VITAMINS AND HORMONES

ADVANCES IN RESEARCH AND APPLICATIONS

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VOLUME XIII

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EDITORS' PREFACE

The Editors are pleased to present this thirteenth volume of *Vitamins and Hormones.*

This volume contains nine chapters by authors in four countries, five related to vitamins, two dealing with other nutritional topics, and two concerned with hormones. This distribution of chapters is comparable to that of Volume XII, which contained four chapters on vitamins, one on other aspects of nutrition and three on hormones; however, it is the reverse of Volumes X and XI in which the emphasis was strongly toward the side of hormones. In general, the Editors aim at a rough balance in the number of chapters in each volume, because the rate of advance in the two fields is approximately the same, and because there is increasing interrelation between studies of vitamins, which are external regulators, and of hormones or internal regulators. This aim is, unfortunately, seldom successful, because manuscripts are usually promised several years ahead and often the authors subsequently find that they cannot comply with a fixed schedule. When the Editors, from a continual survey of the field, consider that a subject is mature enough to warrant a critical review, they arrange for a competent author to prepare it, and his manuscript is published as soon as possible after it is received. Irregularities in balance are therefore unavoidable.

The Editors have been impressed by the devotion of scientists who have been willing to interrupt their research activities so that they may serve their colleagues by preparing these reviews.

The two chapters on Vitamin B_{12}, together with the one in the previous volume, comprise a rather complete treatment of the chemistry, physiology and chemotherapeutics of this vitamin, as far as was known up to the spring of this year. (The structure of Vitamin B_{12} has recently been described in *Nature* August 20, 1955, p. 325.) Similarly the two closing articles on hormone action, together with the two final articles in Volume X, give a fairly complete survey of modern views on the mode of action of the steroid hormones. Thirdly, each of the last four volumes has carried a chapter dealing with the relation between nutrition and

specific diseases; these complement one another, though it can hardly be claimed that together they give anything like coverage of this very large field.

The Editors are always glad to receive other suggestions, either of new topics which warrant review, or of fields like the above which, through partial previous coverage, have been brought to the point where an additional review will complete the presentation of a whole area of research.

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The Role of the Vitamins in Antibody Production

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I. Introduction

The search for dietary factors which could influence the resistance-susceptibility of a host to infectious disease has been prosecuted vigorously for many years. These researches have been motivated by the hope that suitable manipulation of diet might influence the incidence and course of the infection for the benefit of the host. The mass of voluminous and often conflicting data in this field will not be treated further, since it has already been the subject of many reviews (Clausen, 1934; Robertson, 1934; Aycock and Lutman, 1944; Schneider, 1946; Clark *et al.*, 1949; Schneider, 1951; Axelrod, 1952). Antibodies have been identified as important determinants of immunity, particularly acquired immunity. Investigations on diet and resistance to infection have, therefore, been logically extended to include studies on the relationship between dietary components and antibody production. Interest in this field stems also from the following considerations. The mechanisms of antibody synthesis are, at present, obscure. It will be shown in this paper that the dietary

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intake of certain vitamins can markedly affect the extent of antibody synthesis. This ability to regulate antibody production offers possibilities as a powerful tool for unraveling some of the mysteries of antibody synthesis. Since antibodies are considered to be highly specialized proteins, these studies are essentially probing into the mechanisms of protein synthesis. It will also be pointed out that the extent of antibody synthesis may, under certain conditions, represent a very sensitive criterion of dietary adequacy. Thus, the nutritionist may be able to include the magnitude of antibody response to an antigenic stimulus among his measurements for the evaluation of nutritional status.

The present paper will be confined to a discussion of the role of the vitamins in antibody production. Actually, with the possible exception of the proteins (Cannon, 1945), very little attention has been given to the relationships of other dietary constituents to antibody synthesis. A review of the older literature in this field reveals an extensive volume of conflicting data with no unequivocal evidence to indicate that any specific vitamin deficiency invariably leads to impaired antibody production. An accurate assessment of many of these earlier researches is difficult because of the questionable specificity of the deficiency under study as well as the failure to utilize adequate inanition controls. These studies have been reviewed in the literature references cited above, and it is not felt that detailed discussion of them is warranted at this time. We will concern ourselves with the more recent studies in this field, which have been conducted mainly with modern techniques designed to produce well-defined deficiency states and which have utilized adequate controls. It shall be our purpose to survey the present trends in this field, to indicate the gaps in our knowledge, and to suggest further application of nutritional techniques to various immunological problems.

II. Effects of Vitamin Deficiencies on Antibody Production

1. Fat-Soluble Vitamins

Greene (1933) has reported that vitamin A-deficient rabbits produce lower hemolysin titers than controls. Agglutinins against *B. typhosus* were, however, present in nearly the same amounts in both groups. Blackberg (1928) has presented very meager data which indicate a similar depressant effect of vitamin A deficiency in rats. Ludovici and Axelrod (1951a), using human erythrocytes as the antigen, found a moderate reduction in the hemagglutinin production of vitamin A-deficient rats. Lassen (1930, 1934) observed a slight reduction in agglutinin titers of vitamin A-deficient rats with a concomitant large decrease in resistance to infection. Natvig (1942) similarly reported an impaired resistance to

infection with no effect on agglutinin production. Others have failed to find any effect of a vitamin A deficiency upon antibody synthesis (Zilva, 1919; Werkman, 1923; Cramer and Kingsbury, 1924; Simola and Brunius, 1933; Jusatz, 1936; Feller *et al.*, 1942; McCoy and Sensenich, 1954). The variable nature of the reported data on the relationship of vitamin A to antibody synthesis is apparent. Certainly, the weight of evidence does not permit the assignment of any major role in antibody synthesis to vitamin A.

With the exception of the inconclusive experiments of Blackberg (1928), there is substantial agreement on the inability of a vitamin D deficiency to affect antibody production (Greene, 1933; Jusatz, 1936; Natvig, 1942; McCoy and Sensenich, 1954; Ludovici and Axelrod, 1951a).

Vitamin E deficient chicks produced normal amounts of antibodies to porcine gamma globulin (McCoy and Sensenich, 1954).

2. Ascorbic Acid

Hartley (1942) concluded that the control of dietary factors was very important during the biological standardization of diphtheria toxoid in guinea pigs. The differences observed between various laboratories were ascribed to the use of either mangolds (forms of *Beta vulgaris*) or cabbages as sources of ascorbic acid, the latter having a considerably higher ascorbic acid content. These experiments indicated that a dietary inadequacy of ascorbic acid interfered with the immunological response to diphtheria toxoid. In a further study in which graded doses of ascorbic acid were fed to large groups of guinea pigs, Hartley (1948) noted that the diphtheria antitoxin production was significantly higher in the groups receiving the larger dosages of the vitamin. Long (1950), in a well-controlled experiment, found a very slight reduction in diphtheria antitoxin production in ascorbic acid-deficient guinea pigs on primary injection of the toxoid but a very marked decrease in secondary antitoxin production. Several investigators have reported increased antibody production following the administration of ascorbic acid to animals receiving adequate diets (Jusatz, 1936; Hochwald and Schwarz, 1937; Madison and Manwaring, 1937; Raffel and Madison, 1938). Natvig (1942) found an adverse effect of ascorbic acid deficiency on resistance to infection with no reduction in agglutinin titer. Others (Zilva, 1919; Simola and Brunius, 1933) concur in the lack of effect of ascorbic acid deprivation upon antibody production. No positive conclusions regarding the relationship between ascorbic acid and antibody production appear to be warranted at the present. The experiments of Hartley and Long offer strong support for such a role of ascorbic acid. Further experimentation in this field involving the use of inanition controls is required.

3. Individual Members of the Vitamin B Complex

Since many workers in this field have investigated the effects of more than one vitamin within a uniform experimental framework, it seems advisable to discuss the experimental work of each group separately rather than to attempt a subdivision on the basis of the individual vitamins. It is felt that a more coherent picture can be presented in this manner. Those recent researches purporting to show the absence of any effects of vitamin deficiency states upon antibody production will be discussed first.

Saslaw *et al.* (1946) reported that a "vitamin M" (folic acid) deficiency in monkeys did not affect the neutralizing humoral antibody response to influenza virus A or the precipitin response to infection with *Streptococcus hemolyticus*, Group C. The small number of animals employed together with the paucity of actual data makes it difficult to draw any positive conclusions regarding antibody response in this deficiency state. Ruchman (1946) has investigated the effects of several aspects of malnutrition upon neutralizing antibodies in mice following the antigenic stimulation of formalinized Western equine encephalomyelitis. He observed that the removal of either thiamine or riboflavin from the diet led to no significant changes in immune response. These experiments were unfortunately complicated by the necessity of adding suboptimal amounts of thiamine to the thiamine-deficient diet in order to keep the animals alive. Further, the antibody response of the controls in this experiment was lower than that usually obtained. In the author's words, "until more is done on this problem, one cannot be too certain about the complete lack of effectiveness of the removal of thiamine and riboflavin to inhibit the immune response after vaccination."

Leftwich and Mirick (1949) observed that the feeding of a pyridoxine-deficient diet to weanling mice for 6 days subsequent to inoculation with pneumonia virus of mice (PVM) did not affect the humoral response of neutralizing antibody. The short period of time available for antibody production is to be noted as well as the lack of effect of the pyridoxine deprivation upon growth of the mice. In a subsequent paper (Mirick and Leftwich, 1949), these authors determined the neutralizing antibody content of sera from *uninoculated* weanling mice maintained on a pyridoxine-deficient diet for varying periods of time up to six weeks. No effect of the deficiency upon the antibody response to the *latent* PVM was noted. The growth inhibitory effect of the deficiency was very slight. In neither experiment was the antibody response very pronounced.

Unequivocal correlations between vitamin intake and antibody production on the basis of the inconclusive data presented thus far are not

possible. The remainder of this section will be devoted to those studies which have indicated the need for the adequate intake of certain of the B vitamins in antibody formation.

During the course of investigations on the resistance of rats to infection with the nematode, *Nippostrongylus muris*, Watt (1944) has made certain observations which suggest a role for thiamine and riboflavin in antibody production. Thus, rats fed diets deficient in riboflavin or partially deficient in thiamine showed a marked reduction in resistance to a secondary infection with *N. muris*. This decreased resistance may be a reflection of impaired antibody production to the antigenic stimulus of the primary infection. Similar indications were given by experiments demonstrating that the plasma of hyperimmunized rats deficient in thiamine or riboflavin was much less protective against *N. muris* than that of control rats. In the absence of inanition controls, the specificity of action of thiamine or riboflavin remains questionable.

Little *et al.* (1950) have noted that a folic acid deficiency in young chicks inhibited the agglutinin response to *Brucella abortus*, *Pasteurella multocida*, and *Salmonella typhosa*. The ability of vaccinated chicks to resist infection with *P. multocida* was also impaired.

Stoerk and his collaborators, using well-defined deficiency states and adequate inanition controls, have studied the effects of various B vitamin deficiencies in the rat upon the antibody response to sheep erythrocytes. In their first paper these authors observed that pyridoxine-deficient rats developed antibody (hemagglutinin and hemolysin) levels far below those of either inanition controls or *ad libitum*-fed controls (Stoerk and Eisen, 1946). In a later paper (Stoerk *et al.*, 1947), the effect of a pyridoxine deficiency on hemagglutinin production was confirmed, whereas no effects of thiamine, riboflavin, or pantothenic acid deficiencies were noted. That growth retardation per se had no influence upon antibody production was indicated by (1) the ability of paired-weighed controls to attain antibody titers even higher than those of the *ad libitum*-fed controls, and (2) the fact that growth retardation of pyridoxine-deficient rats was no greater than that observed in litter mate animals deficient in riboflavin, thiamine, or pantothenic acid in which antibody response was not impaired. A pooled sample of sera from two pyridoxine-deficient rats was found on electrophoretic analysis to have lower percentages of α- and of γ-globulin than sera from animals deficient in other B factors and from controls. Further studies (Stoerk, 1950) showed that rats and mice rendered acutely pyridoxine-deficient with deoxypyridoxine over a three-week period prior to the secondary antigenic stimulus failed to give the usual anamnestic (secondary) response to sheep erythrocytes. The primary injection of this antigen was made before the induction of the

deficiency state. Electrophoretic measurements of pooled sera from these rats failed to reveal a measurable reduction of γ-globulins or of any of the other fractions.

Wertman and co-workers (Wertman and Sarandria, 1951a, 1951b, 1952; Wertman *et al.*, 1952) have conducted a series of investigations designed to ascertain whether the increased susceptibility of vitamin-deficient rats to the viable rickettsiae (*Rickettsia typhi*) of murine typhus fever could be correlated with the ability of these rats to produce circulating antibody following the antigenic stimulus. Rats with specific vitamin deficiencies were immunized with formalinized suspensions of *Rickettsia typhi*, and circulating antibodies were demonstrated by complement fixation. Both *ad libitum*-fed and inanition controls were utilized. An impaired production of circulating complement-fixing antibody was observed in pantothenic acid, thiamine, pyridoxine, riboflavin, folic acid, and vitamin B_{12} deficiencies. No effect was noted in niacin deficiency. Certain points of interest emerge from Wertman's studies. There was the further demonstration that antibody formation was not impaired in animals suffering from a severe degree of inanition. The lack of correlation between the ability to produce antibodies and growth impairment was strikingly shown in those rats receiving either a totally B-complex–deficient diet or one containing $\frac{1}{10}$ of the optimal amount of the B factors. Despite their poor growth, these animals showed no diminution in antibody-forming capacity. The ability of rats *totally deficient* in the B complex to produce antibodies is worthy of note in view of the deleterious effects of specific *individual* deficiencies. These authors have also emphasized the significance of the dosage of immunizing agent in determining the antibody response in certain deficiency states. Thus, in a number of cases, differences in antibody response between deficient and normal rats were more apparent after a single injection of antigen than after repeated injections of the same suspension of formalized rickettsiae. One wonders whether repeated administration of the crude rickettsiae could have provided the deficient rats with a sufficient quantity of a metabolite needed for antibody production. There is also the possibility that the response in a deficient animal after repeated injection is anamnestic in nature. This would suggest that the metabolic events prerequisite for the secondary response can occur following a primary injection which elicits only a poor response in circulating antibodies. In experiments to be discussed later we have frequently observed that a poor primary response to diphtheria toxoid in control rats may be followed by an excellent anamnestic response to this antigen.

McCoy and Nair (1954), utilizing urease as the antigen in studies with white, leghorn, cockerel chicks, have observed a marked decrease in

serum antiurease formation in pyridoxine and pantothenic acid deficiencies. No effects were noted in riboflavin, niacin, and biotin deficiencies. With porcine γ-globulin as the antigen in the chick, McCoy and Sensenich (1954) noted an impairment of antibody formation in pyridoxine, pantothenic acid, and riboflavin deficiencies. Deficiencies in vitamins A, D, and E, and in thiamine, biotin, and folic acid were without effect. These authors make particular note of the fact that thiamine-deficient chicks possessed normal antibody titers despite their almost comatose state. In rats, McCoy and Sensenich (1954) noted that antibody formation in response to ovalbumin as antigen was decreased in pyridoxine and pantothenic acid deficiencies. No effects were observed in thiamine and riboflavin deficiencies.

Büsing (1950) found that deprivation of pyridoxine reduced the total and globulin nitrogen in rat serum as well as hemolysin production following immunization with sheep blood. Agnew and Cook (1949) showed that the hemagglutinin response to sheep erythrocytes and the agglutinin response to formalinized *B. typhosus* was diminished in pyridoxine-deficient rats. Paired-fed controls receiving pyridoxine produced normal amounts of antibody.

The remainder of this section will be devoted to studies conducted by the authors of this review and their co-workers.

A series of experiments was performed to study antibody formation in specific vitamin deficiencies utilizing a *constant* antigen-host model system. Group O, Rh+ human erythrocytes served as the antigen in the albino rat of the Sprague-Dawley strain. Nonimmunized rats of this strain did not possess any hemagglutinins for this antigen. Paired-fed, paired-weighed, and *ad libitum*-fed animals were utilized as controls. Hemagglutinin production and, in one experiment, hemolysin production constituted the measure of antibody synthesis. Hemolysin production was low but followed the same pattern as that of the agglutinins. It was determined in preliminary experiments that the antibody response of control rats receiving the purified, "synthetic" diet employed in these studies was equal to that of rats fed a commercial foodstuff compounded from natural materials. The decreased antibody response in pyridoxine-deficient rats noted by Stoerk and Eisen (1946) was confirmed (Axelrod *et al.*, 1947). In addition, an equally severe impairment of antibody synthesis in pantothenic acid deficiency and a moderate inhibition in riboflavin deficiency were observed. The discrepancy between these latter findings and those reported by Stoerk *et al.* (1947), particularly in the case of pantothenic acid deficiency, may be attributed to the fact that the human erythrocytes utilized by Axelrod and his co-workers furnished a far stronger antigenic stimulus for hemagglutinin production in the rat

than did the sheep erythrocytes employed by Stoerk and associates. It is conceivable, therefore, that pantothenic acid and riboflavin did not become limiting factors for hemagglutinin production when the antigenic stimulus (sheep erythrocytes) was of low magnitude. This postulated interrelationship between the magnitude of the antigenic stimulus and the effect of a vitamin deficiency upon antibody production should be subjected to a critical analysis in experiments where antibody formation is determined by quantitative measurements of antibody nitrogen (Kabat and Mayer, 1948). Stoerk (1948), however, was inclined to attribute the differences observed in the two laboratories to the more severe pantothenic acid deficiency state which obtained in the studies of Axelrod *et al.* (1947). Accordingly, experiments were conducted to determine the effects of varying degrees of pantothenic acid deficiency upon hemagglutinin production utilizing "paired-weighed" inanition controls (Ludovici *et al.*, 1949). A deleterious effect upon antibody synthesis was noted in rats with a relatively mild pantothenic acid deficiency. It seems unlikely, therefore, that the variation between the two laboratories could be resolved on the basis of differences in the degree of deficiency. Continued experimentation by our group has furnished convincing proof for the role of pantothenic acid in antibody formation. At this point, it is relevant to mention that we have never noted any alteration in the effects of the vitamin deficiency states when variable amounts of antigen (human erythrocytes) have been employed. In an experiment in which the dosage of antigen was apportioned on the basis of the body weight of the rat, the results were identical with those obtained with a constant amount of antigen. Increasing the dosage of antigen far beyond that usually employed was also without effect. It will be recalled that Wertman and co-workers (Wertman and Sarandria, 1951a; Wertman *et al.*, 1952) observed an effect of variable amounts of antigen (*Rickettsia typhi*) upon antibody formation in certain deficiency states.

The relationship between vitamins and antibodies has been extended to include the effects of deficiencies of thiamine, biotin, folic acid, niacin-tryptophane, vitamin B_{12}, vitamin A, and vitamin D upon the hemagglutinin response of the rat to the antigenic stimulus of Group O, Rh+, human erythrocytes (Carter and Axelrod, 1948; Ludovici and Axelrod, 1951a). It should be re-emphasized that identical immunologic procedures were employed throughout these investigations. From these results, it was possible to classify roughly the effects of these deficiencies upon circulating antibodies into three groups as follows: Group I, severe impairment of antibody response (pantothenic acid, pyridoxine, and folic acid deficiencies); Group II, moderate impairment of antibody response (riboflavin, thiamine, biotin, vitamin A, and niacin-tryptophane

deficiencies); Group III, no impairment of antibody response (vitamin B_{12} and vitamin D deficiencies). Ludovici and Axelrod (1951b) have also shown that panthenol (alpha, gamma-dihydroxy-N-(-3-hydroxypropyl)-beta, beta-dimethyl butyramide), the alcohol analogue of pantothenic acid, was as effective as pantothenic acid in promoting antibody synthesis in pantothenic acid-deficient rats. These results were in accord with previous studies demonstrating the quantitative conversion of panthenol to pantothenic acid in the rat (Burlet, 1944; Rubin *et al.*, 1948).

During the course of these studies on the relationship of nutritional status to antibody formation, we have observed instances where the dietary requirements of a given factor for growth did not parallel its need for antibody synthesis. Thus, methionine was capable of sparing the requirement of pantothenic acid for antibody synthesis, but not for growth (Ludovici *et al.*, 1951a). In contrast, supplementation of a niacin-tryptophane–low diet with niacin produced a marked growth response with no effect upon further antibody synthesis. The administration of tryptophane promoted growth as well as antibody production. The efficacy of tryptophane is explicable in terms of its ability to serve as a direct precursor of niacin or niacin derivatives (Heidelberger *et al.*, 1949) as well as its essential function in protein synthesis. Krehl *et al.* (1946) have observed that niacin improves the utilization of tryptophane. Niacin may also exert a "sparing" effect upon the requirement for tryptophane. Whatever the mechanism, it seems clear that niacin alone cannot satisfy the requirement for maximal antibody synthesis on this basal diet. With the administration of niacin, it would appear that tryptophane becomes the limiting factor for antibody formation, although apparently sufficient amounts of this amino acid are available under these circumstances for growth purposes. The production of a more pronounced tryptophane deficiency state by employing a diet completely devoid of tryptophane would aid in clarifying the role of this compound in antibody synthesis. Of significance to the nutritionist is the observation that the process of antibody synthesis may in some instances represent a sensitive criterion of dietary adequacy which may be utilized to advantage in the evaluation of nutritional status.

In confirmation of previous findings, these studies have emphasized the failure of inanition to modify the antibody response. The lack of correlation between growth inhibition and suppression of antibody response was again apparent. This was strikingly evidenced in the thiamine and folic acid deficiencies. Thus, despite the more marked growth-inhibitory effect of the thiamine deficiency, the antibody response of the thiamine-deficient rats was much greater than that of the folic acid-deficient animals.