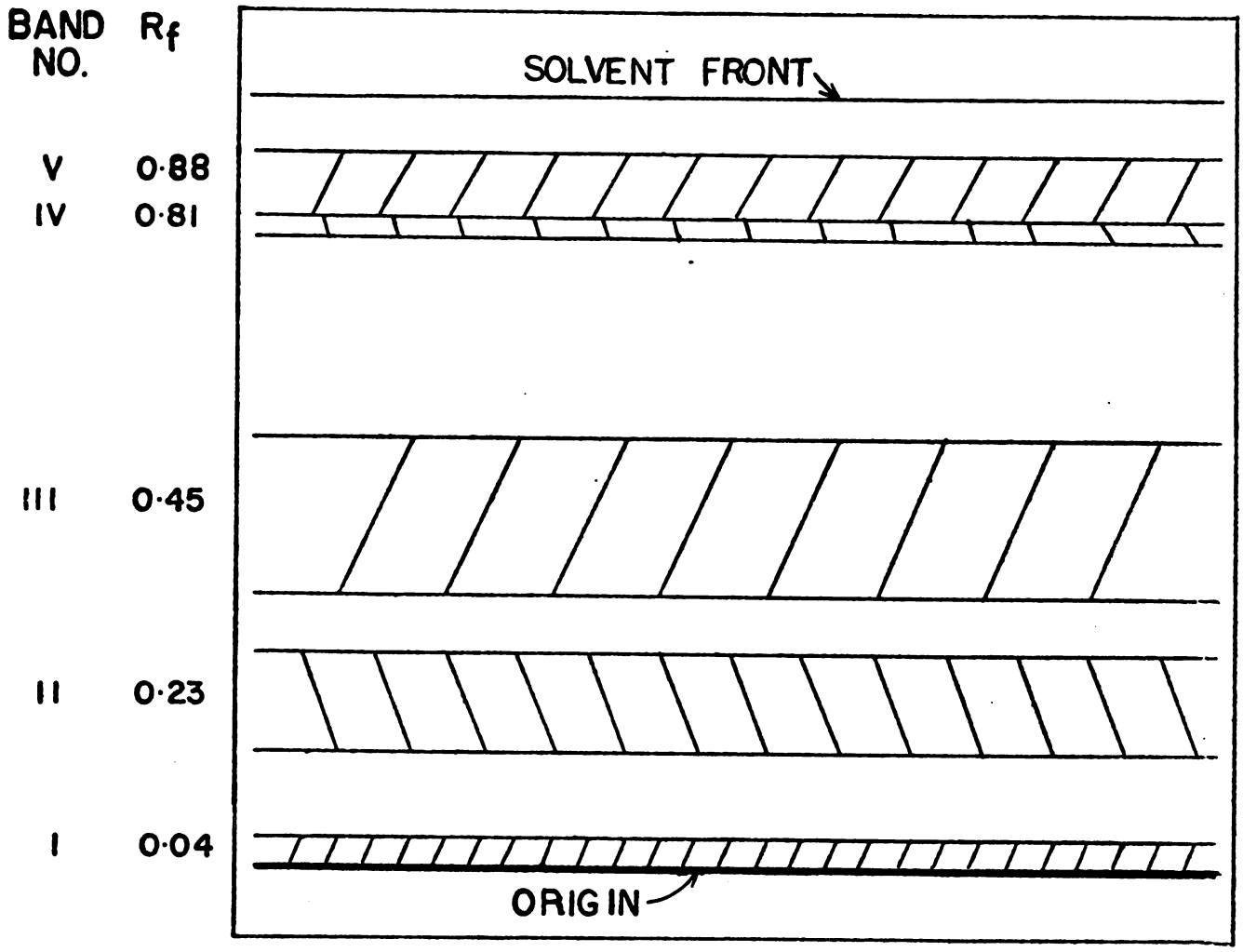
Isolation of stable compounds generated in an aqueous solution of kaempferol, quercetin, or myricetin at pH 7.6 was not achieved. Large reaction volumes were necessary because of the limited water solubility of the flavonols. Extraction with organic solvents, usually butanol or ether, and separation on preparative layer chromatographic plates were done. In each experiment the products were quite unstable and continually degraded during the procedure. As many as eight different bands would develop. Rechromatography of the extract of a single band resulted in the appearance of a multitude of compounds, never a single

FIGURE 24: Mass spectra of myricetin (A) and the reaction products following incubation of myricetin (5 X 10^{-5} M) at pH 7.6 for 30 minutes and extraction with ether (B). The source temperatures were 240° C and 190° C for A and B, respectively, at 0.5 torr.


A.
240
260
280
300
320
B.
240
260
280
300
320
340

spot. Figure 24a shows an example of a silica gel preparative layer chromatogram (butanol:acetic acid:water, 4:1:5) of myricetin following incubation at pH 7.6 (10 mg/100 ml), acidification, and extraction with butanol.

FIGURE 24a: A silica gel preparative layer chromatogram of myricetin following incubation at pH 7.6 and extraction with butanol (butanol:acetic acid:water, 4:1:5). Located by ultraviolet absorbance.


BAND NO.
R_f
SOLVENT FRONT
V 0·88
IV 0·81
III 0·45
II 0·23
I 0·04
ORIGIN

DISCUSSION

The answer to the question first asked in this investigation, "do any of the commonly occurring flavonols inhibit *in vivo* tumor growth?" is yes. The effect is not dramatic when compared with the potency of some currently used anticancer agents, but it is surprising for compounds of such low toxicity to mammals. Although it is unlikely that many of the flavonols discussed here would significantly alter the growth of advanced, established tumors, an influence on very small cancers is quite probable. The data in Table 2 and the experiments of Rudali (33), in which the oral administration of some flavonoids depressed the development of metastases in mice bearing mammary tumors, support this suggestion. Secondary or small primary tumors tend to be rapidly growing (79) and more sensitive to cycle-specific cytotoxic drugs (80). Common dietary factors are of low toxicity and would be likely to influence only the most susceptible or smallest cancers.

The 3-hydroxyl and 4-keto groups are important determinants of the *in vivo* antileukemic activity of the flavonoids: apigenin (XIV) and l-epicatechin (XXII) are inactive, but kaempferol (XV) and taxifolin (XXI), the 3-hydroxyl and 4-keto analogs, respectively inhibited P_{388} growth by over 75%. Initially the reasons for the importance of these functionalities was unclear, although they are known to influence flavonoid metabolism. Fission of the heterocyclic ring is catalyzed by intestinal microflora (81,82) and the type of phenolic aromatic acids produced depends on the presence of the 3-hydroxyl group and whether the 2-3 double bond is saturated (83). The 5-hydroxyl group, which is not required for the anticancer effect of the flavonols, is necessary for the ring fission mediated by intestinal flora (81), suggesting that the

bacterial degradation products are not the active compounds.

The pattern of hydroxylation influences both the effectiveness of the flavonols as inhibitors of P_{388} leukemia growth and their lipid solubility. The use of partition coefficients, which are ratios representing the distribution of a substance between an organic and aqueous phase, in the analysis of structure/activity relationships has been advocated (84). The relationship between the log P (P = partition coefficient) and the relative biologic response of a drug is often linear (85) or parabolic (86). Hansch et al. (87) have shown that the antileukemic activity of the nitrosoureas and imidazole carboxamides are parabolically related to their lipophilic character, although the optimum log P values for the two series are quite different. Preliminary data suggest that the relationship between the log P values (obtained by G. Tischenko in the laboratory of Dr. M. Apple) and the antileukemic activity of the flavonols may also be parabolic. Morin (log P = .6) is less lipophilic, kaempferol (log P = 1.4) more lipophilic, and both are less active than myricetin (log P = 1.1), which is the best inhibitor. The optimum log P value may represent the balance between dissolution in the peritoneal fluid and cellular permeability which results in the highest intracellular concentrations.

The cell cycle of growing cells, whether it is normal or cancerous, is the period between the formation of the cell by the division of its mother cell and the time when the cell itself divides to form two daughters. The cell cycle is divided into phases during which different biochemical processes take place. In the S phase, for example, DNA synthesis occurs, and in the M phase the cell undergoes mitosis. Chemical agents can interrupt this normal progression and induce cyto-

toxicity (88). Many drugs appear to exert their effects by inhibiting the synthesis of DNA or RNA, either directly or indirectly. Inhibition of cellular nucleic acid synthesis might account for the cytotoxicity of the flavonols and, perhaps, the dihydroflavonols.

Quercetin, and to a greater extent, myricetin, interferes with the synthesis of DNA, RNA, and protein in P_{388} leukemia cells. Replication, in particular, and transcription appear much more sensitive to the effects of the flavonols than translation. Quercetin (1×10^{-4} M), for example, did not inhibit ^{3}H-leucine incorporation at 10 minutes (Figure 10-B), whereas ^{3}H-thymidine (Figure 7-B) and ^{3}H-uridine (Figure 9-B) incorporation were decreased by about 80%. These data suggest that the effects on protein synthesis may be secondary to changes in nucleic acid metabolism.

Taxifolin, which is moderately active against *in vivo* P_{388} leukemia growth, also inhibits the synthesis of DNA, RNA, and protein *in vitro*. The dihydroflavonol is less potent than the flavonols; and, perhaps, metabolic activation is important. Taxifolin and quercetin are known to be metabolized similarly (83) and it has been reported that the antifungal properties of the former are dependent on its degradation (19). These reactions are dependent on microbes and would only occur in the intact animal. The conversion of dihydroflavonols to flavonol by mammalian cells has not been demonstrated, but O-methylation and glucuronide formation have been documented (83). Although the relevance of these reactions to the cytotoxicity of taxifolins or any other flavonoid is unknown; eupatorin, a flavone which is both methoxylated and hydroxylated, inhibits cancer cell growth (29).

l-Epicatechin is inactive against *in vivo* tumor growth and has very little influence on DNA or RNA synthesis in P_{388} cells. Thus the

active anticancer flavonoids myricetin, quercetin, and to a lesser extent, taxifolin, inhibit cellular nucleic acid synthesis; the non-toxic flavanol does not.

A number of flavonols inhibit Na^+K^+ (38) and mitochondrial (39) ATPases. In addition, they can regulate the high aerobic glycolysis that is caused by excessive ATPase activity (40). The importance of this effect on the inhibition of P_{388} leukemic cell growth by quercetin (41) has not been clarified, but it may depend on the extent of aerobic glycolysis in individual cells types. Shank and Smith (89) found the growth of mouse lymphoblasts to be directly related to sodium pump activity. In response to 5×10^{-4} M ouabain protein synthesis decreased as the Na^+ gradient dissipated. A 2 hour exposure to the drug did not affect ^{3}H-thymidine incorporation but, as shown in Figure 8, myricetin completely blocked DNA synthesis in the same length of time. Inhibition of ATPase activities by flavonols may decrease DNA synthesis indirectly; a more direct effect also appears likely.

The flavonols are toxic to a wide diversity of organisms and nucleic acid synthesis is a common, vital process for all of them. In view of this, the inhibition of DNA and RNA synthesis in P_{388} leukemia cells is especially interesting, since it could represent a unifying mechanism of action. No data is available on the effects of the flavonols on nucleic acid synthesis in bacteria, fungi, or virally infected cells. The discovery that E. coli DNA-dependent RNA polymerase and Rous sarcoma virus RNA-dependent DNA polymerase are inhibited by a variety of flavonoids (Table 3) does, however, support the idea.

The flavones that inhibit in vivo tumor growth and cellular nucleic acid synthesis most effectively, such as myricetin, are also the best

inhibitors of polynucleotide polymerases *in vitro*. The B-ring hydroxylation pattern influences activity, potency increasing from the 2', 4', to the 3', 4', to the 3', 4', 5' trihydroxylated compounds. The 3-hydroxyl group is also important. Apigenin (XIV), which lacks a hydroxyl function at the 3 position, did not affect tumor growth (Table 2), or the polymerases, whereas kaempferol (XV), which differs only in the addition of that group, inhibited both. Neither the *in vivo*, antiproliferative or enzyme inhibitory effects of the flavonols were dependent on 5-hydroxylation. In general, a consistent pattern of structure/activity relationships was apparent for flavonol inhibition of cellular growth and nucleic acid synthesis and of the two transcription enzymes.

Taxifolin, a dihydroflavonol, does not fit the pattern as well. Although it decreased leukemia growth and the synthesis of DNA and RNA, the flavonoid did not alter DRP or RDP activities *in vitro*. As previously discussed, it is not known whether metabolic activation of taxifolin is required, or if the dihydroflavonols act at a site other than the nucleic acid polymerases.

Flavonol inhibition of RNA polymerase is increased by incubating the drug in aqueous alkali prior to the assay. The UV spectral changes associated with the incubation and the flavonol inhibition of the enzyme reaction are both oxygen dependent. These results indicate that the favonols are converted to an intermediate which appears to be the active enzyme inhibitor. The highly correlated relationship between the ability of flavonols to inhibit RDP and the rate at which they are converted to an intermediate is shown in Figure 23. It is clear from the data that although the presence of the 5-hydroxyl group is not essential for activity, this functionality does influence enzyme

inhibition, probably by altering the rate of conversion to the intermediate. The same reasons are likely to account for much of the difference in the activities of morin, 2', 4'-dihydroxylated, and quercetin, 3', 4'-dihydroxylated in the B-ring. The structural requirements for flavonol polynucleotide polymerase inhibition as well as for oxidation to the intermediate are the same.

UV spectral changes of 3, 4'-hydroxylated flavones in basic media have been used for structural identification (90). But the nature of the intermediate involved in this process is not reported. Of the several possible reaction pathways for alkaline degradation of flavonols the following may be considered: A) oxidation of the B-ring to an orthoquinone; B) nucleophilic attack of the base at the 2-position and the subsequent ring opening; and, C) oxidation of the flavandione followed by nucleophilic attack and ring opening (Figure 20).

The first possibility is likely to occur in compounds having a catechol or pyrogallol moiety. A number of investigators have proposed that O-quinone formation from hydroxylated B-ring flavonoids may be responsible for certain of their pharmacological actions (35,37,91). In view of the inactivity of the dihydroflavonols, which may be expected to be susceptible to this oxidation, formation of B-ring O-quinones alone is insufficient to account for the activity. The 2-hydroxyflavanols (cf XIII) have recently been identified as the products of an enzymatic transformation of flavonols (92). These compounds and the intermediates of the alkaline degradation of 4'-hydroxyflavonols have similar UV spectra, but the former regenerate the parent flavonols upon acidification, a property not shared by the latter. The nonoxidative alkaline degradation pathway of flavonols (path B) may be unimportant as the enzyme inhibition depends on the presence of oxygen.

The intermediate (XXXIII) obtained on oxidative alkaline breakdown of flavonols (path C) may be in equilibrium with the propanetrione (XXXIV) and the 2-aroyl-2-hydroxy-3 (2H)-benzofuranone (XXXV) (71). Subsequent oxidation of these intermediates containing catechol or pyrogallol moieties might result in the formation of O-quinones. Exposure of flavonols to strong enough base can lead to complete breakdown, producing polyhydroxylated benzoic acids and acetophenones (93). Path C is consistent with the observed requirement of oxygen and an alkaline medium for the formation of active polymerase inhibitors from the flavonols, and with the activity of the synthetic propanetrione (II).

The requirement for both the 3 and 4'-hydroxyl groups is the primary characteristic of the in vitro inhibition of polymerases by flavones. The instability of these compounds in alkali and the importance of the reaction product for enzyme inhibition has been discussed. The oxidative pathway, outlined in Figure 20-C, may, however, provide a clue as to why the 4'-hydroxyl is needed. Oxidation of the flavandione (XXXI) to compound XXXII is dependent on the presence of a hydroxyl function at the 4' position. Without this group the oxidation, the subsequent nucleophilic attack at the 2-position (XXXIII), and the ring opening (XXXIV) cannot proceed.

The flavyliums (XII-XV) are good inhibitors of both DRP and RDP (Table 3). In aqueous alkali 3, 4'-hydroxylated flavyliums form an anhydrobase which is subject to further transformation through a nucleophilic addition of water at the 2 position (94). The possibility of intermediates quite similar to those of the flavonol reaction accounting for the activity of the flavylium salts remains to be investigated.

The mechanism of action of polynucleotide polymerase inhibition by

the flavonol oxidation products is unknown, but is an area which deserves further study. Recent reviews (95, 70) of inhibitors of reverse transcription suggest some possible modes of action, including binding directly to the enzyme, as exemplified by rifamycin derivatives (96). An agent might also act as a substrate, template, or primer analog or by binding to the template. Since DNA polymerases are zinc metalloenzymes and are dependent on either Mg^{++} or Mn^{++} for activity, chelating agents may be inhibitors.

The flavonol active intermediates, if they are oxidized to quinones, may react with sulfhydryl or amino groups (97). Hurwitz and Leis (98) showed that p-hydroxy-mercuribenzoate is capable of inhibiting a RNA-dependent DNA polymerase in the absence of sulfhydryl reagents in the reaction mixture. In this case direct interaction with the enzyme would be anticipated.

Thiosemicarbazones, chelators of metallic ions, are inhibitors of RDP (99). The chelating ability of some of the flavonoids is well known (100). It is improbable that this action is important for polymerase inhibition because of the following reasons. Pelargonidin, a flavylium lacking chelation potential, is a good inhibitor of both forward and reverse transcription. The 5-hydroxyl group, which greatly enhances the metal binding ability of flavonols, is not required for enzyme inhibition and finally, the oxidation reaction, which is necessary for enzyme inhibition by flavonols, would not increase chelation.

There are no data to suggest that the flavonol intermediates might act as substrate or template analogs. The planar nature of the flavone molecule does not, however, rule out the possibility of binding to the template, perhaps as an intercalator. All of the above possibilities

should be investigated.

These data support the hypothesis that inhibition of nucleic acid polymerases may account for the anticancer effects of the flavonols. The most potent inhibitors of *in vivo* P_{388} leukemia growth strongly inhibit cellular nucleic acid synthesis and are also the best DNA polymerase inhibitors *in vitro*. Inhibition of certain polymerases may explain some of the selective toxicity of the flavonols. The active intermediates formed by oxidative alkaline degradation of flavonols represent a new class of selective polynucleotide transcription inhibitors, and might provide prototypes for new chemotherapeutic agents.

The question of whether the flavonoids are the naturally occurring cancer suppressing influences affecting world-wide cancer incidence has not been answered; that is impossible without additional epidemiological evidence. The flavonols, the most widely distributed of these plant compounds, have, however, now been demonstrated to possess many of the pharmacological actions necessary to support such a possibility.

APPENDIX I

Inhibition of *in vivo* P_{388} leukemia growth: In the first column the experimental group and the number of animals in each group (N) are given for each experiment. The cell count, expressed as the mean ± standard deviation for each group is in the second column. To convert to the total tumor burden multiply the cell count by 2×10^4; for example, in the first experiment the control group would be 5.2×10^8 cells/animal. The percent inhibition of growth is presented in the last column.

APPENDIX I

Experimental Group	N	Cell Count	Percent Inhibition
Experiment 1			
Control	12	26378 ± 5675	
Myricetin	6	615 ± 145	98
1-Epicatechin	6	23223 ± 6528	12
Morin	6	15318 ± 5097	45
Experiment 2			
Control	11	24544 ± 6050	
Quercetin	5	10286 ± 3695	58
Fisetin	6	5930 ± 1807	76
Taxifolin	6	4465 ± 2265	82
Robinetin	6	5139 ± 3771	79
Kaempferol	6	6090 ± 6899	75
Morin	6	7291 ± 3911	71
Experiment 3			
Control	11	16295 ± 2626	
Apigenin	5	21520 ± 5746	0
Fisetin	6	3420 ± 1096	79
Kaempferol	6	3413 ± 2274	79
Taxifolin	6	4542 ± 2948	72
1-Epicatechin	6	15324 ± 5068	6
Experiment 4			
Control	10	25669 ± 7301	
Myricetin	5	311 ± 503	99
Robinetin	5	2357 ± 1317	91
Quercetin	5	3228 ± 2067	87
Apigenin	5	30291 ± 12818	0

APPENDIX II

Enzyme inhibition curves of both *E. coli* DNA-dependent RNA polymerase and Rous sarcoma virus RNA-dependent DNA polymerase by a series of flavonoids are presented. Each curve is compiled from at least two independent experiments and thus in the figure legends control (100%) values and the tritium counting efficiencies are given as averages. Each point on the curves represents the mean ± standard deviation of three independent samples. If no bars are present, the standard deviation is within the size of the symbol.

FIGURE 25: Inhibition of Rous sarcoma virus RNA-dependent DNA polymerase by myricetin (○) and quercetin (□): control values averaged 1980 cpm and were counted at an efficiency of about 35%.

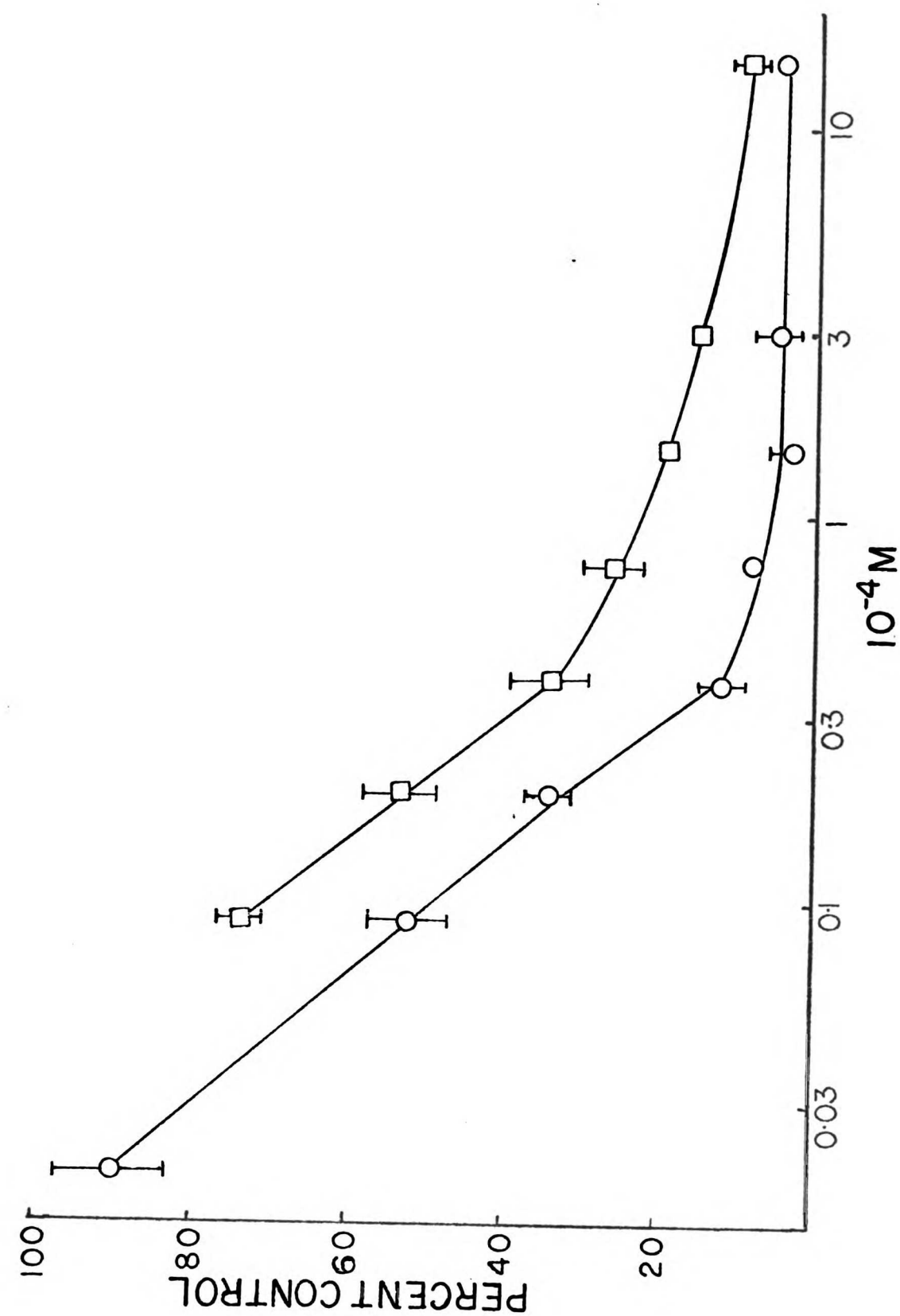

PERCENT CONTROL
100
80
60
40
20
0·03
0·1
0·3
1
3
10
10^{-4} M

FIGURE 26: Inhibition of Rous sarcoma virus RNA-dependent DNA polymerase by robinetin (○), fisetin (□), and morin (△): control values averaged 2770 cpm in the robinetin and fisetin assays and 2420 cpm in the morin assay. The counting efficiencies were approximately 35%.

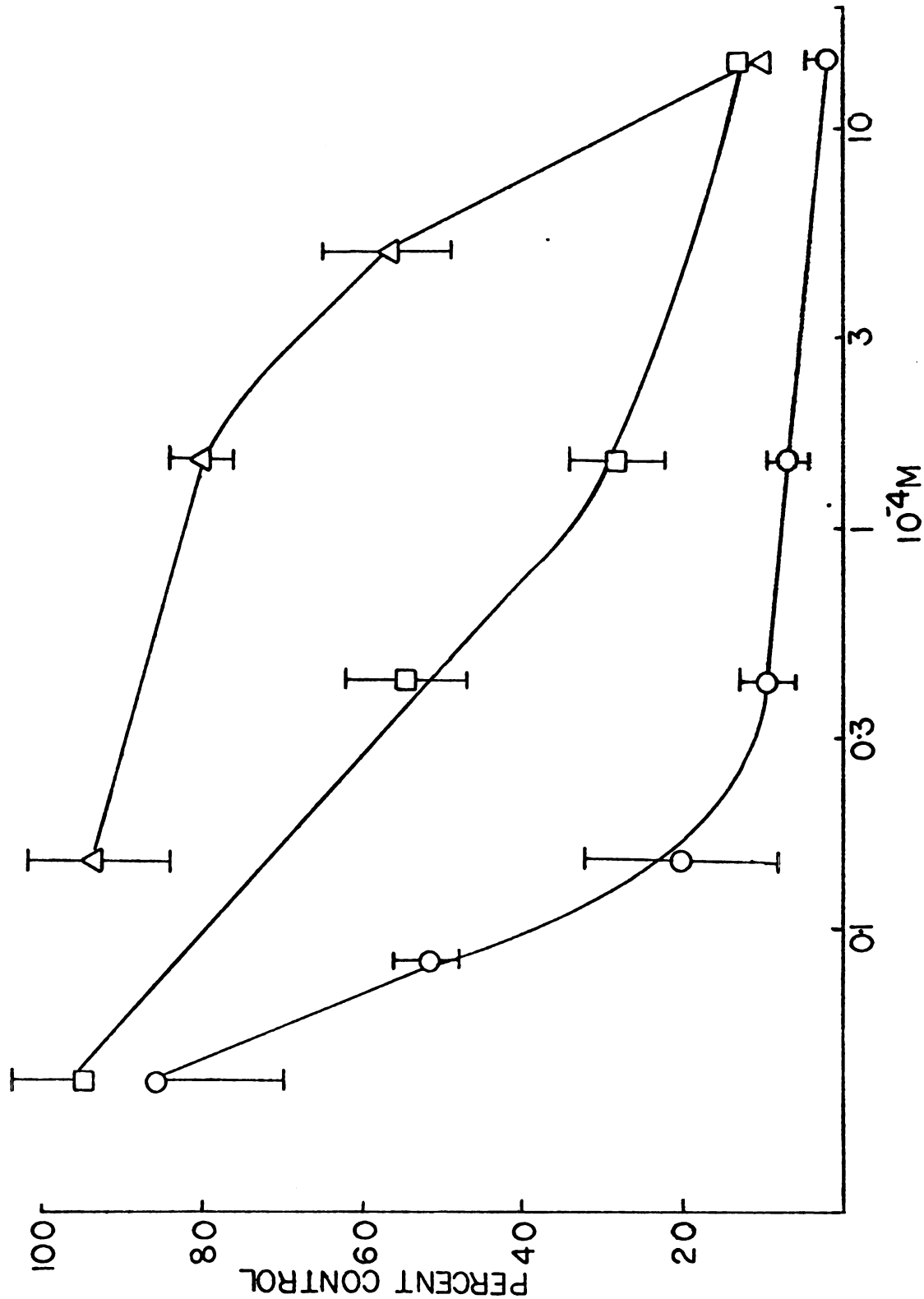
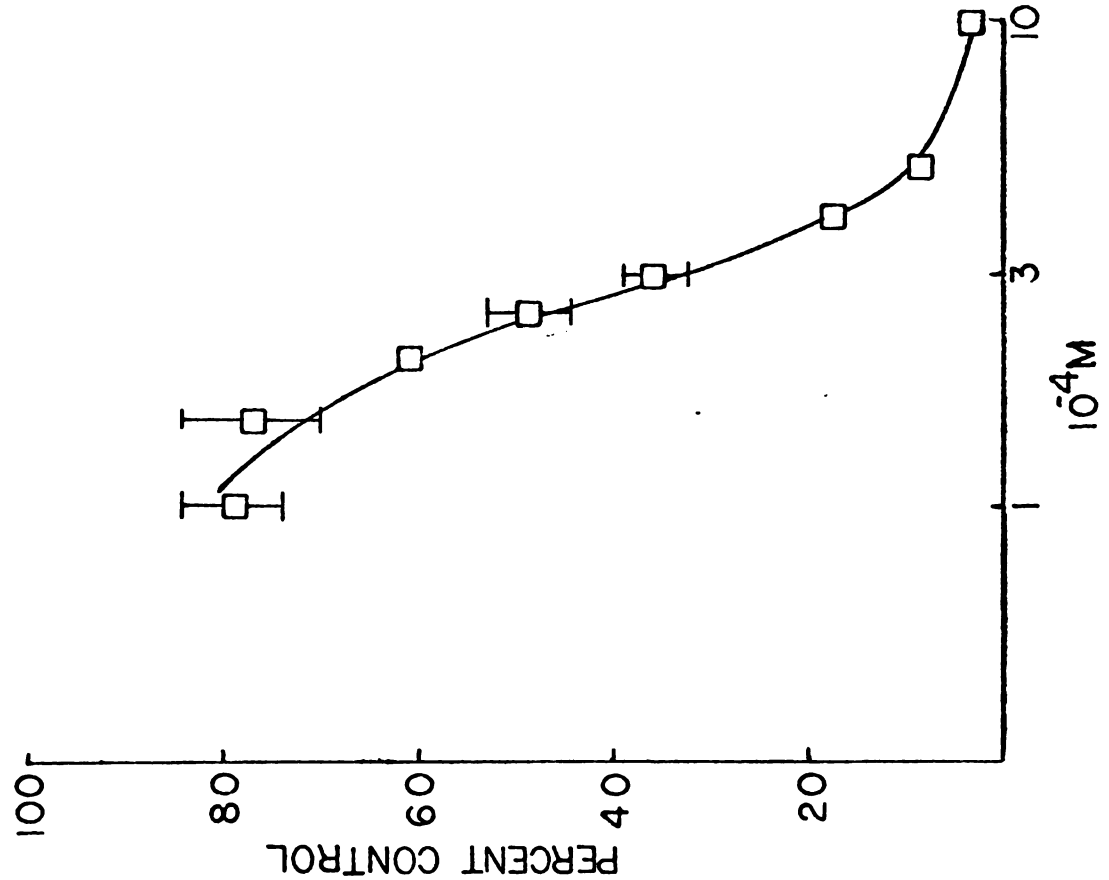

PERCENT CONTROL
100
80
60
40
20
0·1
0·3
1
3
10
10^{-4}M

FIGURE 27: Inhibition of E. coli DNA-dependent RNA polymerase by kaempferol (○) and quercetin (□): control values for the assays, which were run with 5% DMSO, averaged 3150 cpm for kaempferol and 2800 cpm for quercetin. Counting efficiencies were about 33%.


PERCENT CONTROL
100
80
60
40
20
1
3
10
10^{-4}M

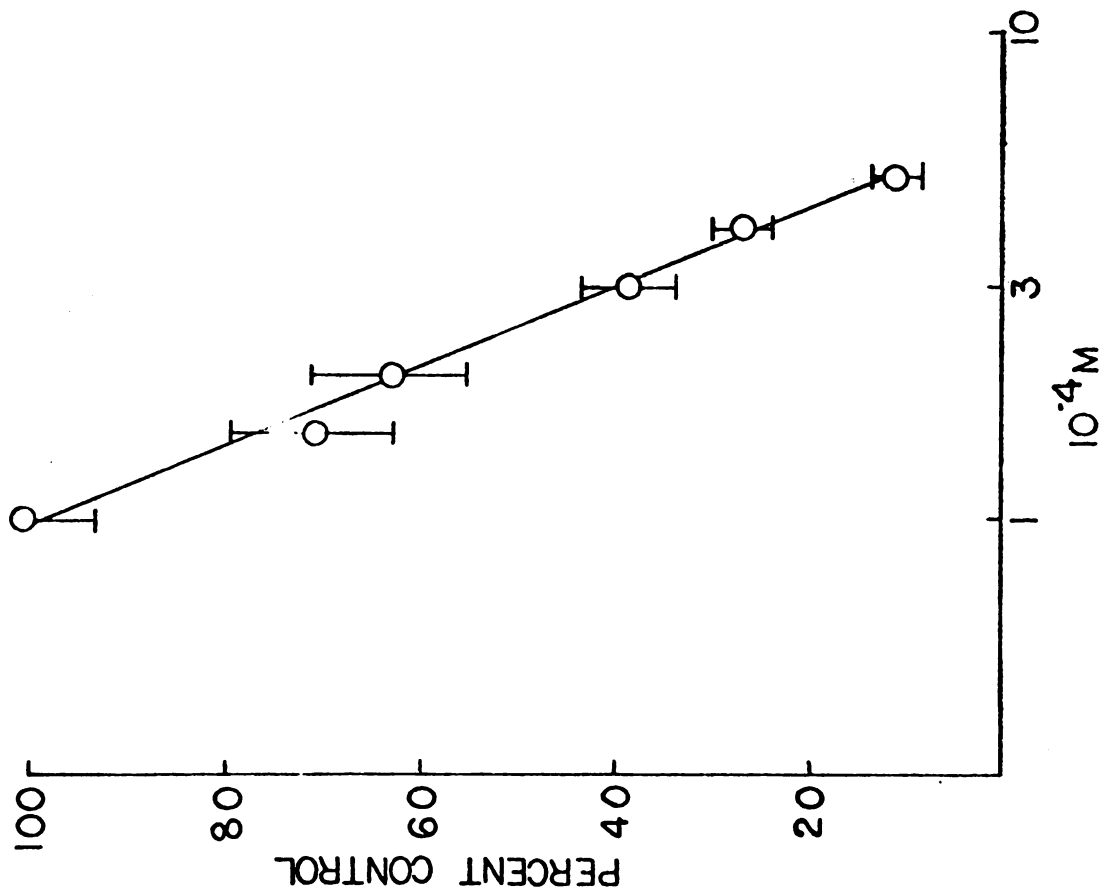
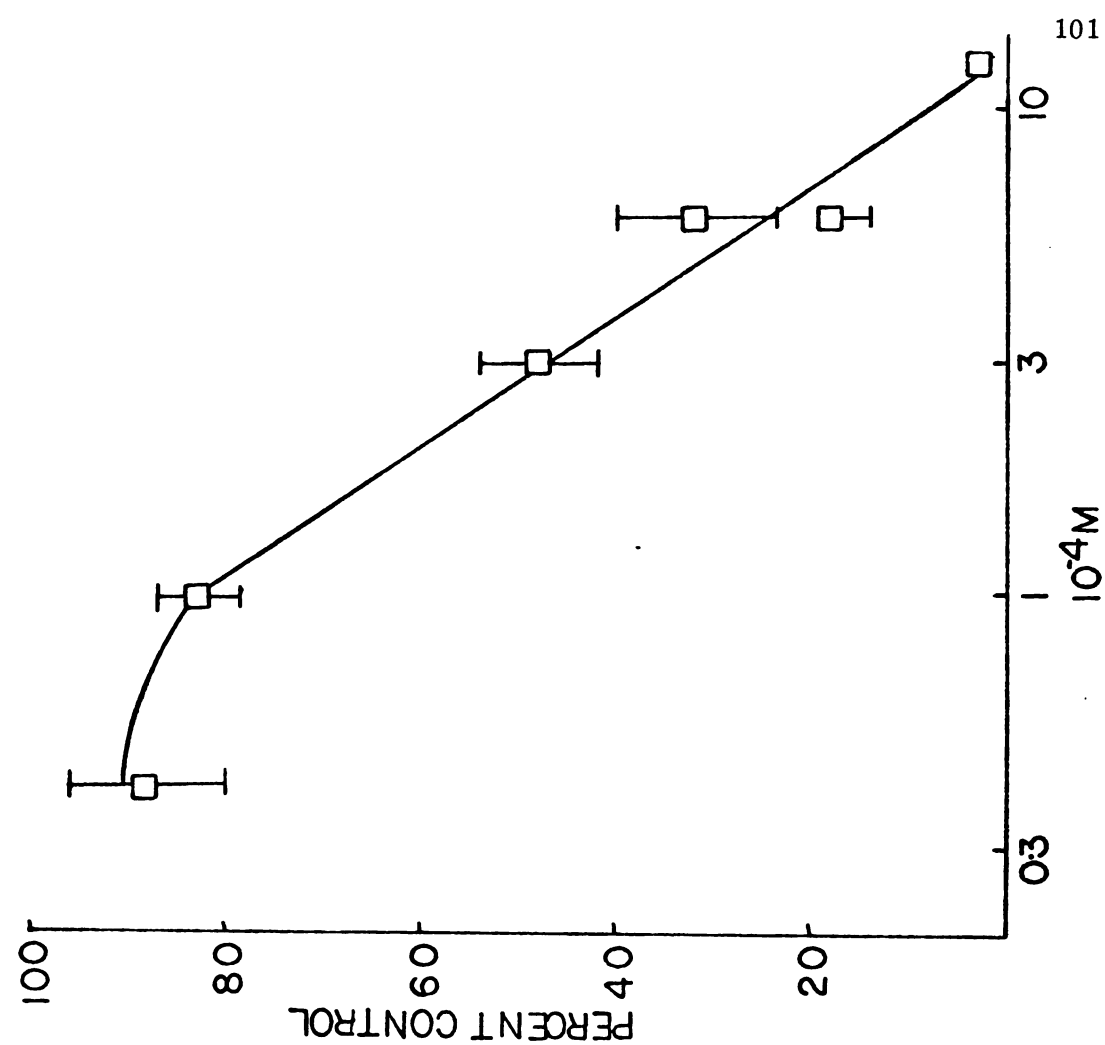

PERCENT CONTROL
100
80
60
40
20
1
3
10
10^{-4}M

FIGURE 28: Inhibition of E. coli DNA-dependent RNA polymerase by myricetin (○) and fisetin (□): The myricetin reactions were run with 5% DMSO and the control values averaged 2040 cpm counted at an efficiency of 33%. The fisetin assays, done at 1% DMSO, averaged 2070 cpm for the control reactions and were counted at an efficiency of 32%.


PERCENT CONTROL
100
80
60
40
20
0·3
1
3
10
10^{-4} M

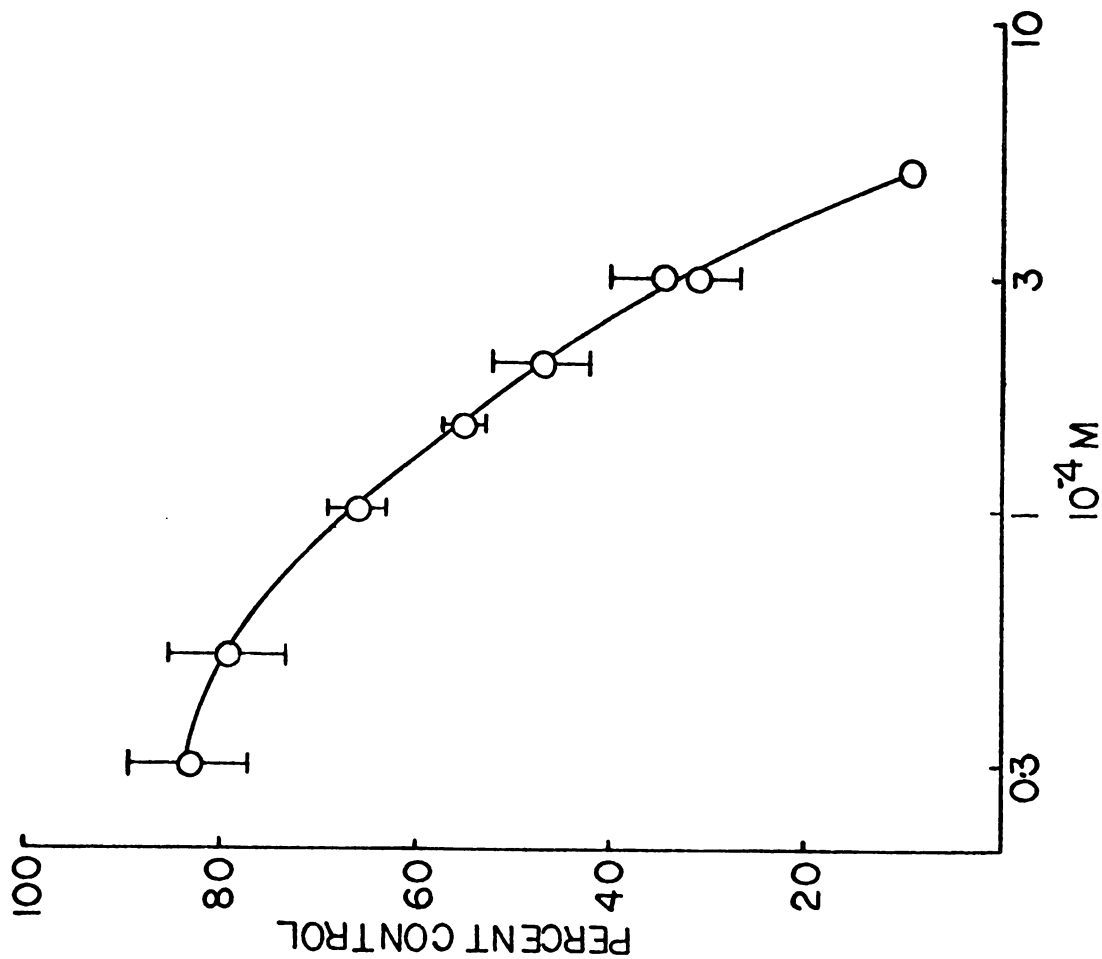

PERCENT CONTROL
100
80
60
40
20
0·3
1
3
10
10^{-4} M

FIGURE 29: Inhibition of E. coli DNA-dependent RNA polymerase by delphinidin (○), cyanidin (□), and pelargonidin (△): control values for these assays averaged 2250 cpm for delphinidin, 2460 cpm for cyanidin, and 2170 cpm for pelargonidin. The counting efficiencies were approximately 18%; the lower percentage was caused by the inclusion of triton X-100 in the scintillation cocktail.

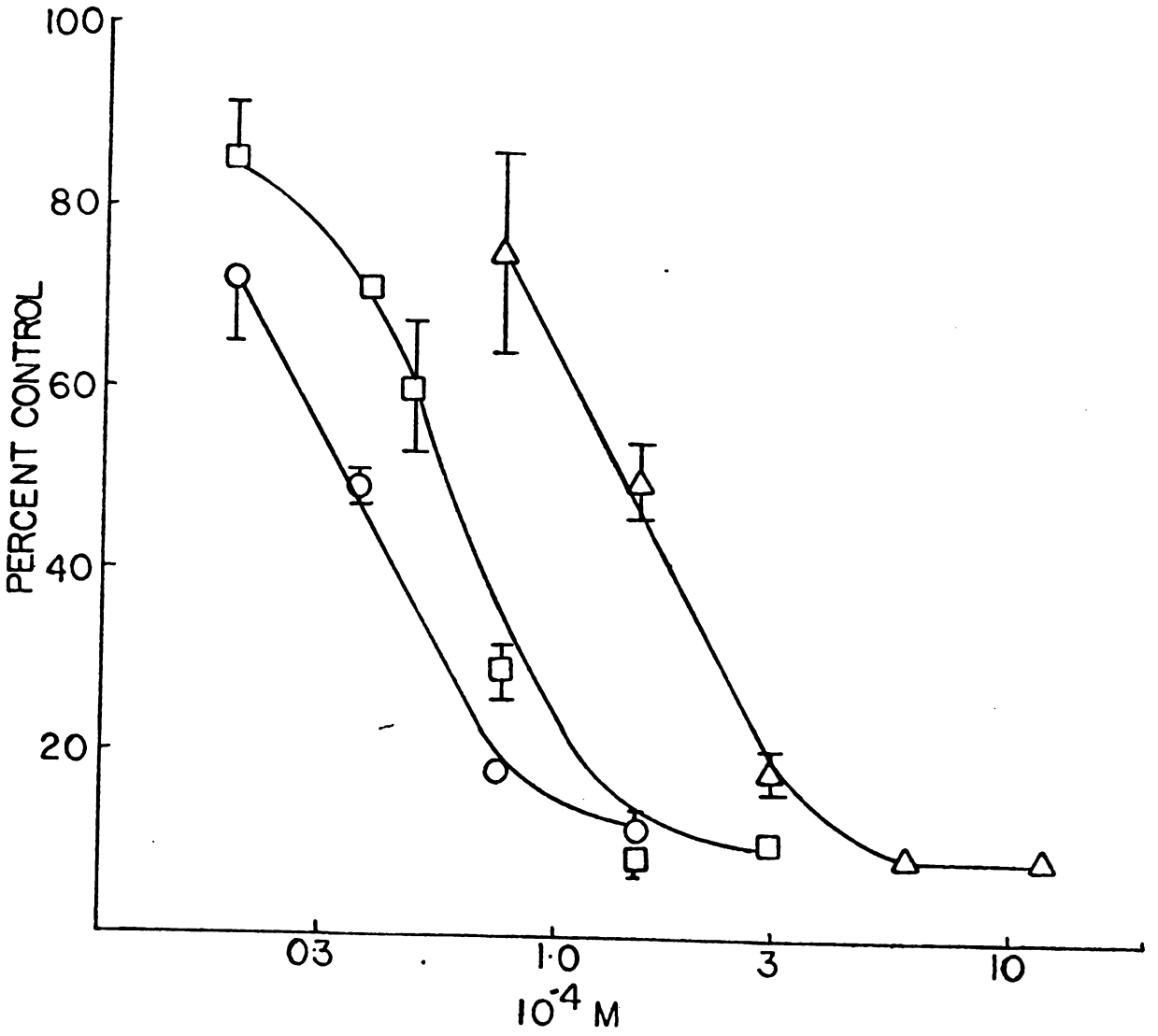
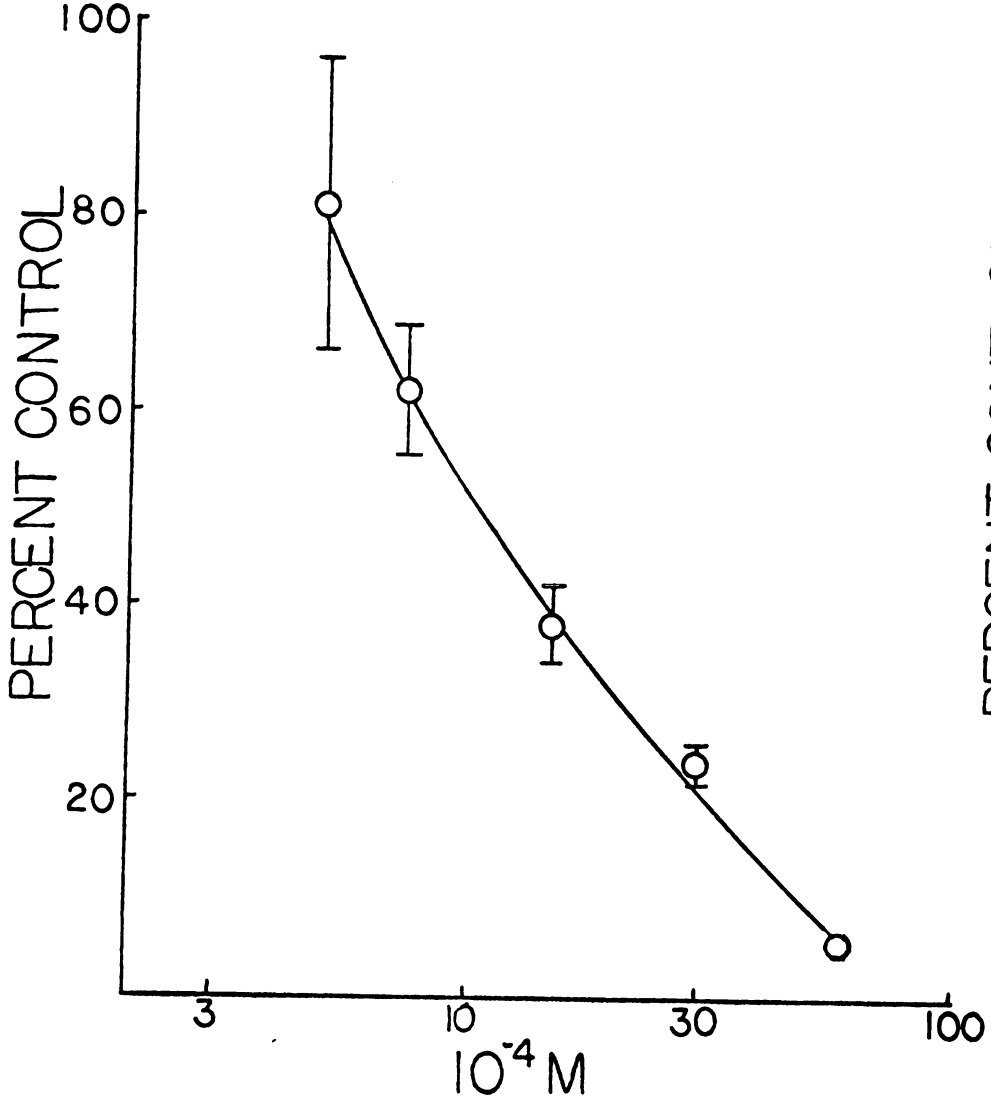
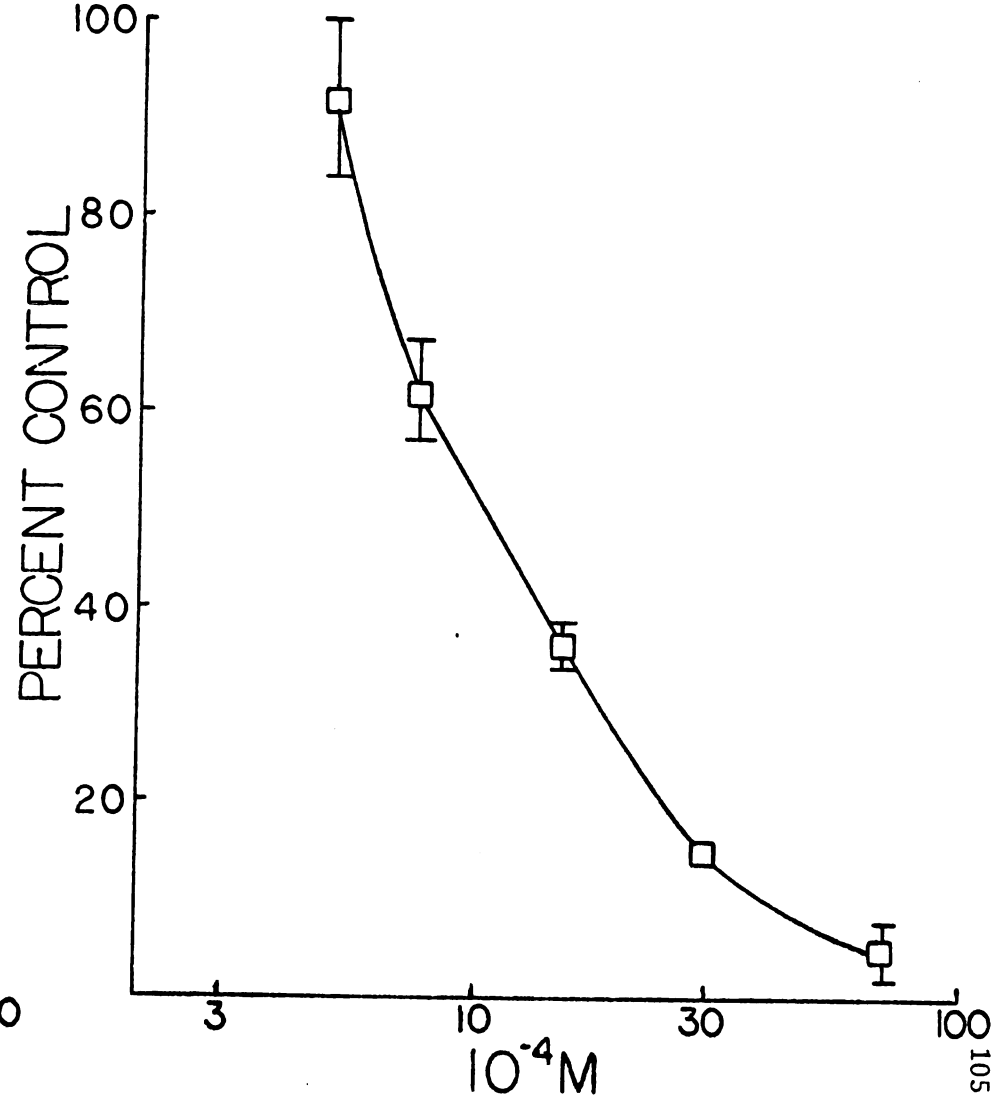

100
80
60
40
20
PERCENT CONTROL
0·3
1·0
3
10
10⁻⁴ M

FIGURE 30: Inhibition of E. coli DNA-dependent RNA polymerase by O-hydroxydibenzoylcarbinol (○) and 2-aroyl-2-hydroxy-3(2H)-benzofuranone (□): for these reactions, run at 5% DMSO, the control values averaged 1810 cpm for the carbinol and 1500 cpm for the benzofuranone. The counting efficiencies were approximately 33%.


100
80
60
40
20
PERCENT CONTROL
3
10
30
100
10^{-4} M


100
80
60
40
20
PERCENT CONTROL
3
10
30
100
10^{-4} M

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