HISTOCHEMICAL AND HISTOLOGICAL STUDIES IN NORMAL AND FOLIC ACID AND VITAMIN B_{12} DEFICIENT RATS

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ABSTRACT

The distribution of alkaline and acid phosphatases, RNA and DNA was studied by histochemical technic in normal and vitamin B_{12} and folic acid deficient rats. Tissues were also stained with haematoxylin and eosin for histological study.

The alkaline phosphatase was increased in kidney, adrenals, spleen, testes, and thyroid in all animals made deficient in the two vitamins. In the deficient animals the acid phosphatase activity increased in liver and adrenal, and disappeared completely in the pituitary.

The vitamin B_{12} and folic acid deficient rats showed diminished RNA contents in liver, pancreas, adrenals, spleen, and testes ; diminished DNA contents in pancreas, adrenals and testes ; and increased DNA content in the spleen.

The deficent animals showed marked histological changes in liver, thyroid, testes, and spleen.

INTRODUCTION

Reproductive failure in vitamin B_{12} deficient rats, and gross underdevelopment in various tissues of newborn rats from vitamin B_{12} deficient mothers have been reported by Dryden, Hartman and Cary (1951) ; Lepkovsky *et al.* (1951) ; Richardson, Witten and Couch (1951), and Jones, Brown, Richardson and Sinclair (1955). Ferguson and Couch (1954) observed degenerative changes in heart, liver, thyroid, and kidney in chick embryos from hens deficient in vitamin B_{12}.

Sebrell and Harris (1954) have cited several references to implicate vitamin B_{12} in the process of nucleic acid synthesis. Stern, Taylor and Russell (1949) have reported a diminution in liver cytoplasmic basophilia in vitamin B_{12} deficient rats. Dempsey and Wislocki (1946) have pointed out that basophilia, shown to be ribonucleoprotein, are intimately related to protein synthesis. Hence in vitamin B_{12} deficient animals formation of nucleoprotein through the nucleoside is limited, and consequently protein synthesis in the body would be retarded. Rasch *et al.* (1955) have reported a diminution in liver nucleic acids in vitamin B_{12} deficiency. Rose and Schweigert (1952) have observed degenerative changes in testes and thyroid in vitamin B_{12} deficient rats. Biochemical studies of liver (Rose and Schweigert, 1952 ; Schweigert, Scheid and Downing, 1954) have shown both DNA and RNA to be decreased per gram of liver in vitamin B_{12} deficient rats. Reports of tissue abnormalities of folic acid deficiency have not been reported so far.

It is presumed that phosphatases play an exceedingly important rôle in the metabolism of carbohydrates, lipids, and nucleoproteins. Colowick *et al.* (1947) have reported that certain hormones (e.g., of adrenal cortex, pituitary, and pancreas) act on hexokinase, which are very closely related to phosphatases. Kellerman (1955) has indicated a rôle of phosphatases in dephosphorylation, and now it is understood that phosphatases act as catalyst in the process of dephosphorylation of certain phosphate esters. Possible relationship of phosphatases to various hormones is also indicated by Dempsey, Greep and Dean (1949) and Mathies, Goodman and Palm (1952).

Ling and Chow (1952, 1953, 1954) have presented evidences to show that vitamin B_{12} deficiency causes derangements in carbohydrate and lipid metabolism. Williams, Nichol and Elvehjem (1949) have indicated the rôle of folic acid in carbohydrate metabolism.

The present work deals with the studies on the distribution of alkaline and acid phosphatases, ribonucleic acid, and desoxyribonucleic acid in the various tissues of normal and folic acid and vitamin B_{12} deficient rats. Histological studies of the tissues of these animals were also undertaken.

Materials and Methods

Weanling male rats weighing between 30 and 35 gm. were divided into two groups with approximately the same weights. They were fed *ad-libitum*, a purified vitamin B_{12} and folic acid free diet (Fatherpaker *et al.*, 1955). Casein was made vitamin free by hot alcohol extraction. Microbiological tests showed no detectable amounts of the two vitamins in the casein preparation used. The salt mixture No. 2 (U.S.P.) was finely powdered and mixed uniformly with the diet. Rats receiving the folic acid and vitamin B_{12} free purified diet with an oral supplement of 0·5 μg of vitamin B_{12} and 8 μg of folic acid per animal per day, were taken as normal controls. The experimental group received 0.3 per cent of a preparation of iodinated casein and sulphasuxidine at a level of 1 per cent in the diet itself, without any supplement of folic acid and vitamin B_{12}. After about 3–4 weeks, the animals without folic acid and vitamin B_{12} supplements, started losing weight. The animals were killed by decapitation after about five weeks on the experimental diet, when they were deficient with regard to the above vitamins as revealed by hematological studies and microbiological assay of the vitamins in the liver. The studies were conducted with six normal and six deficient animals.

The tissues were immediately removed, cut into 2–3 mm. thick pieces and fixed overnight in chilled acetone, chilled 80 per cent alcohol, 10 per cent neutral formalin, and Zenker-formol. They were then dehydrated and embedded in paraffin. Alkaline phosphatase content of the acetone fixed tissues were measured by the histochemical method of Gomori (1946). Acetone fixed tissues were also used for the estimation of acid phosphatase by the modified histochemical method of Gomori (1950). DNA and RNA in alcohol and neutral formalin fixed tissues were measured by the method of Kurnick (1952). Feulgen reaction (Coleman, 1938 ; Stowell, 1945) for DNA were also carried out in the alcohol and neutral formalin fixed tissues. Zenker-formol fixed tissues were used for the histological study.

The following tissues were studied : liver, kidney, adrenal, spleen, pancreas, testes, thyroid, and pituitary.

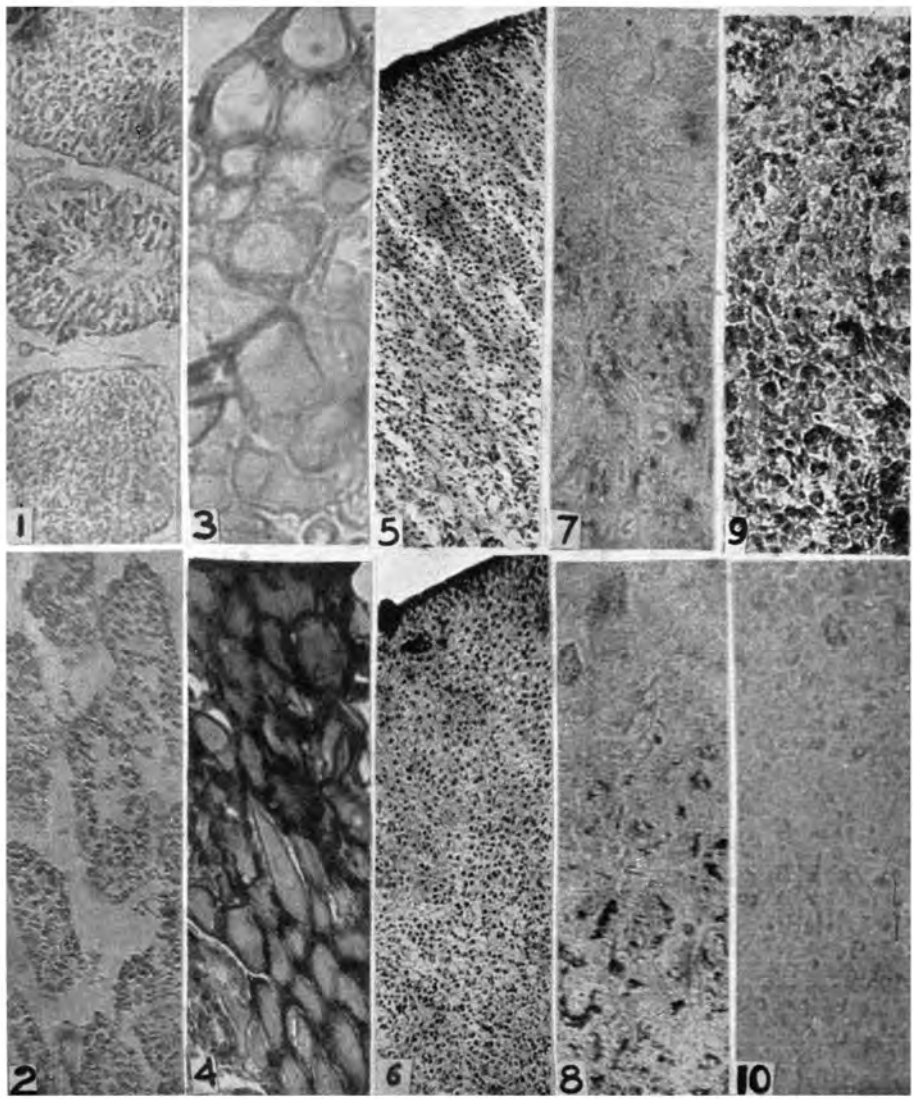
Results

In the different groups of animals studied, changes observed did not vary from animal to animal. The results of the investigations carried out are summarized in Table I.

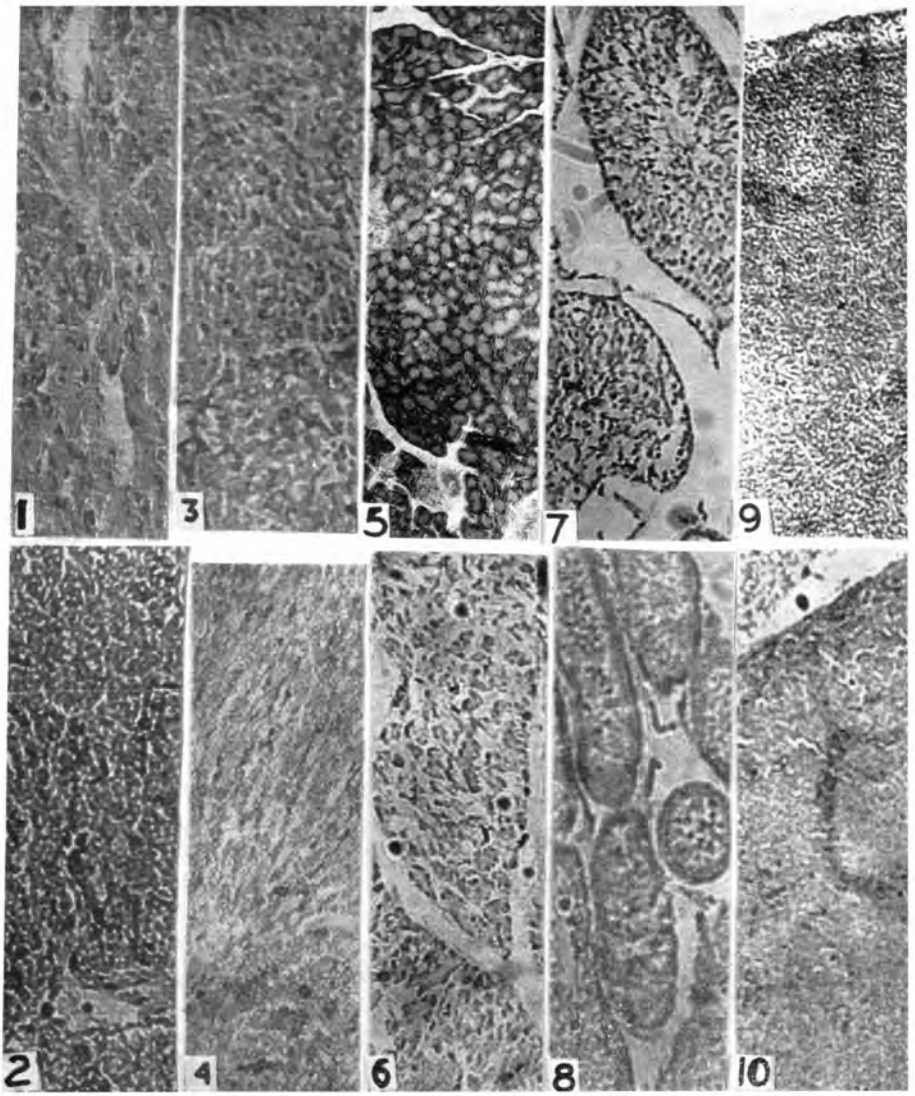
Alkaline and Acid Phosphatases

Liver : Very little alkaline phosphatase was found to be present in the normal liver and no significant change could be observed between the liver of normal and of 'deficient' animals.

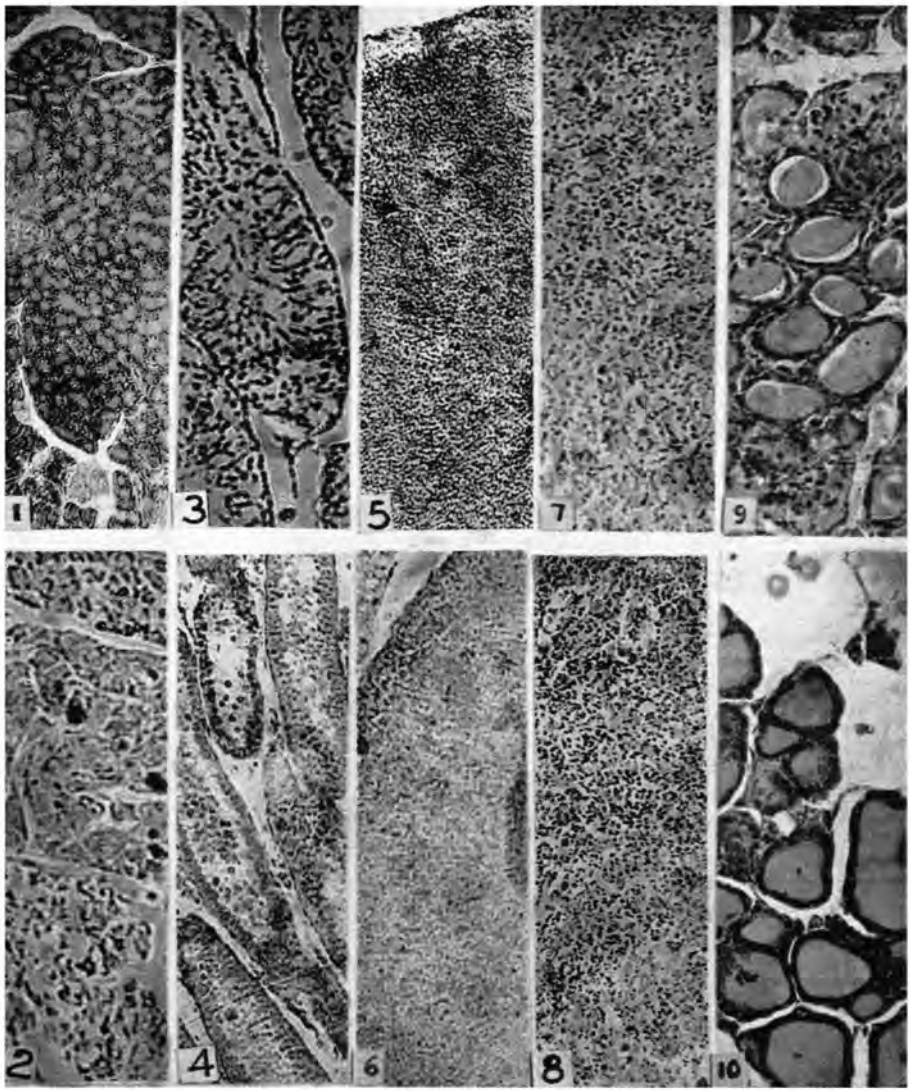
The acid phosphatase gave an intense reaction in the hepatic cells and it seems to have been increased slightly in the liver of 'deficient' animals.



1. Testes of normal rat showing distribution of alkaline phosphatase. × 110.
2. Testes of deficient rat showing distribution of alkaline phosphatase. × 110.
3. Thyroid of normal rat showing distribution of alkaline phosphatase. × 220.
4. Thyroid of deficient rat showing distribution of alkaline phosphatase. × 220.
5. Adrenal cortex of normal rat showing distribution of alkaline phosphatase. × 110.
6. Adrenal cortex of deficient rat showing distribution of alkaline phosphatase. × 110.
7. Kidney of normal rat showing distribution of alkaline phosphatase. × 110.
8. Kidney of deficient rat showing distribution of alkaline phosphatase. × 110.
9. Anterior pituitary of normal rat showing distribution of acid phosphatase. × 110.
10. Anterior pituitary of deficient rat showing a negative reaction for acid phosphatase. × 110.



1. Liver of normal rat showing distribution of acid phosphatase. × 110.
2. Liver of deficient rat showing distribution of acid phosphatase. × 110.
3. Liver of normal rat showing concentration of RNA. × 110.
4. Liver of deficient rat showing concentration of RNA. × 110.
5. Pancreas of normal rat showing concentration of RNA. × 110.
6. Pancreas of deficient rat showing concentration of RNA. × 110.
7. Testes of normal rat showing concentration of RNA. × 110.
8. Testes of deficient rat showing concentration of RNA. × 110.
9. Adrenal cortex of normal rat showing concentration of RNA. × 110.
10. Adrenal cortex of deficient rat showing concentration of RNA. × 110.



1. Pancreas of normal rat showing concentration of DNA. × 110.
2. Pancreas of deficient rat showing concentration of DNA. × 110.
3. Testes of normal rat showing concentration of DNA. × 110.
4. Testes of deficient rat showing concentration of DNA. × 110.
5. Adrenal cortex of normal rat showing concentration of DNA. × 110.
6. Adrenal cortex of deficient rat showing concentration of DNA. × 110.
7. Spleen of normal rat showing concentration of DNA. × 110.
8. Spleen of deficient rat showing concentration of DNA. × 110.
9. Thyroid of normal rat. × 220.
10. Thyroid of deficient rat showing enlarged follicles and flat secretory cells. × 220.

Kidney : The maximum amount of alkaline phosphatase was located in the brush border of the kidney tubules. Considerable amount of the enzyme was present in the kidney cortex evenly distributed throughout, except that in the glomeruli it was much less. An increase, more pronounced in the brush border of the tubules, was observed in the kidney of the deficient animals.

The acid phosphatase showed an even distribution and no change could be observed between the kidney of normal and of deficient animals.

TABLE I

Distribution of alkaline and acid phosphatases, RNA and DNA in the tissues of normal rats, (N) and rats deficient (D) in vitamin B_{12} and folic acid

Tissues	Alkaline Phosphatase		Acid Phosphatase		RNA		DNA	
	(N)	(D)	(N)	(D)	(N)	(D)	(N)	(D)
Liver	+	+	++	+++	++	+	++	+
Kidney	++	++++	++	++	++	++	++	++
Adrenal	++	++++	++	++	++	+	++	+
Spleen	++	+++	++	++	++	+	++	+++
Pancreas	++	++	++	++	++	+	++	+
Testes	++	++++	–	–	++	+	++	+
Pituitary	++	++	++	–	++	++	++	++
Thyroid	++	++++	++	++	++	++	++	++

Adrenals : The glomerular zone of the adrenal cortex showed the most intense activity for alkaline phosphatase. The intensity of the enzyme activity was found to be in a descending order in zona fasciculata and zona reticularis respectively. The adrenal medulla contained minimal amounts of alkaline phosphatase. In the adrenal of deficient animals the enzyme was found to be considerably increased, and though the distribution appeared to be on a similar pattern as in the normal adrenal, the increase was more pronounced in the zona glomerulosa and zona fasciculata.

The intensity of acid phosphatase was found to be more or less the same in the adrenals of both normal and deficient animals, though probably, there is a very slight increase in the adrenals of deficient animals.

Spleen : Alkaline phosphatase was evenly distributed in the spleen pulp. Minimal activity was observed in the Malpighian corpuscles. In the spleen of deficient animals the alkaline phosphatase activity was found to be increased to some extent.

No change was perceptible in the acid phosphatase activity in the spleen of normal and deficient group of animals.

Pancreas : Pancreas gave a positive reaction for alkaline phosphatase. The activity in the acinar cells is much more pronounced than in the islets. No change could be observed in the alkaline phosphatase activity in normal pancreas and in pancreas of deficient animals.

The acid phosphatase also did not show any difference in the normal pancreas and pancreas of deficient animals.

Testes : The alkaline phosphatase activity of the seminiferous tubules of the testes of deficient animals was considerably increased as compared with the normal controls.

The acid phosphatase did not show any variation in the normal and deficient testes. The normal testes gave a negative reaction for acid phosphatase, and in

the testes of deficient animals the activity could be located at some isolated spots, which does not seem to be significant.

Pituitary : There seems to be a selective distribution of both alkaline and acid phosphatases in the pituitary, which, possibly, may be due to the different cell types. The intensity of alkaline phosphatase is more pronounced in the posterior lobe of the pituitary, whereas the acid phosphatase is more distinctly distributed in the anterior lobe. The pars intermedia gives a diffuse reaction for both of these enzymes. No change could be observed in the alkaline phosphatase activity of normal pituitary and pituitary of deficient animals.

The pituitary of the deficient animals gives a more or less negative reaction for acid phosphatase, while the normal pituitary shows a pronounced activity of acid phosphatase.

Thyroid : The secretory cells of the thyroid give an intense reaction for alkaline phosphatase and it is considerably increased in the thyroid of deficient group of animals.

The acid phosphatase does not seem to indicate any perceptible variation.

Ribonucleic Acid and Desoxyribonucleic Acid

The RNA and DNA studied in all the above tissues, in general, seem to have been decreased significantly in the tissues of vitamin B_{12} and folic acid deficient animals. The more perceptible changes were observed in liver, pancreas, adrenal, spleen, and testes which showed a decrease in RNA content in the tissues of the dificient group of animals.

Thyroid, pituitary, and kidney from normal animal and animals rendered deficient in vitamin B_{12} and folic acid did not show any significant change in the RNA content.

The DNA was found to be decreased in pancreas, adrenal and testes. In the liver of deficient animals also the DNA seems to be slightly decreased though it does not seem to be very distinct.

Contrary to the above results, spleen of the deficient animals showed a marked increase in the DNA content as compared with the normal. The number of nuclei is greatly increased. Thyroid, pituitary and kidney did not show any variation as regards DNA content.

Histological Study

The haematoxylin and eosin stained sections on microscopic observation showed changes in liver, spleen, testes, and thyroid only. In the rest of the tissues no variations from the normal could be observed.

Liver : The clumped nature of the cytoplasmic basophilia of the normal liver was not retained in the liver of the deficient group of animals. The granules were evenly and scarcely distributed and a marked decrease in the cytoplasmic basophilia was observed, which is in agreement with the work of Stern, Taylor and Russel (1949). The liver showed heavy haemorrhage, and fat infiltration was also observed to a slight extent.

Spleen : In most of the animals rendered deficient in vitamin B_{12} and folic acid the size of the spleen is significantly increased. The number of nuclei showed a marked increase in the deficient tissue which correspondingly increases the DNA content also.

Testes : Testes show gross degenerative changes in deficient tissues, which confirm the works reported by Rose and Schweigert (1952). The seminiferous tubules were markedly shrunken, thereby increasing the intertubular space. The interstitial cells show marked signs of degeneration. Spermatogenetic activity

is considerably reduced and only a few spermatogonia remained in the germinal epithelium.

Thyroid : The thyroid follicles of the deficient tissue were enlarged and were full of colloid, which takes a deep stain. There were very few or no vacuoles in the follicles.

The secretory cells lining the follicles were low cuboidal or flat. A marked reduction in the size of the secretory cells occur, the reduction being primarily in the cytoplasm of the cells. The gland as a whole was highly vascular. These changes in the thyroid were similar to those reported by Wang *et al.* (1954).

Discussion

There seems to be a general increase in the alkaline and acid phosphatase contents of the various tissues of rats made deficient in both folic acid and vitamin B_{12}. The increase in alkaline phosphatase is more pronounced in kidney, adrenal, spleen, testes, and thyroid. The acid phosphatase is increased in liver and adrenals. There is total disappearance of acid phosphatase from the deficient pituitary.

Our knowledge concerning the biochemical function of phosphatase is still limited, though it is presumed to play an important rôle in the metabolism of carbohydrates, lipids, and nucleoproteins. The derangement in carbohydrate metabolism in vitamin B_{12} and folic acid deficiencies have been reported by Ling and Chow (1952, 1953, 1954) and Williams, Nichol and Elvehjem (1949). The changes observed by us in the alkaline and acid phosphatase content of tissues in vitamin B_{12} and folic acid deficiency may add to the evidence of the rôle of the two vitamins in carbohydrate metabolism. Kellerman's (1955) report on the rôle of phosphatase in dephosphorylation and the work of Piccardo and Salvetti (1955) and other workers, suggesting the close relationship between phosphate turnover and vitamin B_{12}, seem to explain the changes in alkaline and acid phosphatase observed by us.

Moog (1946) and Dempsey and Wislocki (1946) have suggested that adrenal alkaline phosphatase plays an important rôle in the metaboolism of lipoids and so possibly in the synthesis of adrenal cortical hormones. Boxer *et al.* (1955) have observed a considerable increase in the coenzyme A contents of liver and kidney, which, in turn, may affect the Kreb's cycle. The considerable increase observed in the alkaline phosphatase content of the deficient adrenal may indicate a disturbed physiological function of the gland, which can be presumed from the reports on deranged lipid metabolism and the changes in coenzyme A concentrations in vitamin B_{12} deficient animals. The rôle of acid phosphatase in the adrenals is not clearly known.

Kar (1950, 1951) suggests that testicular alkaline phosphatase is in some way related to testicular function. Kellerman (1955) has indicated the relationship between hexokinase and phosphatase in guineapig testes. The observed increase in the testicular alkaline phosphatase in our studies might be due to a changed physiological function of the organ.

The rôle of phosphatase in spleen, thyroid, and pituitary is not clearly known and hence the changes observed can not be explained from the results at hand. It only indicates a changed physiological function of the above organs. Concerning the hormonal regulation of enzymes, it is supposed that the enzymes are functioning under extremely complicated situations. Brachet and Jeneer (1948) and Bourne (1943) have suggested a rôle of alkaline phosphatase in nucleic acid metabolism which will be considered later. In view of the evidences at hand it would seem too premature to expound any theory on the rôle of phosphatases from the results of histochemical technics only.

The ribose and desoxyribose nucleic acids are found to be decreased in all the tissues except thyroid, pituitary, and kidney. The rôle of vitamin B_{12} in

nucleoprotein metabolism is quite evident from the reports available. Sebrell and Harris (1954) have cited references to show that vitamin B_{12} has an important rôle in nucleic acid synthesis, and consequently, in the deficient animals the nucleic acids are markedly diminished. Taking into account the reports of Brachet and Jeneer (1948) and Bourne (1943), the increased alkaline phosphatase may as well explain the diminution in nucleic acid synthesis, though the exact mechanism of action of phosphatase in nucleic acid synthesis is not clearly known. Our work regarding the nucleic acid content of tissues is in agreement with the works reported by Schweigert *et al.* (1954), Rasch *et al.* (1955), Rose and Schweigert (1952) and Wang *et al.* (1954).

Histological changes in the tissues of vitamin B_{12} deficient animals have been observed by various workers. They are much more pronounced in the second generation as reported by Ferguson and Couch (1954). The degeneration observed by us in the testes may explain the gross degenerative changes and undergrowth in the second generation.

The changes observed in the thyroid might be due to the presence of iodinated casein in the diet.

The diminution of cytoplasmic basophilia in livers of rats deficient in vitamin B_{12} and folic acid, confirms the reports of Stern, Taylor and Russell (1949). A heavy haemorrhage and fat infiltration to some extent were also observed which may be due to the depletion of the vitamin B_{12} stores in the body, which is a lipotropic agent. The spleen shows a considerable increase in the number of nuclei in deficiency, which seems to account for the increase in the DNA content of the deficient spleen.

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