Riboflavin and Cancer: A Review

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SUMMARY

The relationship of riboflavin to cancer is intriguing but many gaps remain in our knowledge. Several studies indicate that riboflavin deficiency inhibits tumor growth in experimental animals and possibly in man, but the precise mechanisms involved have not been elucidated. Azo dye carcinogenesis in liver appears to be a special case in that riboflavin deficiency increases the potency of these drugs in tumor causation, probably in large measure because flavin cofactors are involved in their metabolic degradation. Riboflavin influences uptake of chemotherapeutic drugs in at least one instance (methotrexate) into neoplastic cells. The fact that folic acid metabolism depends upon flavin cofactors may have implications for the use of folate antagonists in cancer chemotherapy.

Neoplastic liver appears to lack certain mechanisms that regulate riboflavin metabolism in normal tissues. In addition, it is more resistant than normal liver to riboflavin deficiency, as reflected in the relatively higher concentrations of flavin mononucleotide and flavin adenine dinucleotide measured in samples of tumor than in the host rat liver. By contrast, the concentration of free riboflavin is greatly depressed in Novikoff hepatoma grown in riboflavin-deficient animals. The loss of this relatively dispensable flavin fraction may be one of the mechanisms that enable tumor to retain the more critical flavin mononucleotide and flavin adenine dinucleotide fractions when dietary riboflavin is diminished. The provocative observation that certain patients with cancer excrete less riboflavin than do normal individuals requires further confirmation. Much remains to be learned about the uptake, turnover, binding, and removal of riboflavin and its derivatives from neoplastic tissue.

INTRODUCTION

Animals and humans with tumors may display profound cachexia during the course of illness. In some instances weight loss may be an initial manifestation of cancer, occurring before the neoplasm is widespread. Anorexia, nausea, vomiting, and diminished food intake may be important factors in contributing to the development of cachexia in far-advanced cancer. In addition, anitation may arise from diminished intestinal absorption, blood loss, tissue ulceration and necrosis, or infection and other factors. Disturbances in the metabolism of lipids, amino acids, carbohydrates, and electrolytes have all been described in patients with cancer but the precise mechanisms underlying weight loss and malnutrition remain uncertain. Tumors have been referred to as "nitrogen traps," which derive their amino acid supplies at the expense of the animal or human host. It appears that, when the supplies of dietary nitrogen are limited, tumor competes more successfully than normal host tissues for them.

There is considerable experimental evidence that both initiation and further development of tumors in animals may be dependent to some extent upon nutritional factors. Transplanted tumors are particularly sensitive to total caloric restriction and frequently have a slowed rate of growth when the intake of food is reduced. In C3H mice, reduction in caloric intake resulted in a greatly diminished development of spontaneous mammary carcinoma. Similarly, in mice of a strain with a high incidence of spontaneous lymphoid leukemia, underfeeding of the animals dramatically reduced the overall incidence of the neoplasm and delayed its emergence. Tumors may, however, continue to grow under certain circumstances despite progressive weight loss of the host. Force-feeding of experimental animals with tumors does not necessarily slow the evolution of metabolic alterations in the host, such as anemia and changes in the activities of certain enzymes. Similarly, in man metabolic abnormalities such as hypoalbuminemia are not prevented by incrementing the dietary intake of nitrogen. There are reports that force-feeding of patients with diets high in calories and nitrogen may even accelerate the growth of the cancer. These studies suggest that, under certain conditions, dietary intake may be of importance in regulating the growth of tumors but that other manifestations of cancer appear to be less dependent upon nutritional factors.

The relationship of vitamin metabolism to cancer is equally complex. Vitamins appear to influence the genesis and growth of cancer, but it has frequently been difficult to differentiate the effect of a specific vitamin lack from the accompanying reduction in dietary intake. Not only do vitamins affect the rate of neoplastic growth, but tumors, in turn, may affect the metabolism of vitamins. Vitamins also affect the inactivation of drugs and may influence the response to chemotherapy. The present review considers the various relationships of cancer to a single vitamin, riboflavin.

SPONTANEOUS AND TRANSPLANTED CANCERS

The investigations of Morris and Robertson in 1943 demonstrated that the growth and spread of spontaneous
mammary cancers in mice are markedly diminished in animals that have become riboflavin deficient. In these studies animals were placed on riboflavin-supplemented or riboflavin-deficient diets as soon as mammary tumors were first observed. The loss of body weight exhibited by these riboflavin-deficient mice during a 9-week period was nearly the same as that of similarly deficient animals that were not bearing tumors. The growth rate of tumors that appeared in riboflavin-deficient animals, however, underwent a gradual and progressive decrease compared to those tumors grown in mice on a diet containing normal amounts of riboflavin. The decreased rate of tumor growth was most marked in mice during the later stages of deficiency. Riboflavin refeeding of deficient animals rapidly led to a restoration of the normal growth rate of the mammary tumor.

The results of one of these experiments are shown in Chart 1. In animals on a normal diet (curve A), tumor growth increased at a uniform rate throughout the 4-week experimental period. In riboflavin-deficient animals (Curve B), the decrease in the rate of tumor growth first appeared at 1 to 3 weeks of deficiency. From 3 to 6 weeks of deficiency, virtually no increase in tumor size occurred. In animals that had received a high-riboflavin diet after 3 weeks of riboflavin deficiency (Curve C), the tumor growth rate showed a rapid recovery after the initial lag in growth and, for the following several weeks, was at least as great as in normal animals. In a 4th group of animals (Curve D) fed a deficient diet for 3 weeks, followed by a high-riboflavin diet for 2 weeks, and then a low-riboflavin diet again, the tumor growth rate closely reflected each dietary manipulation.

These studies clearly showed that rates of growth of mammary tumors occurring spontaneously are greatly depressed in riboflavin-deficient animals. Other studies by Morris (47) indicated that the size of spontaneous tumors that is eventually obtained is less in the deficient than in the control animals. A direct relation was found between the dietary riboflavin content and the number of mammary tumors observed grossly. C3H mice feeding on a regular diet were selected for study shortly after spontaneous tumors were first noted. Animals were placed at random on low- and high-riboflavin diets; the total quantity of food consumed was essentially the same in all groups, although exact figures were not reported. The results of these experiments are given in Chart 2. In mice on a high-riboflavin diet, the mean number of tumors per animal increased from slightly more than 1.0 to more than 2.5 during the 5-week experimental period. By contrast, in mice on a deficient diet, the average number of tumors per animal did not exceed 1.5 at any time.

A depression of tumor growth has also been observed with pantothenic acid deficiency (47). Thiamine deficiency did not suppress tumor growth when the effect of diminished food intake was taken into account. Pyridoxine deficiency has been reported to produce regression of mouse Sarcoma 180 and other tumors (32, 43) or to have no effect upon tumor growth (15). In the latter study, 17 patients with severely progressive cancers including carcinoma of the breast, esophagus, larynx, and lung and lymphosarcoma were treated with a pyridoxine-deficient diet for periods of 10 to 80 days. Nine of these patients also received a pyridoxine antagonist, 4-deoxypyridoxine, for varying intervals to accentuate the metabolic deficiency state that had been induced in these patients, but no inhibitory effects on tumor growth were noted (15).

Further investigations concerning the antitumor effects of riboflavin deficiency have used either a deficient diet or structural analogs of riboflavin. In one study, a marked reduction in the size of previously developed lymphosarcoma transplants in mice was observed (62). The findings of this study differed from the results obtained by Morris (47).
in one respect. Accelerated growth of tumors was not observed once the animals had undergone refeeding with riboflavin. Holly et al. (21) showed that several additional isoalloxazine derivatives also cause regression of 1 form of lymphosarcoma. Studies with a structural analog of riboflavin, diethyl riboflavin, which antagonizes the biological effects of the vitamin, have shown that this compound diminishes the growth rate of Walker carcinoma in rats (1). In rats treated with diethyl riboflavin, tumor weight, expressed per body weight, was decreased to two-thirds of that observed in normal animals bearing Walker tumors. An analog of riboflavin, 7-methyl-8-ethyl flavin, which does not produce riboflavin deficiency but instead replaces the vitamin in a variety of biochemical functions, does not cause regression of previously established Walker carcinoma 256 (30).

In a culture medium the growth and metabolism of ascites tumor cells is inhibited by riboflavin. The addition of small quantities of riboflavin, but not of several related compounds, to the culture medium has been effective in substantially reducing the fermentation rate of these neoplastic cells. This effect appears to be light mediated, since it is not demonstrable when cells are grown in complete darkness (69, 70). It is not certain how these observations are related to tumor growth in the deficient animal as a whole.

In summary, these studies generally indicate that riboflavin deficiency decreases the rates of growth of a variety of tumors from experimental animals. This is true with respect to certain forms of mammary tumors, lymphosarcoma in mice, and Walker carcinoma in rats. Because tumor weight relative to body weight has been used in the past as the major index of tumor growth, it is not possible to determine from these early reports whether riboflavin deficiency decreases tumor cell size as well as cell number or whether it produces other morphological changes in the tumors studied. The mechanism of the inhibitory effects of riboflavin deficiency has not been elucidated.

Some evidence exists that experimental riboflavin deficiency may inhibit certain forms of neoplastic disease in man. In an initial study using a derivative of riboflavin, 6,7-dimethyl-9-(2'-acetoxyethyl)-isoalloxazine (U-2112), in 10 cancer patients, Lane et al. (38) observed neither laboratory evidence of riboflavin deficiency nor regression of the tumors. One possible reason for the failure of the drug to produce riboflavin deficiency under these conditions in man was that in the doses administered the drug appeared to undergo rapid and complete degradation. Within 24 hr of administration of U-2112, more than 90% of the dose could be recovered as metabolites in urine, suggesting that there was virtually no retention of the drug by the body. Clinical studies of another riboflavin derivative, sodium-6,7-dimethyl-9-(2'-hemisuccinoyethyl)-isoalloxazine (U-6538) were limited by the development of flank pain, oliguria, and crystalluria in patients who received large doses. The crystalluria observed was due to a metabolite of U-6538 (39). There was little evidence clinically that riboflavin deficiency was produced by this drug and further studies with it have not been reported.

Later, Lane et al. (37) reported results in 6 patients with advanced cancer treated with galactoflavin, in whom manifestations of riboflavin deficiency were clearly demonstrable 10 to 25 days after the start of drug therapy. One patient was believed to have a decrease in tumor size and 2 others had an apparent decrease in tumor growth rate.

Recent observations by this group suggest that riboflavin deficiency induced by dietary means in combination with galactoflavin may be useful in the treatment of certain patients with polycythemia vera and lymphoma (36). Patients were treated for periods of 1 to 5 months with this regimen. Partial remissions were obtained in 1 of 2 patients with Hodgkin's disease and in 2 of 4 patients with lymphosarcoma. A prolonged remission was observed in 2 patients with polycythemia vera. The long-term implications of the observation that riboflavin deficiency may inhibit certain tumors will be awaited with great interest. Extensive clinical trials will be required before the value of this treatment can be established definitively (40).

Riboflavin antagonists have been useful in experimental studies in both animals and man by producing a deficient state more rapidly than can be achieved by dietary means alone. After restriction of the dietary intake of riboflavin, tissue levels of the vitamin and its derivatives are depleted very slowly, in large measure because of the considerable magnitude of tissue stores and their slow rates of turnover (54). The symptoms and signs produced by antagonist-induced deficiency resemble those of dietary deficiency, but certain features, namely, erythroid hypoplasia, morphological changes in the red cell precursors, and peripheral neuropathy, appear to be more prominent following the use of riboflavin antagonists (40). The therapeutic use of antagonists is largely empirical, inasmuch as the exact mechanism of their action is unknown. Galactoflavin, for example, increases the urinary excretion of riboflavin (37), but it is not known how this is brought about. The potential value of these agents might be greater if more knowledge could be obtained about the normal tissue responses to riboflavin deficiency, the regulation of flavin metabolism in tumors, and the structure-activity relationships of derivatives of riboflavin.

**CHEMICAL CARCINOGENESIS**

A variety of epithelial changes, including atrophy, hyperkeratosis, alopecia, ulceration, and dermatitis, are produced in both animals and man as a result of riboflavin deficiency. Wynder and Klein (74) documented the histological changes that developed in the epithelium in mice during progressive riboflavin deficiency. The earliest change noted was atrophy of the epithelium of the esophagus and stomach, which developed during the 3rd to 5th week of deficiency. From the 7th to the 9th week of deficiency, epithelial hyperplasia and hyperkeratosis were prominent. The hyperkeratosis led to nearly complete obstruction of the esophageal lumen. The authors concluded that the epidermal atrophy and hyperkeratosis observed in riboflavin-deficient mice may...
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have been related to accompanying starvation, since normal animals with greatly restricted food intake also exhibited these changes. Hyperplasia of the epidermis, however, was not noted in starved animals and appeared to represent a more specific effect of riboflavin deficiency.

In subsequent studies by these workers (73), riboflavin deficiency in young mice was observed to increase the rate of chemical carcinogenesis in the epithelium. These experiments were performed by placing 3- to 4-week-old mice on a riboflavin-deficient diet for a 4-week period, after which they received a normal diet. Papilloma development following local application of DMBA\(^2\) and croton oil was studied in normal animals and in animals at intervals following recovery from riboflavin deficiency. In all the animals that had previously received a deficient diet, skin tumors developed earlier and in greater numbers than in control animals.

The results of other experiments, in which tumor development was studied in animals on a normal diet, a riboflavin-deficient diet, and a high-riboflavin diet, are shown in Chart 3. Animals on a normal and on a riboflavin-supplemented diet had nearly an identical number of tumors per animal and identical percentage of tumor-bearing animals among the total. By contrast, in animals on a deficient diet, both the total number of tumors per animal and the percentage of animals that developed tumors, were greatly increased. Tumors also arose more rapidly after application of the carcinogen to the skin of animals on a deficient than on a normal or high-riboflavin diet. Tumors did not appear until 4 weeks in the animals on a normal or a high-riboflavin diet but were noted after only 2 weeks on the low-riboflavin diet. In animals that underwent caloric restriction alone, the tumor incidence was similar to that of control animals fed a normal diet \textit{ad libitum}. Roe (58) also observed that the feeding of a diet with a very high level of flavin had little if any effect upon inhibiting the development of skin papillomas in mice treated with 9,10-dimethyl-1,2-benzanthracene.

When the carcinogenic hydrocarbon, DMBA, was placed on the skin of deficient animals and a diet containing normal amounts of riboflavin was fed, an increase in skin tumor formation occurred (10). Under these conditions, there was a marked increase in the activity of aryl hydrocarbon hydroxylase, an enzyme that metabolizes carcinogens. The authors suggested that increased activity of this enzyme could enhance the formation of reactive intermediates of DMBA and lead to increased binding of carcinogenic compounds to DNA.

Other systematic investigations of chemical carcinogenesis in relation to riboflavin have largely been restricted to the azo dyes. Numerous reports indicate that hepatic carcinogenesis by azo dyes is potentiated by riboflavin deficiency and that riboflavin administration to deficient animals inhibits the development of hepatomas (18, 29, 34, 44, 49, 63). These studies suggest a specific relationship between nutrition and the growth of a tumor in experimental animals.

The accelerated rate of growth of azo dye-induced tumors that occurs in riboflavin deficiency has been observed in animals treated with dietary vitamin restriction alone, with structural analogs of riboflavin, or with a deficient diet in combination with the riboflavin analog (18, 34, 44). Each method appears to be suitable provided that the hepatic flavin concentration is significantly depleted below normal levels. The hepatic flavin concentration appears to be the critical factor, and with any given diet the incidence of hepatomas in experimental animals after treatment with azo dyes is inversely proportional to the concentration of riboflavin in liver (18). Furthermore, a given dose of azo dye carcinogen that is too small to produce tumors in normal animals is highly effective in animals that have been treated previously with a particular riboflavin analog (49).

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4 The abbreviations used are: DMBA, 7,12-dimethylbenzanthracene; DAB, 4-dimethylaminoazobenzene; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FIGLU, formiminoglutamic acid.
More recent studies on the relationship of riboflavin to azo dye carcinogenesis have been conducted by Lambooy et al. (4, 34, 35). Further documentation of the protective effect of riboflavin against a variety of tumors induced by azo dyes has been provided. In addition, it has also been shown that, after treatment of experimental animals with an azo dye carcinogen of extremely high potency, 3'-methyl-4'-ethyl-4-dimethylaminoazobenzene, riboflavin does not apparently inhibit tumor formation (4).

The increased carcinogenicity of azo dyes in riboflavin-deficient animals may be due in part to the fact that the hepatic metabolism of these drugs appears to involve flavin-containing enzymes. The demethylation of DAB and other azo dyes, and the cleavage of the azo linkage, are both believed to require flavin cofactors (45). Suggestive evidence is that the addition of flavins in vitro to the tissue preparations greatly enhances the activities of the enzymes degrading the azo dyes. Definitive proof that FAD is the cofactor for these enzymes remains to be obtained, however. The hepatic concentration of FAD is decreased to one-third of normal in riboflavin-deficient animals, and there is therefore less FAD available to stabilize a variety of flavoprotein apoenzymes (54). It is likely that, under these circumstances, inactivation of the enzymes that degrade azo dyes would occur. As a result of such inactivation, the actual dose of carcinogen delivered to the liver of riboflavin-deficient animals may be greatly increased over that of animals on a normal diet.

The observation that azo dye reductase activity is diminished in livers of riboflavin-deficient rats has been confirmed by Williams et al. (72) who called attention to the fact that considerable enzyme activity is detectable in cecal contents. The activity per mg protein in cecal contents in fact was 6 times higher than in liver and also showed a significant decline in riboflavin-deficient animals. These data suggest that bacterial flora as well as the host liver degrade azo dye carcinogens and that dietary riboflavin regulates enzyme activity at both sites.

In addition to influencing the activities of enzymes that inactivate azo dyes, riboflavin may influence azo dye carcinogenesis by another mechanism. Tung and Lin (67) observed a quenching effect of 2 azo dyes, DAB and 4-aminoazobenzene, on riboflavin fluorescence and proposed the formation of a complex between these drugs and riboflavin in aqueous solution. Riboflavin has been effective in solubilizing the azo dyes. These investigators suggested that the direct complexing of riboflavin with the azo dyes may block their action as carcinogens at tissue receptor sites. The postulated riboflavin-azo dye complex has not been isolated or characterized further and requires further substantiation.

Another and perhaps related effect of the administration of azo dyes is to lower the hepatic flavin concentrations (28, 44, 67) and the activities of FAD-dependent enzymes, such as xanthine oxidase (71). Kensler et al. (29) showed that the total flavin concentration in livers of animals treated with DAB is reduced to less than 60% of that of control animals. Several compounds that are structurally related to DAB but are not carcinogens do not appear to lower the hepatic flavin concentration (18). The carcinogenicity of a number of azo dyes has in fact been correlated with the extent to which they decrease the flavin levels in liver (17). The decreased hepatic flavin concentrations that are observed after azo dye treatment may arise by at least 2 mechanisms. (a) Increased urinary excretion of riboflavin has been suggested to occur after treatment with azo dyes (52). The factors responsible for the riboflavinuria need to be determined more precisely. (b) Enhanced enzymatic degradation of FAD. During a study of the reduced hepatic flavin content produced by DAB, Yang and Sung (75) noted that treatment with this drug lowers the hepatic FAD fraction proportionately more than the hepatic FMN or riboflavin fractions. Rubenchik (59) also observed that FAD was particularly depressed in concentration in DAB-treated animals.

To explore a possible mechanism accounting for the marked depletion of FAD in tumors from DAB-treated animals, Yang and Sung (75) developed an assay for nucleotide pyrophosphatase, which besides other reactions catalyzes the degradation of FAD to FMN. The results of this assay of the various flavin fractions in normal liver and in hepatic carcinoma produced by DAB treatment are shown in Table 1. Nucleotide pyrophosphatase activity was 3 times as great in the carcinoma as in the control liver. In the carcinoma, FAD concentration was reduced to one-seventh that of liver, but FMN and free riboflavin concentrations measured together were two-thirds as great as in liver. The total nitrogen concentration was nearly the same in liver and in carcinoma. These authors also found relatively low activity of FAD pyrophosphorylase, which synthesizes FAD from FMN. An increase in the rate of enzymatic FAD

| Table 1 |

| Nucleotide pyrophosphatase activity and flavin concentration in rats treated with DAB |
|-----------------|-------------------|------------------|------------------|
|                  | Nucleotide pyrophosphatase activity (μM FAD degraded/g tissue/hr) | Flavin concentration (μg/g tissue) | Nitrogen concentration (g/100 g tissue)* |
|                  | Total | FAD | FAD + riboflavin | Total |
| Liver            | 2.8   | 21.77 | 18.91 | 2.86 | 2.85 |
| Carcinoma        | 8.4   | 4.53  | 2.70  | 1.83  | 2.49 |

* Measured by the micro-Kjeldahl method.

Data are derived from the work of Yang and Sung (75). Liver carcinoma was induced by the feeding of a diet containing 0.06% DAB in brown rice for more than 6 months to rats of the Long-Evans strain.
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degradation relative to synthesis would be expected to diminish the FAD fraction particularly.

In a recent report (9), riboflavin deficiency reduced the activity of the hepatic enzyme that metabolizes 3,4-benzpyrene, a potent carcinogen, to less than one-third of that observed in normal animals. Diminished activity of this degradative enzyme might be expected to alter the rate of carcinogenesis by 3,4-benzpyrene, but no information is available on this point in vivo in riboflavin-deficient animals.

In summary, riboflavin deficiency potentiates carcinogenesis by azo dyes. This effect may be due in part to diminished activities of presumed flavin-dependent enzymes that inactivate the azo dyes. The enhanced growth rates of tumors induced by azo dyes is in sharp contrast to the reduced rates of growth of spontaneously developing and transplanted tumors noted above in riboflavin-deficient animals. Azo dyes, in turn, decrease the hepatic concentration of FAD in host animals.

**DRUG METABOLISM IN CANCER**

The potentiation of azo dye carcinogenesis appears to be one of a number of effects of riboflavin deficiency upon drug metabolism. The finding that the hepatic activity of microsomal TPNH-cytochrome c reductase, a major drug-metabolizing enzyme which has FAD as its cofactor, is reduced in livers of riboflavin-deficient rats (57) suggests that the effects of many other drugs both toxic and therapeutic may be enhanced under these conditions. The hepatic activities of drug-metabolizing enzymes appear to be quite variable in riboflavin-deficient animals. The observation that the pharmacological effect of hexobarbital, as measured by sleeping time in mice, is markedly increased in riboflavin-deficient animals is compatible with diminished drug-metabolizing enzyme activity in vivo (9). Kato et al. (27) have shown that many of the drug-metabolizing enzymes are reduced in activity in livers of tumor-bearing animals. If the effects of riboflavin deficiency and of bearing a tumor upon drug metabolism are additive, then it is likely that riboflavin deficiency produced in a tumor-bearing animal would depress drug inactivation rates to an extreme degree. At the present time knowledge of the metabolism of drugs in riboflavin-deficient animals bearing transplanted tumors and in nutritionally depleted patients with cancer is scanty.

The effects of riboflavin upon folic acid metabolism may have implications for the use of folic acid antagonists in cancer chemotherapy. In riboflavin deficiency, the concentration of folic acid is reduced in serum (14). Injection of tritium-labeled folic acid to riboflavin-deficient animals results in a much greater fraction of the isotope excreted in urine than that observed in normal animals (50). Suggestive evidence of an alteration in the metabolism of folic acid in riboflavin deficiency has been the observation that, in animals that have received a deficient diet for 11 to 30 days, the urinary excretion of FIGLU following a histidine load is markedly reduced. The excretion of FIGLU is decreased whether the histidine is administered p.o., i.p., i.m., or i.v. (51). Riboflavin-deficient animals have a defect in the conversion of folic acid to N5'-methyltetrahydrofolate in liver (23). Riboflavin deficiency appears to decrease the hepatic activities of 2 enzymes involved in folic acid metabolism, N5'-methylene tetrahydrofolate reductase and N5'-methyltetrahydrofolate transferase, and this may result in accumulation in liver of tetrahydrofolate compounds other than N5'-methyltetrahydrofolate (50). Another group of investigators (7, 53) have observed an increase rather than a decrease in the urinary excretion of FIGLU following a histidine load in riboflavin-deficient animals and have suggested that the major defects are in the conversion of folate to tetrahydrofolate and in tetrahydrofolate dehydrogenase activity. Thus, it is likely that defects in the metabolism of folic acid occur in riboflavin deficiency, since flavin cofactors are involved in certain important metabolic transformations of folic acid. The nature and specificity of these defects require further clarification.

Another effect of riboflavin upon drug metabolism in cancer is that upon the transport of certain drugs into neoplastic cells. Hakala (19) showed that, in Sarcoma 180 cells grown in tissue culture, riboflavin inhibits the net rate of uptake of amethopterin (methotrexate). Riboflavin in the concentration usually present in tissue culture medium is sufficient to cause this effect. By contrast, Goldman et al. (16) reported that riboflavin enhances the net transport of methotrexate into L1210 mouse leukemia cells. These differences in results obtained appear to have been resolved by the subsequent studies of Lichtenstein and Goldman (41), who reported that riboflavin and methotrexate are involved in 2 separate processes. (a) Riboflavin competitively inhibits methotrexate influx. Methotrexate and riboflavin were mixed at the moment of contact with cells of L1210 mouse leukemia grown in cell culture. As shown in Chart 4, measurements of the time course of uptake of methotrexate revealed that riboflavin at a concentration of 290 μM appreciably inhibits influx of the drug. In subsequent

![Chart 4. Time course of uptake of 0.1 μM MTX in L1210 mouse leukemia cells in the presence (●) and absence (O) of 290 μM riboflavin. Both compounds were mixed together at the instant of exposure to the cells. From Lichtenstein and Goldman (41) with the permission of the publisher.](chart.png)
experiments, increasing the riboflavin concentration in the extracellular medium while maintaining a constant methotrexate concentration resulted in progressive reduction in methotrexate influx. Analysis of kinetic data suggested that the mechanism of inhibition was competitive. (b) Riboflavin appears to undergo a photochemical reaction with methotrexate. In the dark, the spectrum of methotrexate was not affected by the addition of riboflavin to the solution. By contrast, when methotrexate and riboflavin in solution were exposed to light, a spectral shift occurred. These and other experiments suggested that incubation of methotrexate with riboflavin resulted in the production of new compounds the elution pattern of which on column chromatography differed from that of methotrexate (41).

The degradation products of methotrexate that are produced photochemically have not been characterized fully. They appear to be transported across the cell membrane by a carrier system different from that of methotrexate. In addition, unlike methotrexate, these derivatives are not bound to dihydrofolate reductase. It is likely that these 2 competing processes may govern the net uptake of methotrexate and its derivatives by tumor cells in vitro in tissue culture.

Thus, riboflavin may determine to some extent the effectiveness of folic acid antagonists in chemotherapy by regulating the metabolic transformations. Additionally, the transport of methotrexate across cell membranes is affected by riboflavin.

EFFECTS OF CANCER ON TISSUE FLAVIN LEVELS

In tumors that have been induced by azo dyes, FAD concentrations are markedly reduced, as noted above, an effect that may be due in part to increased riboflavin excretion and to enhanced FAD degradation (75). In this instance a carcinogen has a direct effect upon tissue flavin levels.

A more general effect of cancer on flavin levels was revealed by the studies of Morris and Robertson (47, 48) noted above, who demonstrated for the 1st time that neoplastic tissue differs from normal tissue in the rate at which total riboflavin concentration decreases with increasing duration of riboflavin deficiency. These investigators observed that, in mammary tumor grown in riboflavin-deficient mice, the flavin levels in the tumor decreased at a much slower rate than that recorded in either liver or muscle of the host animal. It was suggested that the time at which the greatest decrease in tumor flavin levels occurred, the 3rd to 5th week of vitamin deficiency, corresponded to the point at which the tumor growth rate was first observed to decrease. In this study neoplastic tissue compared to normal tissues exhibited a relative resistance to riboflavin deficiency. These studies comprised measurements of the total flavin content of the various tissues and did not distinguish among FAD, FMN, and free riboflavin.

Clinical studies on riboflavin metabolism have demonstrated that patients with cancer tend to excrete lower than normal amounts of total riboflavin in urine (25). Measurements of total urinary riboflavin excretion of 1000 patients with a wide variety of cancers, including cancer of the stomach, breast, skin, uterus, and lung, and in other nonneoplastic diseases was reported by Kagan (25). He observed that patients with cancer regardless of the site of origin, or whether the lesion is primary or metastatic, excrete virtually no riboflavin in urine in 80% of the cases, whereas the great majority of normal individuals have detectable amounts of the vitamin in urine. Furthermore, administration of an p.o. dose of riboflavin results in markedly less urinary excretion of riboflavin in cancer patients than in normal individuals. These results are compatible with increased uptake and/or increased retention of riboflavin by neoplastic tissue but do not exclude other effects, such as altered intestinal absorption or greater uptake and binding by host tissues. Unfortunately, the actual quantity of riboflavin excreted in the urine was not indicated in this report nor was the dietary intake of the vitamin regulated in the patients studied.

In a clinical study of patients with cancer of the larynx the concentration of riboflavin in urine and blood was determined before and after the administration of a load of riboflavin (31). Results were then compared with those obtained in patients with benign diseases of the larynx, including chronic laryngitis, laryngeal papillomas, and fibromas, as well as in normal subjects. In the patients with cancer, the plasma concentration of riboflavin was one-half as great and the urinary excretion was one-third as great as in the other groups of patients. After receiving a riboflavin load, the patients with cancer had lower concentrations of the vitamin in both plasma and urine than did those of patients with benign disorders. The plasma concentrations of riboflavin could be correlated with the stage of the disease in the patients with laryngeal cancer. After effective treatment, riboflavin concentrations increased to normal and, in patients in whom treatment was unsuccessful or in whom a recurrence of the disease occurred, riboflavin concentrations in plasma remained at a low level.

In animals that have received transplants of sarcomas, epitheliomas, and asciites hepatomas, riboflavin excretion may increase initially, but it decreases below normal when the tumor growth has become more advanced (66). The effects of transplanted tumors upon the flavin concentrations of host tissues were measured by Aptekar (2) who reported that, in rats that have received transplants of ascites hepatoma, the concentration of FAD is reduced approximately one-third in liver and reduced slightly less in kidney and heart. The FMN and riboflavin concentrations in the organs of normal and of tumor-bearing animals do not differ from one another. Transplantation of 4 other tumors, including several varieties of sarcomas, had no effect upon the tissue concentration of FAD, FMN, or riboflavin. In mice that have received transplants of hepatoma and sarcoma, FAD concentrations were decreased by one-fourth in host kidney and liver, but riboflavin and FMN concentrations in these organs were not altered (3).

In a recent study, suspensions of Novikoff hepatomas, a rapidly growing, undifferentiated tumor, were trans-
planted (24) into the peritoneal cavity of normal and of riboflavin-deficient rats. Animals were sacrificed at intervals after transplantation, and simultaneous measurements were made of the concentrations in tumor of FAD, FMN, and riboflavin. As shown in Table 2, the FAD concentrations in tumor, in contrast to those in liver, were completely unaffected by riboflavin deficiency. FMN concentrations in tumor grown in riboflavin-deficient rats were decreased appreciably below levels in tumor grown in normal animals but the concentration of FMN in the tumor of deficient animals (36% of normal) was still decreased significantly less (p < 0.001) than in liver (20% of normal) of these animals.

Free riboflavin concentrations in specimens of Novikoff hepatoma were extremely variable. In tumor grown in deficient animals (Table 2), free riboflavin concentrations were greatly diminished to 16% of those measured in tumor from normal animals. The proportional decrease in free riboflavin concentrations in tumor was significantly greater than that of either FMN or FAD (p < 0.001).

These results indicate that the Novikoff hepatoma shows a remarkable resistance to riboflavin deficiency. FAD concentrations are completely intact and FMN levels are decreased proportionately less than are those of host liver. Aptekar (2, 3), as noted above, reported a decrease in the hepatic concentration of FAD in animals bearing I form of ascites hepatoma but no change in either FAD, FMN, or riboflavin concentrations in animals bearing other varieties of transplanted tumors.

One physiological mechanism that appears to be involved in the adaptation of normal animals to riboflavin deficiency is the increase in hepatic FAD pyrophosphorylase activity. The relatively greater decrease in FMN than in FAD concentrations in liver of deficient animals (8, 9, 13, 33, 46) may be mediated in part by an adaptive increase in the activity of this enzyme, which converts FMN to FAD (13). In the Novikoff hepatoma grown in riboflavin-deficient rats, FMN levels decrease proportionately more than FAD levels but no increase in FAD pyrophosphorylase activity occurs (56). It is likely, therefore, that Novikoff hepatoma conserves FAD by different mechanisms than does liver. These mechanisms may include more efficient uptake, greater trapping, tighter binding to tissue flavoprotein apoenzymes, or a decrease in the rate of enzymatic FAD degradation. Information on these points is not available at present. The fact that the free riboflavin content of the Novikoff hepatoma is depleted to an extremely low level suggests that one of the mechanisms that permit tumor to preserve its concentrations of flavin coenzymes required for vital metabolic processes may be sacrifice of the relatively small and dispensable supply of stored free vitamin.

The thyroid hormonal regulation of flavoprotein enzymes has been examined in both well-differentiated (Morris hepatoma 7800) and poorly differentiated (Novikoff) hepatomas in rats. The induction by thyroid hormone of the FAD-dependent enzyme, mitochondrial \( \alpha \)-glycerophosphate dehydrogenase, 5- to 10-fold in normal liver, is undetectable in Novikoff hepatoma, is diminished but present (2-fold) in Morris hepatoma 7800, and is completely intact in livers of rats bearing either tumor (24). Karsten et al. (26) have also shown no increase in activity of this enzyme in several transplanted hepatomas after treatment with thyroid hormone.

These studies indicate in sum that flavin concentrations and activities of flavoprotein enzymes in tumor tissue are relatively resistant to both hormonal and nutritional regulation. Neoplastic liver differs from normal liver in the total concentrations and relative amounts of riboflavin and its coenzyme derivatives.

### FUTURE PERSPECTIVES

The relationship of riboflavin to cancer is part of a larger problem of elucidating the role of nutritional factors in the initiation and spread of cancer. As noted earlier, the rate of growth of transplanted tumors may frequently be slowed by reducing the intake of food. Similarly, lowering the riboflavin intake alone may reduce the rates of growth of transplanted tumors. We must learn how tumor growth is altered by the lack of a specific nutrient. Because of the wide distribution of flavin coenzymes, involving reactions in fat, amino acid, carbohydrate and vitamin metabolism (54), the effects of riboflavin deficiency upon the metabolism and growth of tumors are likely to be complex. It is essential to learn which of the consequences of riboflavin deficiency are most critical for the maintenance of the neoplastic cell.

The finding that there is increased carcinogenicity of azo dyes in riboflavin-deficient animals, probably in large measure as a result of a flavin requirement for inactivation of these drugs, points out the need to learn more about the pathways involved in carcinogen degradation. One may ask

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**Table 2**

Concentrations of riboflavin, FMN, and FAD in Novikoff hepatoma grown i.p. in normal and riboflavin-deficient rats.

Values in Novikoff hepatoma from normal animals are: riboflavin, 0.069 ± 0.024 μg/g fresh wt.; FMN, 0.460 ± 0.045 μg/g fresh wt.; FAD, 1.82 ± 0.50 μg/g fresh wt. Data are derived in part from work of Rivlin et al. (3).

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Riboflavin</td>
</tr>
<tr>
<td>Normal</td>
<td>22</td>
</tr>
<tr>
<td>Riboflavin-deficient</td>
<td>32</td>
</tr>
</tbody>
</table>

*All values expressed as percentages of the concentrations measured in tumors grown in normal animals (mean ± S.E.)
whether FMN and FAD are involved in the inactivation of other classes of carcinogens. The increased rate of chemical carcinogenesis in the epithelium of deficient animals (73) raises the possibility that flavin coenzymes may be involved in the conversion of certain carcinogens to more active metabolites.

Future studies of riboflavin and cancer will require tracer methodology in order to determine the uptake, binding turnover, and removal of drugs, hormones, and vitamins. Tumors may differ from normal cells in the nature of tissue receptors and in the affinity of binding both intracellularly and at the cell surface. The turnover and pool size of riboflavin and its derivatives must be determined in both normal and neoplastic tissues. The relative resistance of the neoplastic liver to riboflavin deficiency could be due to a number of factors, such as more efficient trapping, more effective synthesis, slower turnover, or tighter binding of flavin derivatives, as well as to the sacrifice of the free riboflavin fraction noted here.

Clinical studies of riboflavin metabolism in patients with cancer will necessitate advances in methodology, if the concentrations of the vitamin in blood and urine and the uptake into tumors are to be determined accurately and rapidly. The reduced excretion of riboflavin by patients with cancer (25) may represent a specific defect and must be studied under controlled conditions. It is hoped that increased use will be made of antagonists of riboflavin both experimentally and clinically. The coordinated efforts of investigators in biochemistry, oncology, nutrition, and clinical medicine will be required to attack these problems.

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