THE SYNTHESIS OF DIPHOSPHOPYRIDINE NUCLEOTIDE IN THE ERYTHROCYTE.

A Thesis

by

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LIST OF ABBREVIATIONS

DPN	Diphosphopyridine nucleotide
DPNase	Diphosphopyridine nucleotide nucleosidase
TPN	Triphosphopyridine nucleotide
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine-5'-phosphate
PRPP	5-phosphoribosylpyrophosphate
PP	Inorganic pyrophosphate
NMN	Nicotinamide mononucleotide
NR	Nicotinamide riboside
SFH	Stroma-free hemolysate
CoA	Coenzyme A
UDPG	Uridine diphosphate glucose
UTP	Uridine triphosphate
ARPPR	Adenosine diphosphate ribose
HMG	β-hydroxy β-methyl glutaric acid
DMA	β, β-dimethylacrylic acid
HIV	β-hydroxy isovaleric acid
ADHase	Alcohol dehydrogenase

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INTRODUCTION

At the outbreak of World War II, work was initiated in the Department of Biochemistry at McGill University, under the direction of Dr. O.F. Denstedt, on a study of the means whereby the then existing methods of blood preservation might be improved. Similar investigations were undertaken in many other laboratories throughout the world, particularly in the United States and Great Britain. The availability of blood and plasma for prompt treatment of the wounded at the fighting front accounted for the tremendous saving of lives in World War II as compared with World War I.

With the cessation of hostilities, active research on the problem of blood preservation appears to have been discontinued for the most part. Research here, however, was continued and augmented with the emphasis on the enzymology and metabolism of the erythrocyte. It became clear as early as 1942 that an insight into the fundamental problem of why the erythrocyte fails during storage in an artificial medium is essential if real progress is to be made in the preservation of blood. The dominant question was whether the failure in viability of the red blood cell is due to metabolic causes or to physical changes in the membrane. The results which accrued from these studies were continually

put into practical application. With the development of the cold war after 1945, interest was again stimulated in blood preservation and work in other countries was resumed, the approach to the main problem being essentially the same as that being carried on in our group in McGill University.

Pappius (1), working in this laboratory, had shown that there is a slow but steady breakdown of the DPN content of the erythrocyte during storage in the cold. Because of the importance of this coenzyme in glycolysis, it was felt that the progressive decrease of DPN during storage may be the dominant factor in the progressive failure of the cells. The writer's main interest, therefore, has been directed towards the study of the capacity of the erythrocyte to replenish DPN. Since the synthesis of DPN in other somatic cells is confined to the nucleus (136), these studies afforded a means whereby the maturation of the red blood cell and the interrelationship between the nuclear and the cytoplasmic activities of the nucleated erythrocyte could be investigated. Inorganic pyrophosphate, a by-product of the synthesis of DPN, is destroyed by the inorganic pyrophosphatase of the erythrocyte, while DPN itself is hydrolyzed by means of a DPN nucleosidase. The writer undertook, therefore, to study some of the properties of these enzymes in order to obtain a more complete picture of the mechanism of the synthesis and degradation of DPN in the red blood cell.

An appendix follows on the synthesis of cholesterol in the red blood cell. This was done in collaboration with Dr. V.J. O'Donnell and Mr. P. Ottolenghi of this laboratory. Although the work reported in this chapter bears no relationship to the major problem with which we were concerned, it is nevertheless of interest since it sheds additional light on the nature and functions of the red blood cell. All of the cholesterol in the red blood cell is contained in the stroma (155) which consists predominantly of membrane material. The nature of the participation of the membrane as a "barrier" to the transport of various organic substances and inorganic ions is still poorly understood, although it is now well known that the alteration in the metabolic activity of the cell contributes to a great extent to the changes in the permeability of the erythrocyte. It was felt that a study of the "turnover" of cholesterol in the erythrocyte might give us some insight into the role which the membrane might play in the transport of the various organic materials and inorganic ions under normal circumstances and when the transport mechanism is impaired.

CHAPTER I

The Synthesis of DPN in the Erythrocyte.

(a) The Discovery of DPN and the Elucidation of its Structure.

In 1906, Harden and Young (2,3) discovered that the fermentation of glucose by yeast-juice required the presence of a certain substance the nature of which was not known except that it was dialyzable and thermostable. They referred to this material as a "coferment" of yeast juice. Meyerhof (4) pointed out that coferment was present in the muscle and the liver of the frog, rat and dog, the lungs and kidney of the dog, frog ovaries, but was absent from blood serum. He suggested furthermore that in respiration (as measured by the uptake of oxygen) in the presence of methylene blue, as in fermentation, the same coferment is required. Meyerhof referred to the coferment as a "coenzyme". The vast bulk of the subsequent work on the function and nature of DPN was contributed by the Stockholm school directed by Dr. H. von Euler. At first, little was understood about the mechanism of glycolysis, and since the Swedish workers used "fermentation" as a measure of coenzyme activity, the requirement for this cofactor was sometimes confused with the requirement of other, hitherto unknown, substances. Nevertheless, many important contributions were made.

von Euler and Myrback (5) showed that dry yeast could easily be washed free from the coenzyme of fermentation, leaving "zymase", which could then be used for the study of "cozymase" in other biological material. With this test system, von Euler and Nilsson (6) found cozymase activity in the blood of the dog, rat, pigeon and toad. Furthermore, they found that the activity was present in the red blood cells and not in the serum. Further studies on the distribution of cozymase in nature are reported by Myrback (7). Using yeast fermentation as the test system, he demonstrated cozymase activity in various yeasts, Streptococcus lactis, B. casei, and B. propionicus and in lesser degree in some of the higher plants. Attempts were made to isolate and purify cozymase obtained from yeast. Myrback showed that on acid hydrolysis of this material, a nitrogen containing base was liberated. Enzymatic deamination of cozymase could be effected, as with muscle adenylic acid, but cozymase proved not to be identical with the adenylic acid. The preparations of cozymase showed a maximum absorption in the ultra violet region at 258-260 mμ, a property in common with adenine-containing compounds.

von Euler and Vestin (8) succeeded in further purifying cozymase. They showed that the cozymase could be inactivated by heating at 100°C in 0.03 N sodium hydroxide for four minutes. Studying the role of cozymase in glycolysis in muscle, these workers suggested that

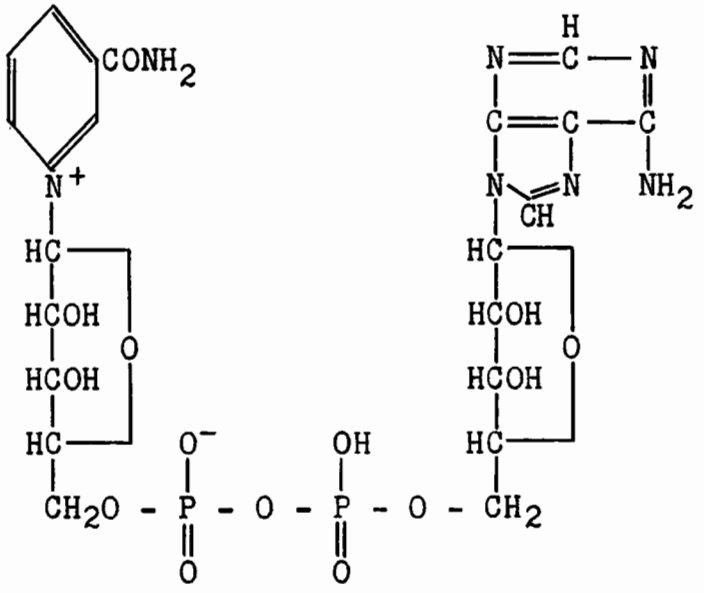
cozymase has two active groups - one which is labile and which influences oxidation - reduction and fermentation processes, and the other which is involved in lactic acid formation and the uptake (esterification) of phosphate. In this latter respect, their evidence clearly indicated a connection between phosphorylation and cozymase activity, but the assumption that the cozymase served as a "co-phosphorylase" or phosphate carrier was somewhat conjectural at the time. But as subsequent events have shown, they may indeed be quite correct in their deductions (32).

In 1935, von Euler, Albers and Schlenk (9) succeeded in purifying cozymase still further and observed that acid hydrolysis of this material liberated adenine and nicotinamide. The following year, Schlenk and von Euler (10) were able to propose a structural formula for cozymase; the structure differed only slightly from that now accepted. Meanwhile, Schlenk (11) observed that alkaline hydrolysis of cozymase liberated nicotinamide and adenosine-diphosphate-ribose. In a review of the subject of cozymase, von Euler (12) in 1936, pointed out that this material functions as a hydrogen carrier. The reduced form proved to be unstable in acid and after reduction with sodium hydrosulfite, a peak in the absorption of the reduced coenzyme appeared at 340 mμ. He now called the coenzyme "dehydrase" and the apoenzyme - "apodehydrase". von Euler's review lists some of the enzymes requiring cozymase as a cofactor for their activity.

von Euler (12) suggested a method for the measurement of cozymase. Using aqueous ethanol as the substrate, and the suitable apodehydrase, the increment in the absorption at 340 mμ (due to the reduction of cozymase) signified the concentration of the coenzyme present. In the same year, Warburg (13) isolated cozymase from the red blood cells of the horse. He observed that in the functioning of the coenzyme as a hydrogen carrier, the pyridine component of the molecule is reduced to dihydropyridine. In addition, he named the "hydrogen-transferring coferments - pyridine nucleotides" and proposed the name "diphosphopyridine nucleotide" for cozymase. In 1938, Warburg (14), pointed out that the pyridine nucleotides served as prosthetic groups for the "pyridine proteins", i.e., the apoenzymes concerned with dehydrogenase activity.

Schlenk (15), in 1942, prepared nicotinamide riboside by hydrolysis of DPN. He identified the carbohydrate component as being d-ribose and he showed further that both ribose phosphoric acid molecules of DPN have the phosphoric acid radical linked to carbon 5 of the pentoses. From these studies, and from previous work of his own and that of other workers, Schlenk was able to deduce the structural formula for DPN as illustrated below.

FIGURE 1



Codehydrogenase I (cozymase)

Most of the subsequent work on DPN, and indeed, a good deal of the previous work, was concerned mainly with the role of this coenzyme as a co-catalyst in various biochemical reactions. In 1955, however, Kaplan et al. (16) added to our knowledge of the distribution and structure of DPN by demonstrating that purified preparations of DPN contained an α-isomer to the extent of 10-15%. This material contained an α-ribotide linkage at the nicotinamide end of the molecule, in contrast to the usual β-ribotide linkage. The biological significance of the isomer was not known.

The history of the discovery of DPN, the elucidation of its structure and function is intimately connected with that of TPN, although in the latter instance, the work was begun much later. We are indebted to Warburg for a great deal of the fundamental work on this problem, which he accomplished in a surprisingly short length of time.

(b) The Discovery of TPN and the Elucidation of its Structure.

In 1931, Warburg and Christian (17) found a "ferment" and "coferment" in the blood of the dog, guinea-pig, rat and horse which catalyzed the oxidation of hexose monophosphate (Robison ester or glucose-6-phosphate) in the presence of methylene blue. By 1935, Warburg and Christian (18, 19) and Warburg, Christian and Griese (20) succeeded in purifying coferment from the red blood cells of the horse, and showed that for every molecule of coferment, they obtained, upon hydrolysis, one molecule of adenine, one of nicotinamide, three of phosphoric acid and two of pentose. From 100 liters of horse blood, they were able to isolate 10-20 mgm. of nicotinamide. These authors reported that the coferment was widely distributed in nature, was not identical with, but closely related chemically to cozymase. Like cozymase, coferment was found to be stable in acid, but unstable in alkali, while the reverse was true for the reduced form. Reduction of the coferment

could be effected with sodium bisulfite. While the oxidized form showed an absorption maximum at 260 mμ, the reduced coferment gave a new peak at 345 mμ. In the following year, Warburg and Christian (13) showed that coferment, like cozymase, functions by virtue of its hydrogen-carrying capacity, and that in the process, pyridine is reduced to dihydropyridine. Warburg suggested the name "triphosphopyridine nucleotide" for his "hydrogen transferring coferment".

For some years following this work, there was considerable controversy over the exact chemical formulation of triphosphopyridine nucleotide, mainly with respect to the position of the third phosphate group, since it was acknowledged that the remainder of the structure was identical with DPN. In 1950, Kornberg et al. (21) presented good evidence to show that TPN could be considered as a dinucleotide of nicotinamide ribose-5'-phosphate and adenosine-2',5'-disphosphate. TPN was cleaved at the pyrophosphate bond by means of a purified nucleotide pyrophosphatase, to yield a diphosphoadenosine fragment which was separated as a lead salt. The phosphate esterified to carbon-5' of the diphosphoadenosine fragment was specifically hydrolyzed by an adenosine-5'-phosphatase preparation from potato to yield adenosine monophosphate which was then purified by ion exchange chromatography. With the aid of ion exchange chromatography, paper

chromatography and of a potato phosphatase preparation, the adenosine monophosphate fragment from TPN was shown to be identical with adenylic acid 'a' (presumptively adenosine-2'-phosphate) and different from adenosine-3'-phosphate.

Kaplan et al. (148) isolated a deaminase from takadiastase which catalyzed the deamination of DPN. TPN was inactive in this system. 5'-adenylic acid and adenylic acid 'b' were also directly deaminated, but the amino group of adenylic acid 'a' was resistant to the action of the enzyme. These authors considered that Kornberg's observations pointing to the presence of adenylic acid 'a' in TPN was confirmed by the fact that their deaminase preparation did not deaminate TPN or adenylic acid 'a'.

Later, Heppel et al. (22) presented additional evidence which indicated that TPN contains a monoester phosphate attached probably to C(2') of the adenosine residue, and that it does not contain a 2':3' cyclic phosphate group, an alternative possibility which these authors had considered. Kaplan et al. (23) followed the acid-catalyzed interconversion of the 2' and 3' adenylic acid moieties of TPN and isolated the 3' isomer. This isomer of TPN reacted in systems which function with either DPN or 2' TPN, but not in systems specific for one or other of these.

Although the preceding two sections have been separated for the sake of clarity, the discovery of DPN and TPN and the elucidation of their structure proceeded hand-in-hand.

Their interconversion and function as coenzymes was developed concurrently and these aspects will now be considered.

(c) The Interconversion of DPN and TPN; their Function as Coenzymes.

In 1938, von Euler and Vestin (24) demonstrated the conversion of coenzyme I (DPN) to coenzyme II (TPN) in the presence of ATP and yeast apozymase. The optimum activity occurred at pH7 in the presence of manganese ions. Later, Adler et al. (25) demonstrated the conversion of coenzyme I to coenzyme II in yeast maceration juice supplemented with hexose diphosphate and inorganic phosphate. The reaction could be coupled with the oxidation of triose phosphate and could occur in the presence of phosphoenol-pyruvate and coenzyme I. Their results suggest that, in effect, the conversion of coenzyme I to coenzyme II required the presence of ATP, and the possibility that this substance had been regenerated by virtue of the glycolytic mechanism or during intermediate steps in fermentation could not be excluded.

Kornberg (26), in 1950, succeeded in partially purifying an enzyme from antolysates of ale yeast which catalyzed the synthesis of TPN by direct phosphorylation of DPN by ATP in the presence of magnesium or manganese ions. The same system converted reduced DPN to reduced TPN.

In 1952, Kaplan and coworkers (27) presented direct evidence for the following transhydrogenase reaction,

catalysed by an enzyme from the microorganism, Pseudomonas fluorescens:

(1) $TPNH_2 + DPN \longrightarrow TPN + DPNH_2$

The reverse of this reaction could be demonstrated only by coupling other systems with it. The transhydrogenase was found also to promote the following reaction:

(2) $TPNH_2 + \text{desamino DPN} \longrightarrow TPN + \text{desamino } DPNH_2$

This experiment ruled out the possibility that the transhydrogenase reaction involves phosphate transfer and indicated that the reaction involves a shift of electrons (or hydrogen atoms). The following reaction was also catalyzed by the transhydrogenase:

(3) $DPNH_2 + \text{desamino DPN} \rightleftharpoons DPN + \text{desamino } DPNH_2$

Using deuterium labelled reduced DPN, Kaplan et al. (28) demonstrated further that the reaction catalyzed by the pyridine nucleotide transhydrogenase of Pseudomonas fluorescens involved a direct hydrogen transfer rather than an electron shift. It is clear that the transhydrogenase reaction shows only an interrelationship between the coenzymes DPN and TPN, but does not show an interconversion of these substances. On the other hand, the work of Kornberg (26), referred to above, does in fact demonstrate the potential conversion of DPN to TPN.

Since Warburg's demonstration that DPN and TPN function by virtue of their hydrogen carrying capacity, the precise mechanism by which the pyridine nucleotides execute this function has been elucidated only partly. The site at which the reduction of the pyridine component of DPN takes place has been fairly well established, but has not yet been worked out for TPN. Karrer and coworkers (29,30) studied the nature of the products obtained upon chemical reduction (sodium hydrosulfite) of N'-methyl nicotinamide and other N'-substituted pyridine derivatives. Comparing the physical and chemical properties of the isolated product with known ortho- and para dihydro compounds, these authors concluded that the isolated compound (in the case of N'-methyl nicotinamide) was N'-methyl-o-dihydronicotinamide. It was assumed for a long time afterward that the reduction of the pyridine ring of the pyridine nucleotides occurred in the ortho position as well. But in 1954, Colowick et al. (31) described experiments, using deuterium as a tracer, which indicated that the reduction of DPN occurs in the para position rather than in the ortho position as previously assumed.

It is nowwell known that the pyridine nucleotides function as coenzymes for large numbers of dehydrogenases, oxidases, reductases and transhydrogenases. The mechanism of action of the pyridine nucleotide-linked enzymes, which includes a discussion on the mode of attachment of the

coenzymes to the apoenzymes, the participation of the coenzymes in the formation of the enzyme-substrate complex, and the role of the coenzymes in oxidative phosphorylation or in the formation of other high energy bonds has been treated extensively in a recent comprehensive review by Racker (32) and therefore will not be considered here.

Before proceeding with a discussion of the synthesis of DPN, it may be helpful, for the sake of orientation, to examine our present knowledge concerning the synthesis of the mononucleotides.

(d) The Biosynthesis of the Purine and the Pyrimidine Nucleotides.

This section will be divided arbitrarily into three parts. In the first, we shall consider the *in vivo* synthesis of purines and pyrimidines from labelled precursors; in the second, the problem of the *in vitro* degradation of the nucleosides and in the third, the *in vitro* synthesis of the purine and pyrimidine nucleotides. In some instances, there is a correlation apparent between degradation and synthesis and where possible, this will be acknowledged.

(i) The Biosynthesis of the Purines and Pyrimidines from Labelled Precursors.

Although considerable research had been done on the problem of purine, pyrimidine and nucleic acid synthesis

prior to the introduction of the use of radioactive materials, the interpretation of the results was fraught with difficulty. The use of isotopically labelled compounds helped to clarify the subject, but in most instances the only information gained was that a certain product was formed from the administered precursor. The pathways by which this conversion occurred might be more difficult to explain and only by examining the possible intermediates and their specific radioactivity could some of the intermediate steps in the synthesis be deduced. Inconclusive as many of these studies may have been, certain information was obtained that could not have been derived without the aid of isotopes.

In 1939, Parnas et al. (33) injected radioactive disodium phosphate (P^{32}) into rabbits and extracted the ATP from the muscle. This was done (on rabbits) 30, 60 and 120 minutes after the injection. He found that the terminal two "labile" phosphates of ATP were exchangeable very rapidly in the living animal with the inorganic phosphate, an equilibrium being established very quickly. The adenylic acid phosphorus of ATP, on the contrary, was found to participate very slowly in the exchange.

Schoenheimer and coworkers (34,35) showed that N^{15} ammonia, when fed to birds was built into muscle adenylic acid to only a small extent, thus confirming Parnas' (33) observation that the turnover of muscle adenylic acid is relatively very slow. Nitrogen labelled ammonia,

administered to rats and pigeons, however, was rapidly incorporated into the purines and pyrimidines of the nucleic acids of the internal organs. This did not occur when isotopic guanine was given. These authors concluded that neither the purines nor the pyrimidines in the diet are utilized by the body for the synthesis of nucleoprotein. Protein nitrogen, they stated, must be continuously used for purine and pyrimidine nitrogen, by way of the large "nitrogen pool" of the body. This view was confirmed by previous observations that animals on a diet in which protein was the sole source of dietary nitrogen showed no reduction in the quantity of purines excreted. These authors suggested that the purines and pyrimidines might be synthesized in the nucleus from smaller molecules and that the purines and pyrimidines might be used for the synthesis of nucleoproteins if they are supplied in the form of nucleosides, nucleotides or nucleic acids. That ingested guanine is not utilized for nucleic acid synthesis was confirmed by Brown et al. (36,37). It was necessary, for the following reason, to modify Schoenheimer's hypothesis that neither purines nor pyrimidines supplied in the diet can serve as precursors of nucleic acids. Adenine, labelled with heavy nitrogen (N^{15}) in positions 1 and 3 of the purine ring was prepared and given to rats in the diet. Brown et al. (36, 37) isolated similarly labelled adenine from the tissue nucleic acids and to a lesser extent from the ATP of the muscles. Some of the ingested adenine

apparently had been converted also to analogously labelled guanine. Neither adenine nor guanine, either free or as a component of nucleic acids, was found to serve as a precursor for the synthesis of pyrimidines.

Buchanan et al. (38,39) administered a group of C^{13} labelled compounds to pigeons. The uric acid was isolated from their excreta, purified and degraded with manganese dioxide. From these studies, they were able to show that carbon atoms 2 and 8 of the acid were derived from the administered formate. Carbon atom 6 was derived mostly from carbon dioxide. Carbon atoms 4 and 5 were derived from the carboxyl and α-carbon atoms, respectively, of glycine. Confirmation of these findings in the human was obtained by Shemin and Rittenberg (40) who administered N^{15}-labelled glycine to an adult man. On degradation of the urinary uric acid, they found that nitrogen atom 7 contained a great deal of radioactivity, while nitrogen atoms 1, 3 and 9 were much lower in specific activity. From these studies and previous work of Buchanan et al., Shemin and Rittenberg concluded that C atoms 4 and 5 and N atom 7 of the uric acid were derived directly from glycine. Since uric acid probably represents the principal end product of purine metabolism, there is undoubtedly a great deal of validity to Schoenheimer's original concept that purines and pyrimidines may be synthesized in the cell nucleus from smaller molecules.

(ii) The Enzymatic Degradation of the Nucleosides in vitro.

Dixon and Lemberg (41), in studies on the xanthine oxidase of milk, pointed out that substrates such as inosine, adenosine, inosinic acid and adenylic acid are oxidizable by xanthine oxidase, though more slowly than hypoxanthine. Guanosine and xanthosine are not oxidized by the enzyme. These authors showed further that the aforementioned oxidations are attributable to the presence of other enzymes which liberated free hypoxanthine from the substrates named. The oxidation of inosine, for example, was thought to be due to the action of a "nucleosidase" which liberates free hypoxanthine. The latter, in turn, is oxidized by the xanthine oxidase. Similarly, adenosine was thought to be deaminated to inosine and then oxidized as already described. Inosinic acid and adenylic acid behaved similarly to inosine and adenosine. It was thought that in these instances, the nucleotides first were converted to nucleosides by means of a phosphatase and then acted upon as previously described. Klein (42) prepared extracts from lung, spleen, liver and heart muscle which contained nucleosidase activity. By adsorbing the enzyme on alumina, eluting it with arsenate or phosphate, they obtained a fraction with increased activity. Arsenate and phosphate were used to activate the nucleosidases, and purine nucleosides were used as the substrate. Klein succeeded in preparing a separate pyrimidine nucleosidase, that was distinct from the purine nucleosidase.

In 1945, Kalckar (43,44) made an important contribution to progress in this field by preparing an enzyme from rat liver which catalyzed the following reaction:

(4) inosine + phosphate $\rightleftharpoons$ hypoxanthine + ribose-1-phosphate.

Phosphate can be replaced by arsenate, but one or the other is necessary in order for the forward reaction to proceed. Kalckar isolated the ribose-1-phosphate, demonstrated the reversibility of the reaction and showed that the equilibrium tends to be shifted far towards the left; that is, favoring synthesis of the nucleosides. He called the enzyme "nucleoside phosphorylase", and showed that phosphorolysis of guanosine occurred as with inosine. The specificity of the nucleosidase was demonstrated by Schlenk et al. (45). They showed that 2-hydroxy,6-aminopurine-D-riboside (crotonoside, isoguanosine) and adenine thiomethylpentoside are neither deaminated by the adenosine deaminase from small intestine, nor split by the nucleosidase of mammalian tissue. The failure of the nucleosidase to split hypoxanthine thiomethylpentoside shows definitely that the carbohydrate component also contributes to the specificity of this enzyme. These authors (46,47) demonstrated furthermore that incubation of purine nucleotides and nucleosides with tissue extracts of liver, kidney, spleen, brain, blood, heart and striated muscle, favors the production of a stable phosphoric acid ester (ribose-1-phosphate is very unstable in acid). Ribose-5-phosphate disappeared

on incubation with these tissues and the authors concluded that, following cleavage of the nucleoside or nucleotide, ribose-1-phosphate was converted to ribose-5-phosphate and that the latter was further metabolized to form a stable phosphoric acid ester. The implications of these findings with respect to the metabolism of pentose by way of the hexose-monophosphate shunt are of interest, but a discussion of this topic is not relevant at this time.

In 1947, Kalckar (48,49,50) introduced sensitive methods that are particularly suitable for the study of purines and purine derivatives in biological materials. These methods greatly facilitated further progress. The procedures combined spectrophotometry with the action of specific enzymes. Kalckar developed methods for the determination of very small quantities of hypoxanthine, inosine, xanthine, guanine, guanosine, uric acid, adenosine, adenosine-5'-phosphate, adenosine-3'-phosphate, ADP and ATP. He also prepared the following enzymes in sufficient purity to be used as analytical reagents for the measurement of the purines on which they act: adenine deaminase, adenylic deaminase, adenyl pyrophosphatase, xanthine oxidase, uricase, nucleoside phosphorylase and guanase. With the aid of these techniques, Kalckar (51) extended the studies he had commenced in 1945 on nucleoside phosphorylase. Using inosine and guanosine as substrates for the nucleoside phosphorylase of rat liver, he

demonstrated the dependence of the reaction on phosphate or arsenate, the reversibility of the reaction and identified the products in all instances. The results were much the same as already referred to, but the measurements were made with greater precision. In 1950, Kalckar (52) drew attention to the odd discrepancy between the results of studies in vivo and those in vitro with regard to the synthesis of purine nucleotide. While radioactive adenine was incorporated into nucleic acids as adenine and guanine (see above), guanine and hypoxanthine were not utilized in this respect. On the other hand guanine and hypoxanthine were the only two purine bases that were utilized in the system in vitro, adenine being inactive. Kalckar postulated that transfer of these bases may occur in vivo. He suggested moreover that the phosphorylase must have primarily a synthesizing function, since the equilibrium of nucleoside phosphorylase activity was greatly in favor of the synthesis of the nucleosides.

But purine ribosides other than inosine and guanosine were soon shown to act as substrates for the nucleoside phosphorylase. Friedkin (149), for example, pointed out that xanthosine (and desoxyanthosine) could act as substrates for the phosphorylase isolated from rat liver. The reaction proceeded at a much slower rate, however, than the phosphorolysis of desoxyguanosine.

Nucleoside phosphorylase activity has been demonstrated in blood as well as in other tissues. In 1930, Deutsch and Laser (150), showed that adenosine is cleaved by beef bone marrow. The activity of the raw extract was greater towards the pyrimidine nucleosides than towards adenosine. These authors observed that the nucleated and the non-nucleated erythrocytes had no pyrimidine nucleosidase activity, but that the leucocytes were active in this respect. Later, Dische (151,152) showed that an uptake of phosphate occurred upon the addition of adenosine to hemolysates of red blood cells. The adenosine was phosphorylated, but no adenosine-3'-phosphate or adenosine-5'-phosphate was found. In the phosphorylation, a ribose ester was formed, and this substance underwent considerable conversion to identifiable and non-identified glycolytic intermediates. In 1955, Gabrio et al. (153) showed that the metabolic degeneration of aged erythrocytes could be reversed completely by incubation with adenosine. Inosine, xanthosine, guanosine and desoxyadenosine produced similar results while the pyrimidine nucleosides were inactive. Adenosine was evidently metabolized via phosphorolytic cleavage with subsequent utilization of ribose-1-phosphate through the hexose monophosphate shunt. A nucleoside phosphorylase was purified from the hemolysates of red blood cells, and was found to have an absolute requirement for phosphate or arsenate. Wintrobe et al. (154) demonstrated purine nucleoside phosphorylase

activity in the erythrocytes of man, dog, rabbit, pig and chicken. There was no activity towards xanthosine and the pyrimidines, uridine or cytidine.

Phosphorolysis among the nucleotides is not confined to the purine ribosides. Manson and Lampen (55) prepared purine nucleoside phosphorylase from rat liver and calf thymus and showed that this enzyme catalyzed the phosphorolysis of hypoxanthine desoxyriboside. Desoxyribose-5'-phosphate was isolated as one of the products of the reaction. These authors (54) showed furthermore that cell-free extracts of Escherichia coli catalyzed the cleavage of guanine and hypoxanthine, but not adenine desoxyribosides - yielding the free base and desoxyribose phosphate.

The pyrimidine nucleosides likewise undergo phosphorolysis. Manson and Lampen (55) demonstrated the phosphorolysis of thymidine, catalyzed by preparations from dog bone marrow and calf kidney. Thymidine was not cleaved by hemolysates of rabbit erythrocytes nor by purine nucleoside phosphorylase prepared from rat liver. Using preparations obtained from Escherichia coli or other microorganisms, Paege and Schlenk (56) showed that the phosphorolysis of uridine resulted in the liberation of uracil and ribose-1-phosphate. Cytidine, they suggested, is probably deaminated to uridine before phosphorolysis occurs, since cytosine did not react with ribose-1-phosphate to

form cytidine. Manson and Lampen (54) using the cell-free extracts of E. coli, as mentioned above, showed that the uracil and thymine - but not cytosine-desoxyribosides, were cleaved yielding the free base and desoxyribose phosphate. More recently, Friedkin et al. (57) purified thymidine phosphorylase, obtained from horse liver. The enzyme was specific for thymidine, phosphate or arsenate being required in the reaction. Uridine could not serve as a substrate. The enzyme was found to be present in the supernatent fraction in rat liver which was homogenized in 0.25 M sucrose and centrifuged at 25,000 x g. for 6 hours.

The purine and pyrimidine nucleosidases do not always require the presence of phosphate or arsenate in order to effect a cleavage of the purine or pyrimidine nucleosides. Wang (58) and Wang and Lampen (59) prepared cell-free extracts of the microorganism Lactobacillus pentosus 124-2. These extracts catalyzed the cleavage of uridine, cytidine, inosine, adenosine, guanosine and xanthosine, but not thymidine. No adenosine or cytidine deaminase was demonstrated in the extract. Since the rate of cleavage of uridine and cytidine was greater in phosphate and arsenate buffer than in 'tris' (hydroxymethylaminomethane) buffer, these authors suggested that the reaction probably proceeds by phosphorolysis (or arsenolysis) rather than by simple hydrolysis. Still, they were unable to demonstrate the formation of phosphate esters during the reaction. The

same authors (60,61), pursuing their problem, and using the same bacterial extract, showed that uridine and cytidine were cleaved more rapidly in phosphate or arsenate, rather than in tris buffer. The stimulatory effect of phosphate or arsenate could be duplicated with sulphate or succinate. The pyrimidine nucleosidase was stable in the presence of divalent anions but was inactivated by monovalent anions. The cleavage of the purine nucleosides and uric acid riboside on the other hand was decreased by the addition of phosphate or arsenate. The purine nucleosidase was found to be stable in tris buffer, but rapidly became inactive in phosphate or arsenate buffers. In all instances, only the free ribose and the nitrogenous base could be identified as products of cleavage of both the purine and the pyrimidine nucleosides. Free pentose was not formed from ribose-1-phosphate or ribose-5-phosphate by the enzyme preparation, nor did the preparation catalyze the arsenolysis of ribose-1-phosphate. The authors concluded that the nucleosidases of Lactobacillus pentosus catalyze the hydrolysis of the nucleosides.

The hydrolytic pyrimidine nucleosidase is not confined to a particular microorganism. Carter (62) obtained this type of enzyme activity in plasmolyzed yeast extracts. The enzyme was specific for uridine. This author was unable to demonstrate hydrolytic pyrimidine nucleosidase activity in mammalian tissues. Heppel and Hilmoe (274) found two

mechanisms for the splitting of ribonucleosides in autolysates from dried baker's yeast; namely hydrolysis and phosphorolysis. The enzyme systems were completely separated, and each partially purified. The phosphorylase catalyzed the cleavage of inosine, guanosine and nicotinamide riboside, while the hydrolase catalyzed the cleavage of inosine, adenosine, guanosine, xanthosine and nicotinamide riboside. Neither of the two enzymes was effective in splitting uridine or cytidine. Tarr (63,64) prepared a riboside hydrolase from fish muscle, which catalyzed the hydrolysis of cytidine and purine ribosides, in the absence of added phosphate.

Another method for the degradation of nucleosides, which is really intermediate between cleavage and synthesis, will be described here. MacNutt (65) showed that dialyzed enzyme preparations from Lactobacillus helveticus were able to catalyze the transfer of the desoxyribosyl group from one purine or pyrimidine to another. Pyrimidine desoxyribosides were formed by the interaction of the corresponding pyrimidine with purine desoxyribosides. Similarly, purine desoxyribosides were formed when the corresponding purine was allowed to react with pyrimidine desoxyribosides. The transfer was catalyzed in phosphate-free buffers by enzyme preparations dialyzed for several days, thereby ruling out the possibility of phosphorolysis with subsequent synthesis of a different nucleoside. The enzyme was considered to

be a trans-N-glycosidase. Other microorganisms catalyzed the transfer but attempts to demonstrate the "trans" reaction in living cells of Leuconostoc citrovorum were unsuccessful. MacNutt (66) added further proof to the foregoing observations by demonstrating an exchange between radioactive adenine and the hypoxanthine of hypoxanthine desoxyribosides catalyzed by the enzyme preparations from Lactobacillus helveticus. The adenine desoxyriboside isolated at the end of the reaction had a molar C^{14} concentration as high as that of the initial adenine. There was no question of a transamination involving the 6-positions of adenine and hypoxanthine. Hoffman (67) using highly dialyzed extracts of Escherichia coli B, showed that the desoxyribose moiety of purine desoxyribosides could be transferred only to purine bases in the absence of phosphate and similarly, that the desoxyribose moiety of pyrimidine desoxyribosides could be transferred only to pyrimidine bases. The presence of inorganic phosphate was required for the transfer of the desoxyribose component of purine desoxyribosides to pyrimidine bases, and the same condition applied for the desoxyriboside transfer of pyrimidine desoxyriboside to the purine bases.

To recapitulate briefly, the writer has presented evidence that mechanisms exist, in a wide variety of biological materials, for effecting the cleavage of purine and pyrimidine ribosides and desoxyribosides. When attempts

have been made to demonstrate the reversibility of the cleavage mechanism, the equilibrium of the reaction is apparently always in the direction of nucleoside formation. Evidence will be presented in the next section that the cleavage as well as the synthesis of the nucleosides may play a part in the biological synthesis of the nucleotides.

(iii) The Biosynthesis of the Purine and Pyrimidine Nucleotides in vitro.

There are three main pathways by which the purine and pyrimidine nucleotides can be synthesized. We shall consider first, the synthesis of the nucleotides from their nucleoside precursors; second, the synthesis of the nucleotides from their respective purine and pyrimidine bases and finally, the synthesis of the nucleotides from small fragments. As yet, little is known as to which of the aforementioned mechanisms plays the most prominent role in the intact organism; it is possible that all three, or even more pathways are equally important.

Ostern et al. (68), in 1937, showed that certain yeast preparations, in the presence of adenosine and phosphate, catalyzed the synthesis of adenosine-5'-phosphate and ATP. It is impossible to tell, from these results, whether phosphorylation of adenosine occurs directly, or whether there is, at first, phosphorolysis of adenosine with subsequent conversion of the purine base to the nucleotide.

But in 1951, Kornberg et al. (69) succeeded in attaining the partial purification of an enzyme from autolysates of brewer's yeast which catalyzed the direct phosphorylation of adenosine and of 2-amino adenosine by ATP according to the following equation:

(5) Adenosine + ATP ⟶ adenosine-5'-phosphate + ADP.

Similarly,

2-amino adenosine + ATP ⟶ 2-amino adenosine-5'-phosphate + ADP

The adenosine phosphokinase was found to be specific for adenosine and 2-amino adenosine among a group of 17 nucleosides tested, and the phosphate was transferred specifically to the 5'-carbon of the nucleoside. Caputto (70), at about the same time, demonstrated the presence of an adenosine-kinase in yeast maceration juice and in extracts of the rat and rabbit kidney and liver.

Brawerman and Chargaff (71-73) reported that various tissues (malt, rat liver, human prostate) contain enzymes which catalyze the transfer of phosphoric acid from low-energy organic phosphates to nucleosides, yielding nucleotides. The enzymes in barley and liver catalyzed the formation of 5'-nucleotide isomers, while the prostatic enzyme (or enzymes) produced all of the possible mononucleotide isomers. These enzymes were called phosphotransferases. Goldwasser (74, 75) using cell-free preparations of pigeon liver, demonstrated the conversion of radioactive adenine to 5'-adenylic acid.

The enzyme activity was confined to the supernatant fraction obtained by centrifugation of the preparation at 81,000 x g. for 1 hour. From the specific activities of the adenine, adenosine and adenylic acid estimated periodically during the incubation, the author concluded that the formation of adenylic acid occurs by two main pathways: a) from adenosine and b) from adenine directly without intermediate formation of the nucleoside. Goldwasser suggested that in the latter instance the adenine might condense with ribose-1,5-diphosphate to form adenylic acid.

Concerning the direct conversion of purine or pyrimidine bases to their respective nucleotides, Wajzer (76) found that a crude preparation of calf liver nucleoside phosphorylase could catalyze the formation of nucleotides from inosine and adenosine provided a previous phosphorolysis of the nucleosides had taken place. This system, therefore, must have contained the components necessary first, to cleave the nucleosides, and subsequently, to synthesize the nucleotide from the purine base and some other substrate. In 1952, Williams and Buchanan (77-79) reported that pigeon liver extract contains a soluble enzyme system capable of catalyzing the conversion of hypoxanthine to inosinic acid in the presence of ribose-5-phosphate and ATP. The system included two enzymesthat could be separated by ethanol fractionation. Fraction II could be replaced by <u>crude</u> nucleoside phosphorylase. Using labelled hypoxanthine and inosine, either alone or together, these authors showed

that hypoxanthine was a better precursor of inosinic acid than inosine, and the nucleoside was not necessarily an intermediate in the conversion of hypoxanthine to inosinic acid. Purified nucleoside phosphorylase was found to have an inhibitory effect on the synthesis of the nucleotide from hypoxanthine. Buchanan suggested that the synthesis occurs by virtue of a reaction between the free base and ribose-1,5-diphosphate. In this connection, phosphoribomutase had no effect on the nucleotide-synthesizing system.

Saffran and Scarano (80) found that the soluble fraction of a pigeon liver extract (after centrifugation at 80,000 x g.) contains two enzymes, which in combination, catalyzed the synthesis of adenylic acid from ribose-5-phosphate, ATP and adenine. The reactions proceed in the following way:

(6) Ribose-5-phosphate + ATP $\longrightarrow$ Ribose-1,5-diphosphate + ADP

(7) Adenine + Ribose-1,5-diphosphate $\longrightarrow$ AMP + phosphate.

Phosphorylation of adenosine was ruled out as a step in the formation of adenylic acid under the conditions employed in the foregoing experiments. Kalckar (81), reviewing the subject of nucleoside and nucleotide synthesis, pointed out that ribose-1,5-diphosphate could arise from ribose-1-phosphate and glucose-1,6-diphosphate in the presence of phosphoglucomutase and that the formation of ribose-1,5-diphosphate does not necessarily occur through the phosphorylation of

ribose-5-phosphate by ATP. As Saffran et al. had pointed out, the enzyme involved in the reaction 7 is heat stable. This observation was confirmed by Buchanan et al. (82) who found that heating of their fraction II (see above) at 60°C for 5 minutes destroyed the nucleoside phosphorylase without interfering with the capacity of the system to synthesize inosinic acid.

Kornberg and coworkers (83-87), in a series of brilliant experiments, succeeded in elucidating the mystery of purine and pyrimidine nucleotide synthesis from the respective free bases. Using a purified enzyme preparation made from pigeon liver, they demonstrated that the following reaction is the initial one in the over-all synthesis.

(8) ATP + ribose-5-phosphate $\longrightarrow$ 5-phosphoribosylpyrophosphate + AMP.

The PRPP was isolated with the aid of ion exchange chromatography. Using ATP labelled in the terminal two phosphate groups, they found that the isolated product, PRPP, had the same degree of radioactivity as the ATP. In addition, these workers, considering the acid lability of the PRPP and the appearance of reducing substance after acid hydrolysis concluded that the compound with which they were dealing was, in fact, 5-phosphoribosylpyrophosphate. Then, using the isolated PRPP as one of the substrates, they demonstrated the synthesis of adenylic acid and orotidine-5'-phosphate. The irreversible decarboxylation of orotidine-5'-phosphate

was also shown.

(9) Adenine + PRPP $\rightleftharpoons$ Adenosine-5'-phosphate + PP

(10) Orotic acid + PRPP $\rightleftharpoons$ Orotidine-5'-phosphate + PP

(11) Orotidine-5'-phosphate $\rightarrow$ uridine-5'-phosphate + CO_2

They found further that ribose-1,5-diphosphate could not replace PRPP in reaction 10, nor could ADP replace ATP in reaction 8.

Kornberg et al. showed that the orotidine-5'-phosphate pyrophosphorylase of yeast did not catalyze the formation of nucleotide from adenine or uracil. But they purified another enzyme from yeast that catalyzed the condensation of adenine with PRPP to form adenylic acid but did not catalyze the formation of orotidine-5'-phosphate from orotic acid.

The enzyme that catalyzed the synthesis of PRPP apparently was present also in mammalian liver and in bacteria. ATP and ribose-5-phosphate were required specifically for the reaction; ribose-1-phosphate, 2-desoxyribose-5-phosphate or glucose-6-phosphate being totally inactive in this respect.

Orotidine-5'-phosphate pyrophosphorylase activity appears to be present in the livers of several animal species. Similarly, adenine pyrophosphorylase occurs in liver but is much less active than in yeast. Kornberg et al. demonstrated the pyrophosphorolysis of hypoxanthine and guanine

and pointed out that the enzymes involved in nucleotide formation from adenine and hypoxanthine are distinct.

Almost simultaneously, Buchanan et al. (88,89) achieved results similar to those of Kornberg and coworkers. Using adenine and hypoxanthine as substrates, they demonstrated the synthesis of adenylic acid and inosinic acid respectively, catalyzed with the aid of purified preparations obtained from pigeon liver. Again, these authors identified the "active" ribose compound as α-5-phosphoribofuranosylpyrophosphate. They showed, furthermore, that 5-phosphoribose-1,2-monohydrogen phosphate was inactive as a substrate for the synthesis of the aforementioned purine nucleotides.

At least two satisfactory mechanisms whereby nucleotide synthesis can occur have been discussed, namely, direct phosphorylation of the nucleoside, and direct synthesis from the free base. The wide distribution of the enzymes which facilitate the synthesis of the nucleotides by the latter route and the ease with which the reaction occurs suggests that this, in fact, is one of the mechanisms of the synthesis of the nucleotides in the intact organism. That this is not the only mechanism will become evident from the following discussion.

Greenberg (90-92) showed that the synthesis of hypoxanthine involves the following over-all reaction:

(12) $3NH_3 + CO_2$ + glycine + "formate" + ribose-1-phosphate $\longrightarrow$
ribose intermediates $\xrightarrow{\text{formate}}$ inosine-5'-phosphate $\longrightarrow$
inosine $\longrightarrow$ hypoxanthine + ribose-1-phosphate.

By estimating the specific activity at intervals, and by means of balance studies, Greenberg demonstrated that C^{14} labelled formic acid is incorporated into inosinic acid prior to the formation of hypoxanthine. It is apparent from the reaction given above, that the components necessary for the synthesis of uric acid were present in the preparation (see previous section). Greenberg showed further that the nucleotide, rather than the purine base, is synthesized first, then undergoes cleavage to liberate the free base. Furthermore, he suggested that the ribosides and ribotides are formed prior to ring-closure in the purines. But, the most unexpected finding was that the hypoxanthine formed by this apparently indirect route is used to synthesize inosinic acid. For optimum synthesis of hypoxanthine, Greenberg found that it was necessary to supplement the system with glutamic acid or glutamine and boiled liver extract. The latter could be replaced by biotin and citrovorum factor. Brown et al. (93) concurred with Greenberg in his postulate that the ring closure in purine formation occurs after the ribose phosphate moiety has become attached to an acyclic precursor.

Buchanan et al. (94-99) approached the problem from a

somewhat different angle. These authors, using pigeon liver extract as a source for the enzyme, showed that hypoxanthine could be synthesized from its elementary precursors. The carbon substrates, glycine, CO_2 and formate were utilized for hypoxanthine synthesis in the molecular proportion of 1:1:2. These studies with an isolated system afforded good corroboration of the findings concerning the biosynthesis of purines, in vivo. These authors then showed that when 4-amino-5-imidazole carboxamide (an acyclic compound resembling hypoxanthine except for the absence of a carbon atom in position 2) was incubated with pigeon liver homogenate or particulate-free extracts in the presence of α-ketoglutarate, there was appreciable utilization of the "carboxamide" for the synthesis of hypoxanthine. Formate and carboxamide interact approximately mole for mole in the synthesis of hypoxanthine. Radioactive carboxamide was not formed from active glycine in pigeon liver homogenates. When either active glycine or carboxamide was incubated with pigeon liver homogenates in the presence of non-labelled inosinic acid and inosine (or hypoxanthine) the specific activity of the inosinic acid, after a short incubation, was found to be considerably greater than that of the residual inosine or hypoxanthine. These results indicated that: a) inosinic acid is an intermediate in the synthesis of hypoxanthine from glycine and from carboxamide, and that the units of ribose and

phosphate are added prior to ring closure. Formic acid is then incorporated into the 2-position of the purine base. b) Although the carboxamide is probably not an intermediate of purine synthesis from glycine, the latter substrate can be converted to a non-purine intermediate, possibly the carboxamide ribotide or its formylated derivative.

Further extending their work, these authors showed that there was a greater than theoretical incorporation of formate than of glycine into hypoxanthine, in the presence of inosinic acid, indicating that there is a mechanism for the incorporation of formate in addition to the de novo synthesis of hypoxanthine from formate and glycine. By incubating labelled formate and inosinate in the presence of the appropriate enzyme system, they demonstrated a labelling of the purine moiety of the inosinate in the 2-position. This suggested that a reaction was involved - called an "enzymatic exchange" by these authors - in which inosinate was in equilibrium with formate and the carboxamide ribotide.

Under conditions which decreased the de novo synthesis of inosinic acid from glycine or when the rate was artificially reduced by the omission of bicarbonate from the incubation medium, it was possible to demonstrate the "enzymatic exchange" referred to above. Incorporation of radioactive formate into inosinic acid was dependent, of course, on the presence of inosinic acid. The latter could

not be replaced by inosine or hypoxanthine. Citrovorum factor, by virtue of its capacity as a "formyl" carrier, exerted a specific action on the enzymatic exchange of formate with carbon-2 of inosinic acid.

Relating these findings to systems in vivo, Buchanan et al. showed that suspensions of yeast, in a relatively quiescent state of growth, were able to incorporate the carboxamide into purine compounds at about the same rate as formate is utilized. Rapidly growing yeast cells likewise utilize the carboxamide for the synthesis of purine compounds, but they preferentially utilize the small carbon and nitrogen units, glycine and formate, under these conditions. The question arises whether the rate of penetration of the carboxamide into rapidly growing yeast cells limits the extent of its incorporation into purines. It was observed that cell-free extracts of yeast could synthesize inosinic acid from carboxamide, and also from hypoxanthine. Buchanan et al. purified a nucleoside phosphorylase from beef liver "acetone powders" which catalyzed the synthesis of 4-amino-5-imidazole-carboxamide riboside from the carboxamide and ribose-1-phosphate. These authors (88), furthermore, demonstrated the synthesis of the carboxamide ribotide from the carboxamide by a mechanism similar to the synthesis of inosinic acid from hypoxanthine and adenylic acid from adenine.

In the preceding section, the discussion dealt with studies pertaining to the synthesis of the purine and pyrimidine nucleotides. The writer has attempted to reconstruct the picture by presenting the evidence pertaining to the synthesis of the purine and pyrimidine bases from small fragments and the condensation of the bases with larger fragments. Finally, the whole system was considered, namely the synthesis of the nucleotides from smaller and larger components mediated by a highly integrated system of catalysts. Without doubt, these studies have a direct bearing on the problem of the synthesis of the pyridine nucleotides, and without the former there would little understanding of the latter. From our present knowledge, it appears that the organism can construct fragments, even as small as the nucleotides, from almost elementary precursors. Whether this is the normal mode of synthesis remains to be seen. In the isolated system, the synthesis of the purine and pyrimidine nucleotides occurs with apparently equal facility whether the system is supplied with larger and more immediate precursors, or with smaller, elementary substrates. It is difficult to believe that the synthesis of the nucleotides from preformed purine and pyrimidine bases represents merely an artefact, or rather, a nonspecific behaviour of the enzyme systems. Although this may very well be so, it is possible at the same time that alternative

routes are operative. Concerning the pyridine nucleotides at least, the synthesis of these substances begins with the pyridine base. This consideration, and what happens thereafter, is the theme of the next section.

(e) The Biosynthesis of the Pyridine Nucleotides.

The synthesis of the pyridine nucleotides in the red blood cell is closely similar to the synthesis of these substances in other tissues. To make an arbitrary distinction between these aspects of the problem would result only in confusion, hence they will be considered as a whole. It is expedient again to begin with the observations from studies in vivo and proceed to a discussion of the results that have been obtained with isolated systems.

In 1938, Kohn (100) showed an increase in the V-factor of red blood cells in normal individuals and in pellagrins who had ingested about 20 mgm. of nicotinic acid per kgm. of body weight per day. According to Lwoff and Lwoff (101) the organism Hemophilus parainfluenzae depends on a factor-V for growth. This substance can be replaced by the codehydrogenases, but adenylic acid, nicotinic acid and nicotinamide proved to be ineffective as growth-promoting factors. In the earlier work on pyridine nucleotide synthesis, the H. parainfluenzae test system was used, and it was frequently assumed that the measure of activity was

a reflection of the DPN or TPN content of the tissue examined. But Gingrich and Schlenk (102) showed that nicotinamide riboside and desamino-DPN could serve also as growth factors for this organism. This fact must be kept in mind in assessing the earlier literature on the subject of DPN synthesis, where the H. parainfluenzae test system was used. In all instances, reference will be made to the changes which occur in the particular tissue as changes in the pyridine nucleotides.

Axelrod et al. (103-105) showed that in the dog or pig which had been made deficient in nicotinic acid, there was a decrease in the pyridine nucleotide content of the muscle and the liver. There was, however, no alteration in the pyridine nucleotide content of the brain, the kidney or blood. In addition, these authors showed that the pyridine nucleotide content of rat tissues could not be increased by the parenteral administration of nicotinic acid. This result is in agreement with their observation that it is difficult (if not impossible) to induce a nicotinic acid deficiency in the rat. Kohn et al. (106) showed that the pyridine nucleotide content of the red blood cells in the dog remained normal during an attack of black-tongue (nicotinic acid deficiency) or during the recovery period following the injection of nicotinamide. This finding is in keeping with the previous observation

that nicotinic acid deficiency in the dog did not result in any change in the pyridine nucleotide content of the red blood cells. But in the human, as Spies et al. (107) pointed out - and which was in agreement with the previous findings of Kohn - there was a decrease in the pyridine nucleotide content of the red blood cells of pellagrins (nicotinic acid deficiency among other things) and that this factor returned to normal after nicotinic acid therapy. Later, Axelrod et al. (108) showed that the DPN content of erythrocytes (as ascertained by the yeast fermentation method) does not decrease significantly in the various stages of pellagra, but that, in general, the DPN content of striated muscle decreases as the nicotinic acid deficiency becomes more severe. In other words, although the DPN content of the erythrocytes was found to be diminished in pellagra, the extent of the change could not be used as a measure of the severity of the disease. The administration of nicotinic acid to pellagrins induced a marked increase in the DPN content of the erythrocytes and the muscle. These authors pointed out, furthermore, that practically all of the DPN content of whole blood was confined to the erythrocytes. Axelrod et al. embarked then on some preliminary studies of the synthesis in vitro of pyridine nucleotides and showed that only nicotinamide and neither pyrazinemonocarboxylic acid, quinolinic acid or coramine,

was effective in the synthesis of DPN in defibrinated blood. The use of the term DPN should be used with some reservation here, since even in the yeast fermentation system, it was possible that a precursor of DPN is first synthesized to this coenzyme before the latter participates in fermentation in yeast. Hoagland *et al.* (109,110) and Handler and Kohn (111) showed that in humans, following the ingestion of nicotinamide or nicotinic acid, there was an increase in the pyridine nucleotide content of the erythrocytes, but in the case of nicotinamide, this substance was less efficient than nicotinic acid in initiating the synthesis of the pyridine nucleotides. Handler and Kohn found this to hold also for the synthesis of the pyridine nucleotides *in vitro*.

Considering now the studies on the synthesis *in vitro* of the pyridine nucleotides, Kohn and Klein (112,113), showed first that with defibrinated blood, incubation with nicotinic acid resulted in the synthesis of the pyridine nucleotides. Much later, Leder and Handler (114) showed that the incubation of human erythrocytes that had been washed with isotonic saline or Ringer's solution, in a medium containing inorganic phosphate, glucose and 2% nicotinamide, resulted in an 8-10 fold increment in the content of cellular pyridine nucleotides of which 75-95% was identified as nicotinamide mononucleotide, and the remainder as DPN. The synthesis did not occur in the presence of glycolytic inhibitors. These authors showed,

furthermore, that NMN in the presence of limiting quantities of DPN, served as a growth factor for H. parainfluenzae, and supported the fermentation of washed brewer's yeast. Using hemolysates of human red blood cells, Leder and Handler (115) again demonstrated the synthesis of NMN. The substrates used were nicotinamide, ATP and fructose diphosphate. There was virtually no synthesis of NMN in the absence of ATP. In 1955, Preiss and Tabachnick (116) showed that NMN could be synthesized by dialyzed hemolysates of human erythrocytes with equal facility from either NR and ATP or nicotinamide, ribose-5-phosphate and ATP. With either substrate mixture, the synthesis was dependent on the presence of inorganic phosphate. The role of inorganic phosphate, however, was uncertain since little radioactive phosphate was incorporated into the NMN. The synthesis of NMN was blocked by the addition of guanine, hypoxanthine, adenine and their respective ribosides. On the strength of their observations, these authors concluded that a simple phosphate transfer to NR as a synthetic pathway does not occur, and suggested that a direct formation of ribotide takes place from nicotinamide and either ribose-1,5-diphosphate or 5-phosphoribose-1-pyrophosphate. If this be so, then the necessity of inorganic phosphate for the synthesis of the nucleotide from nicotinamide, ribose-5-phosphate and ATP is indeed difficult to explain.

Kornberg et al. (117-122) contributed a great deal

to our understanding of the mechanism of the synthesis of the pyridine nucleotides. First, they purified an enzyme from extracts of hog liver "acetone powder" which was found to catalyze the following reaction:

(13) $NR^+ + \text{orthophosphate} \rightleftharpoons \text{nicotinamide} + \text{ribose-1-phosphate} + H^+$

The enzyme had no effect on methyl nicotinamide, NMN, DPN or TPN but did catalyze the phosphorolysis of inosine. They suggested that a single enzyme might be responsible for the phosphorolysis of NR and inosine. The equilibrium constant of the above reaction was about 10, in comparison to a value of 16 obtained by Kalckar for the synthesis of inosine. This suggested a similarity in the energy value of the glycosidic bonds of the pyridinium and purine nucleosides. Using crude liver fractions, these workers followed the synthesis of NMN from NR and ATP, a reaction which proceeds slowly. They could not, however, detect any synthesis of NMN in the presence of nicotinamide, ribose-1-phosphate, ribose-5-phosphate and ATP. Kornberg succeeded in purifying an enzyme preparation from an autolysate of dried brewer's or ale yeast and from an extract of hog liver "acetone powder" which was found to catalyze the following reaction:

(14) $\text{NMN} + \text{ATP} \rightleftharpoons \text{DPN} + \text{PP}$

The value of the equilibrium constant was about 0.3-0.5. NR, ADP and AMP provedto be inactive in the DPN synthesis. The reduced form of DPN underwent pyrophosphorolysis in the

presence of the enzyme and inorganic pyrophosphate, but TPN and flavine adenine dinucleotide were inactive in this system. Magnesium ions were required as a cofactor for the pyrophosphorolysis or synthesis of DPN, and could not be replaced by manganese ions. The reversible enzymatic synthesis of DPN and PP from NMN and ATP was investigated with radioactive inorganic pyrophosphate. Starting with DPN and PP, the ATP formed was isolated and was found to have the same specific radioactivity as the inorganic pyrophosphate. The pyridine nucleotides were recovered quantitatively and were found to be free from radioactivity. The location of P^{32} in the ATP was shown to be exclusively in the terminal two phosphate groups. Kornberg postulated that the synthesis of NMN might occur in one of the following two ways:

(15) Nicotinamide + ribose-1,5-diphosphate $\rightleftharpoons$ NMN + orthophosphate

or,

(16) Nicotinamide + ribose-1-phosphate $\rightleftharpoons$ orthophosphate + NR $\xrightarrow{ATP}$ NMN.

In 1952, Mirsky et al. (123) in a study on the localization and distribution of various enzymes within cellular nuclei and other cellular components, showed that adenosine deaminase, nucleoside phosphorylase and guanase were present in a high proportion in most of the cellular nuclei of the various tissues tested. In view of the role

which nucleoside phosphorylase might play in the synthesis of DPN (see above), these authors suggested that the nucleus might exert specific control on the synthesis of this and other coenzymes.

That this prediction was correct was demonstrated by Hogeboom and Schneider (124,125) who studied the properties of the enzyme that catalyzes the synthesis of DPN from NMN and ATP in various cell fractions of mouse liver. From 69-101% of the enzyme activity was found to be contained in the nuclear fraction. Even better recoveries were obtained under conditions where excess damage to the cell nuclei was avoided. Approximately 90% of the enzyme activity was extracted from the nuclei by treatment with 1 N sodium chloride, and of this activity, approximately 50% was precipitated by dilution to a sodium chloride concentration of 0.17 molar. The authors concluded, therefore, that the enzyme is capable of combining with nucleic acid, but that the latter was not essential for activity, since the enzyme activity was unaffected by incubation with ribonuclease or desoxyribonuclease. In addition, they pointed out that the enzyme is water-soluble. Since the synthesis of DPN was found to take place in the nuclei of these cells, and since a number of DPN-linked dehydrogenases were known to be present in the cytoplasm, Hogeboom et al. suggested that a biochemical interaction existed between the nucleus and the cytoplasm. Baltus (126), working on

the intracellular distribution of a group of enzymes, described a method for the isolation of the nucleoli from starfish oöcytes. He found that the nucleoside phosphorylase and the enzyme that synthesizes DPN (which we shall call DPN pyrophosphorylase) were highly concentrated in the nucleoli of the starfish oöcytes.

The synthesis of nicotinic acid and DPN from small fragments was demonstrated by Hutchens et al. (127). These authors used the organism Chilomonas paramecium which is a cryptomonad flagellate capable of growing in the dark, utilizing ammonia as a source of nitrogen and a variety of compounds including acetate, formate and carbon dioxide as a source of carbon. The synthesis of DPN and nicotinic acid was accomplished by the organisms in a solution containing ammonia as the only nitrogen source and sodium acetate as the sole source of carbon. Although the synthesis of DPN from elementary precursors can take place in the living unicellular organisms, this does not imply that a more complex organism can accomplish the same feat. In fact, deprivation of nicotinic acid in the diet of the dog, the pig and man results in recognizable clinical symptoms and a diminution of the pyridine nucleotides in the tissues. The rat, however, may use other nitrogen sources for the synthesis of the pyridine nucleotides, since, as mentioned before, it is extremely difficult, if not impossible, to produce a nicotinic acid deficiency in this animal.

In the foregoing section, an outline of some of the data pertaining to the biological synthesis of the pyridine nucleotides was given. This, combined with the discussion in the preceding sections on the synthesis of the purine and the pyrimidine nucleotides, affords us a reasonably good picture of how the synthesis of the free bases, the nucleosides and the nucleotides is accomplished. It is now expedient to digress from this subject and discuss the mechanism of erythropoiesis, or maturation of the red blood cell, a topic which has an important bearing on the main problem under study.

(f) Erythropoiesis.

The general problem of erythropoiesis has been, still is and will continue to be an alluring subject for investigation. A general review of the subject was written in 1949 by Dacie and White (128); additional information can be obtained from any standard textbook of hematology. We are concerned principally with the fate of the nucleus during the course of the maturation of the red blood cell.

The erythrocytes originate in the bone marrow from a primitive nucleated precursor. As this cell develops, hemoglobin is synthesized within the cell, the nucleus becomes progressively smaller and the mitochondria and nucleoli are no longer morphologically distinguishable.

Finally, a stage is reached when the nucleus disappears, and the mature nonnucleated erythrocyte is liberated into the general circulation.

There are mainly three theories concerning the fate of the nucleus during the course of the development of the erythrocyte from the nucleated to the nonnucleated level. Howell (129), in 1890, suggested that the nucleus is lost by "extrusion" from the mature nucleated erythrocyte. The immature nucleated erythrocyte does not lose its nucleus, but was found to undergo multiplication. Cooke (130) and Davidson (131) would not subscribe to this theory and in fact, suggested that if the nucleus does disappear by extrusion, the phenomenon is the only example of biological hari-kiri known. These authors suggested that the nucleus of the normoblast (as the mature nucleated erythrocyte is called) disappears by karyolysis (dissolution) or by pyknosis (becoming smaller and denser) and karyolysis, with or without karyorrhexis (fragmentation). Bostrom (132) presented the hypothesis that the mature nonnucleated erythrocytes are formed by a process of "budding-off" of the hemoglobin-containing cytoplasm from nucleated precursors in the bone marrow. Howell had evidently observed this phenomenon and suggested that the apparent act of budding might result from multiplication of an immature cell with loss of the nucleus from the daughter cell before separation of the latter is complete. The controversy as to which of these

three hypotheses is correct has continued to the present day. With new technique, however, the morphological evidence seems to support the nuclear extrusion theory. In 1951, Albrecht (133) showed that mature erythroblasts (nucleated erythrocytes) from guinea pig bone marrow regularly show in the tissue culture an expulsion of their nucleus by active contracting movements. This observation was made with the use of microcinematography and serial photographs. The process of nuclear extrusion could occur rapidly or slowly. Bessis et al. (134,135), using a combination of phase contrast microscopy and microcinematography, observed nuclear expulsion in orthochromatic erythroblasts (nucleated erythrocytes) in hemolytic disease of the newborn and in rabbit bone marrow. They could not say whether the same thing applies in normal adult man, or only in vitro and in pathological states. These authors held that following the expulsion of the nucleus, the cell becomes what is known as a reticulocyte. This cell has no nucleus and is generally believed to be the immediate precursor of the mature erythrocyte. Bessis et al. noted that the nucleated erythrocyte throws out many excrescences, one of them containing the nucleus, and after convulsive movements on the part of the cell, the nucleus is eliminated by extrusion. Only the acidophilic erythroblast (most mature of the nucleated erythrocytes) and the reticulocyte showed these convulsive movements. These authors pointed out also that the convulsive movements were slow and could

not be observed with the conventional microscope. But with the aid of phase contrast and microcinematography, the movements could be observed.

In all of this work, the conclusions were based on purely morphological evidence. The techniques have been improved enormously in the past few years, but there is still ground for a different interpretation of the observed findings. The writer has tackled the problem from a somewhat different approach as described in the following section.

(g) The Synthesis of DPN in the Erythrocyte.

As mentioned previously, the mechanism of the biosynthesis of DPN was clarified to some extent by Kornberg's (12) demonstration that an enzyme purified from autolysates of ale yeast and from extracts of an "acetone-dried" powder from hog liver could catalyze the synthesis of DPN from the substrates NMN and ATP according to reaction 14. Later, Hogeboom and Schneider (125) showed that in mouse liver homogenates, the enzyme activity is confined almost entirely to the nuclei.

In the course of a comprehensive study in this laboratory on the metabolism of the erythrocyte, it was of importance to investigate the biosynthesis of DPN in the red blood cell for the following reasons. Pappius et al. (1) previously had shown that during the preservation of human blood in the cold, DPN in the erythrocyte undergoes a slow but steady

breakdown. Since DPN is required as a coenzyme for two of the enzymes of the glycolytic system (triose phosphate dehydrogenase and lactic dehydrogenase), it was surmised that a failure in the mechanism responsible for the synthesis of DPN might be an initiating factor in the progressive impairment of the energy metabolism of the red blood cells during storage.

The precise nature of the "maturation" of the mammalian red blood cell, with particular reference to the fate of the nucleus, has been a subject of controversy for many years. The three main views as to the genesis of the erythrocyte in this respect have been outlined in the preceding section. To recapitulate, they are: 1) that the nucleus is eliminated by "extrusion", 2) that the nucleus disappears by fragmentation or dissolution and 3) that the mature nonnucleated erythrocyte represents a cytoplasmic bud "nipped off" the mature nucleated erythrocyte. Stern (136) pointed out that of all the so-called "nuclear" enzymes, DPN pyrophosphorylase is the only one that is confined strictly to the nucleus. It occurred to the writer that the presence or absence of this enzyme in the chicken erythrocyte (which is nucleated) or in the mammalian nonnucleated erythrocyte might afford valuable information concerning the maturation of the mammalian red blood cell. If the enzyme activity be present solely in the nucleus of the nucleated erythrocyte and be retained in the nonnucleated erythrocyte, one may infer that

in the development of the cell, nuclear fragments are dispersed throughout the cytoplasm of the mature nonnucleated cell. On the other hand, if DPN pyrophosphorylase activity is not present in the mammalian erythrocyte, then one may surmise that the nucleus is extruded from the cell during maturation. If the enzyme activity be present in the nucleated erythrocyte, it was of considerable importance to ascertain whether the activity is confined solely to the nucleus. Rubinstein et al. (137) have shown that in the chicken erythrocyte, fumarase and malic dehydrogenase activities, though confined largely to the nucleus, are present also to an appreciable extent in the cytoplasm. Since fumarase and malic dehydrogenase are found also in the cytoplasmic (soluble) fraction of the mature mammalian nonnucleated erythrocyte, these authors were unable to say whether, in the evolution of the cell, these enzymes were derived from the nucleus or from the cytoplasm of the nucleated precursor of the erythrocyte.

Although there is a class difference between the bird and the mammal, neverthless, the avian red blood cell is comparable with other somatic cells in many respects (137) and as other studies (138) in our laboratory have shown, is comparable with the mammalian reticulocyte. Furthermore, immature human erythrocytes are difficult to obtain free from other bone marrow elements, and, in any event, would not be available in sufficient quantities for our purpose.

MATERIALS AND METHODS

ATP and DPN (95% purity) were obtained from the Nutritional Biochemical Corporation, and alcohol dehydrogenase from Sigma Biochemical Company, lyophilized snake venom from the species Crotalus adamanteus was purchased from the Ross Allen's Reptile Institute, Silver Springs, Florida, and was used as a source of 5'-nucleotidase for the preparation of nicotinamide riboside. Potatoes were bought at the local market.

NMN was prepared from DPN by means of a crude extract of potato nucleotide pyrophosphatase, prepared according to the method of Kornberg et al. (139). In assaying the activity of the enzyme, one unit of enzyme activity was defined as that amount of enzyme required to hydrolyze one μM of DPN per hour.

The method for the measurement of DPN is essentially that of Racker (140). DPN was incubated in phosphate buffer (0.5M, pH 7.0) in the presence or in the absence of the enzyme for 20 minutes at 37°C. Aliquots of each of the mixtures were placed in cuvettes, and to each cuvette was added: ethanol (95%, 0.3 ml.), glycine (1.5%, 0.2 ml.), sodium pyrophosphate (0.03 M, pH 10.0), water (0.8 ml.). Readings were made in the Beckman Model DU spectrophotometer at a wave length of 340 mμ. Water (0.1 ml.) was added to a 'blank' cuvette, and alcohol dehydrogenase (33.3 micrograms) was added to the experimental cuvettes. The value obtained

with the solution containing DPN but no nucleotide pyrophosphatase served as a control from which the value obtained in the experimental solution was subtracted. The difference represented the amount of DPN that had undergone hydrolysis in the presence of the nucleotide pyrophosphatase. An extinction coefficient of $6.3 \times 10^6 cm.^2 \times mole^{-1}$ at 340 mμ for DPN was used in the calculations (141).

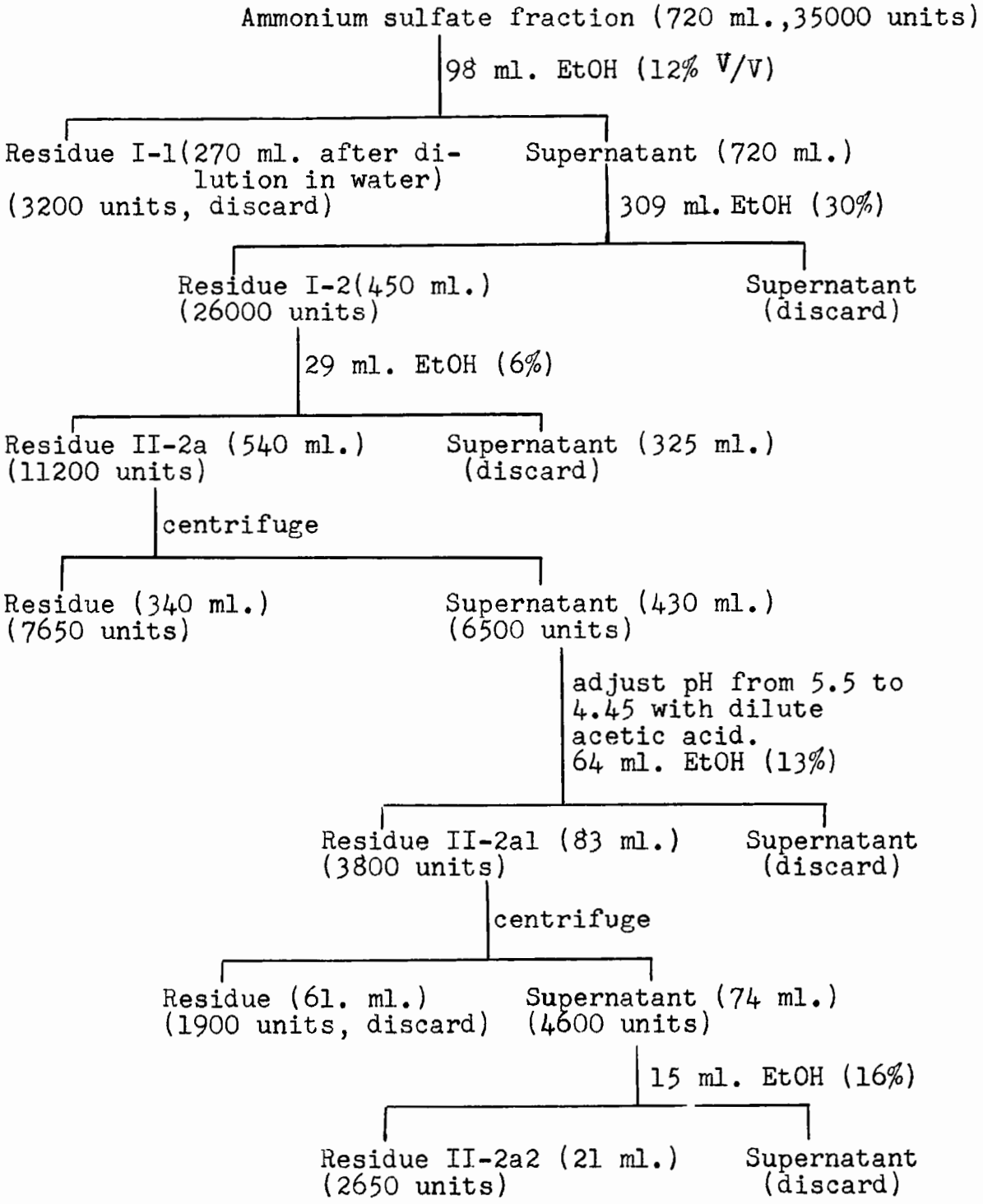
The following procedure was used for the preparation of the nucleotide pyrophosphatase.

Peeled potatoes (7.8 kgm.) were pulped in 15.6 litres of water with a 'Waring Blendor'. The pulp was filtered through a Buchner funnel yielding a total volume of 15.5 litres of filtrate. To the filtrate was added 7 kgm. of solid ammonium sulfate to bring the concentration of ammonium sulfate to 60% saturation. The mixture was left at 4°C overnight. A large volume of precipitate formed which was removed by filtration through a Buchner funnel, the first filtrate collected being returned to the mixture until the filtrate remained clear. It was pale amber in color. The precipitate was collected and dissolved in water, then dialyzed against running tap water at 8°C for 3 hours. The ammonium sulfate fraction contained 35,000 units of activity in 720 ml.

This fraction was then refractionated with 95% ethanol at -5°C, the latter being added slowly. The accompanying flow sheet (figure 2) indicates the procedure used. A

FIGURE 2

The Purification of the Potato Nucleotide Pyrophosphatase



fairly heavy loss occurred in the total activity and only about a 3-fold purification was achieved. However, sufficient activity was obtained in a small volume of solution to be satisfactory for our purposes.

In preparing the NMN, we adopted the following procedure: 500 mgm. of DPN (75% purity) were dissolved in 2.0 ml. of water. The pH was adjusted to 6.0 with dilute sodium hydroxide, and potassium phosphate buffer (0.4 ml. of 0.5 M, pH 7.7) was added. To the solution were added 7 ml. of the crude nucleotide pyrophosphatase preparation, and the mixture was incubated for 120 minutes at 37°C. At the end of the period, DPN could no longer be detected in the incubation mixture.

Basic lead acetate (1M) was added and the reaction mixture was centrifuged. The supernatant was removed, the pH adjusted to 2.6 with dilute nitric acid and to this solution was added 0.5 ml. of 20% mercuric acetate. The precipitate was removed by centrifugation and the supernatant was treated with H_2S. The black mercuric sulfide was removed by centrifugation, and the clear supernatant was assayed by the cyanide method (141), to determine the quantity of the nicotinamide-riboside moiety present. For the calculation, an extinction coefficient value of 6.3×10^6 cm.^{2} x mole^{-1} at a wave-length of 325 mμ, as suggested by Colowick et al. (141), was used. By determining the total phosphate and inorganic phosphate concentration by the method

of Fiske and SubbaRow (142), along with the nicotinamide-riboside component and DPN, it was found that no inorganic phosphate or DPN was present in the preparation. Of the material, 53% was found to be NMN and 47% nicotinamide riboside. The total yield of pyridine nucleotides was 30%, and that of NMN was 15%. The concentration of the solution of NMN and NR was 0.022 Molar. The presence of NR did not interfere with the determinations of the extent of the synthesis of DPN from NMN and ATP, since the proportions of NMN and NR in the solution of these two materials was known, and the extent of the synthesis of DPN from the NMN could therefore be calculated. The yield of NMN in other preparations was similar and contained a similar proportion of NMN and NR. Wherever there was reason to surmise that the presence of NR might affect the synthesis of DPN, the NMN was purified in the following way:

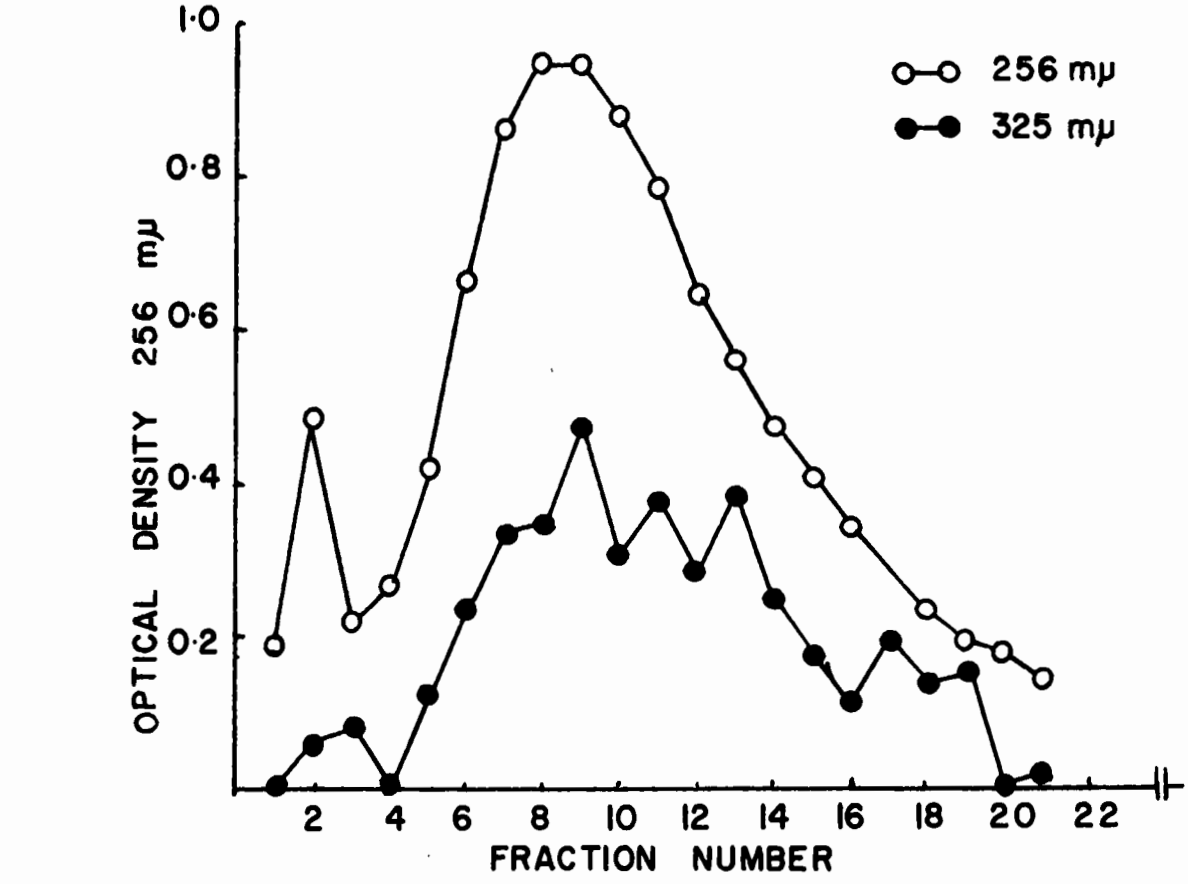
A cation exchange resin, Dowex 50 x 12 (200-400 mesh), was washed 5 or 6 times with distilled water, the fines being decanted after each washing. The resin was then washed alternately 5 or 6 times with 2 M acetic acid and water, discarding the supernatant each time. Then the resin was washed with water and placed in a glass tube (for chromatography) 10 cm. long and 2 cm. in diameter. The column was washed with water, until the washings showed no absorption in the ultraviolet at 256 mμ (143). The NMN was eluted with 0.01 M. sodium acetate buffer, pH 4.9, the flow rate

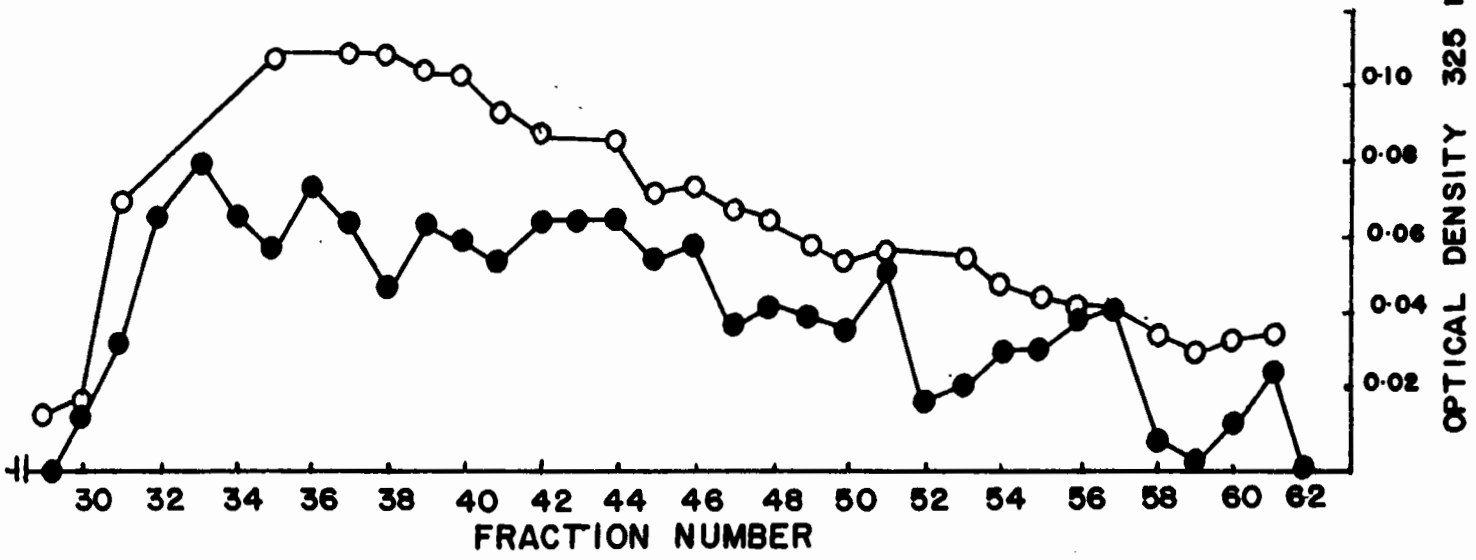
being regulated at 2 ml. per minute. The eluate was collected in fractions of 20 ml. The progress of the elution of the material was determined by measuring the ultraviolet absorption of aliquots at 256 mμ. As a check, sodium cyanide was added to another aliquot, and the absorption at 325 mμ measured. A small absorption peak was detected in the first 3 eluates collected. The substance responsible for the absorption appeared to be DPN and was discarded. NMN appeared in fraction numbers 4-21 in the series, and was eluted rather rapidly and completely as indicated in figure 3, by the symmetrical peak. No material was removed from the column with the 0.1 M acetate buffer, but the nicotinamide riboside was eluted with the 1 M acetate buffer, pH 4.9.

The pH of the combined fractions, numbers 4-24, was adjusted to pH 2.0 with concentrated hydrochloric acid and that of the combined fractions, numbers 30-61 containing the nicotinamide riboside, to pH 1.6. The volumes of the two lots were reduced by vacuum distillation at a temperature below 30°C. As expected, a large amount of sodium chloride precipitated out in the fraction containing the NR. The content of the nicotinamide-riboside component, total and inorganic phosphorous, and of DPN, of both lots was determined. In the sample containing the NMN, no NR was found, but the sample was found to be contaminated with DPN to the extent of about 3%. The sample containing the NR contained

FIGURE 3

The Purification of Nicotinamide Mononucleotide and Nicotinamide Riboside by Cation Exchange Chromatography.


1·0
0·8
0·6
0·4
0·2
OPTICAL DENSITY 256 mμ
256 mμ
325 mμ
2
4
6
8
10
12
14
16
18
20
22
FRACTION NUMBER


0·10
0·08
0·06
0·04
0·02
OPTICAL DENSITY 325 mμ
30
32
34
36
38
40
42
44
46
48
50
52
54
56
58
60
62
FRACTION NUMBER

no DPN nor phosphate - hence appeared to consist of pure NR along with a considerable amount of sodium chloride. The concentration of the NMN sample was found to be 0.003 M.

When required, purified NR was prepared by the procedure of Rowen and Kornberg (118). To a mixture of NMN and NR (4 ml., 0.0033 M with respect to NR) which had previously been prepared with the aid of the nucleotide pyrophosphatase, but had not been fractionated into the components NMN and NR - were added 3 ml. of glycine buffer (0.25 M, pH 8.5), magnesium chloride (0.7 ml., 0.15 M), water (0.3 ml.) and snake venom as a source of 5'-nucleotidase (20 mgm. in 2.0 ml. of potassium chloride, 0.01 M). The mixture was incubated at 37°C for 60 minutes. After cooling, the solution was extracted three times with 10 ml. volumes of aqueous phenol (60 gm. in 14 ml. water). The phenol was extracted three times with 30 ml. quantities of diethyl ether and the aqueous phase was re-extracted with ether. The ether phase was washed twice with water, and the aqueous washings combined with the previous aqueous phase. The aqueous solution was aerated to remove the residual ether. The volume was reduced *in vacuo* below 42°C to about 100 ml. Since some phenol still remained in the solution, the latter was re-extracted three times with 40 ml. volumes of ether. The aqueous phase was then distilled *in vacuo* below 45°C, and reduced to a volume of 3 ml. The concentration of nicotinamide riboside thus prepared was 0.001 M, with a maximum contamination of 3% NMN.

The composition of the reaction mixtures used in the studies on the DPN pyrophosphorylase will be found in the footnotes accompanying the tables. Following incubation of the experimental reaction mixtures, trichloroacetic acid was added and the specimens were centrifuged. The supernatant solution was neutralized with sodium hydroxide (2N), using phenol red as an internal indicator. A small volume of trichloroacetic was treated in the same manner as a control. Into each cuvette were introduced ethanol (95%, 0.3 ml.), sodium pyrophosphate (0.1 M, pH 9.5, 1.6 ml.), an aliquot of the filtrate and water to make the volume up to 2.9 ml. Readings of the optical density were taken with the Beckman spectrophotometer at 340 mμ, using the sample containing only trichloroacetic acid to "zero" the instrument. Then to each cuvette was added 83 micrograms of alcohol dehydrogenase. The increase in optical density at 340 mμ afforded a measure of the quantity of DPN in the reaction mixture. When the cyanide method was used, the removal of the protein was achieved as described with the tables accompanying the appropriate experiments. To an aliquot of the supernatant material (usually 0.1 ml.) in the cuvette, sodium cyanide (1 M, 2.9 ml.) was added and the optical density at 325 mμ was determined.

Preparation of erythrocytes:

Chicken blood was collected into a heparin solution.

The samples were centrifuged and the plasma and 'buffy layer' removed. The erythrocytes were washed three times with 0.9% sodium chloride, then diluted with this medium to the volume of the original blood sample. The red blood cells were then hemolysed by alternate freezing of the specimen in a dry-ice-alcohol mixture and thawing in a bath at 37°C, repeating the treatment three times. The hemolysate was centrifuged at 3000 rpm. (International Refrigerated Centrifuge, Model PR-1), and the particulate fraction containing nuclei and cellular membrane, was separated from the supernatant or stroma-free hemolysate (SFH). The particulate fraction was washed with saline 5 or 6 times until almost white in appearance. To ensure the complete removal of finely divided particulate material the SFH was centrifuged further 5 or 6 times at 3000 rpm. Both the particulate fraction and the SFH were then made up to the volume of the original hemolysate with saline.

Reticulocytes were obtained from rabbits in which an intense (95%) reticulocytosis had been induced by giving injections of acetylphenylhydrazine. These blood specimens were kindly provided by a colleague, Mr. P. Ottolenghi. The reticulocytes were washed, hemolysed, and divided into the particulate and SFH fractions.

Mature erythrocytes from rabbits and humans were collected in heparin and similarly fractionated into the particulate material and the SFH.

EXPERIMENTAL

Chicken blood was studied first as a source of DPN pyrophosphorylase. With NMN and ATP as substrates, both the whole hemolysate and the particulate fraction were found to synthesize DPN very readily. The analytical data are given in Table I. Sodium fluoride appeared to inhibit the reaction in the whole hemolysate, but not in the particulate fraction. The explanation for this apparent anomaly is that inorganic pyrophosphatase, the enzyme that catalyzes the hydrolysis of inorganic pyrophosphate, is confined entirely to the soluble fraction (SFH) of the red corpuscles of the chicken, rabbit and the human. This enzyme is inhibited by small concentrations of sodium fluoride. These facts will be reported in greater detail in Chapter II. In the synthesis of DPN by the hemolysates, therefore, inorganic pyrophosphate undergoes hydrolysis by the action of inorganic pyrophosphatase and thus is removed. But in the presence of sodium fluoride, the inorganic pyrophosphatase is inhibited, with the result that inorganic pyrophosphate accumulates and the synthesis of DPN (reaction 14) is retarded. That the enzyme is not directly inhibited by sodium fluoride is indicated by the observation that with the particulate preparation from chicken erythrocytes, DPN synthesis was not inhibited by sodium fluoride.

This interesting interrelationship between nuclear and

TABLE I

DPN Synthesis in the Chicken Erythrocyte.

Preparation	NaF	DPN Synth. (μM)	Inhibition (per cent)
Hemolysate	-	0.162	-
Hemolysate	+	0.126	22%
Particulate fraction	-	0.090	-
Particulate fraction	+	0.088	2%

Incubation medium consisted of: 0.1 M ATP, 0.1 ml.; 0.002 M NMN, 0.2 ml.; 0.15 M $MgCl_2$, 0.1 ml.; 0.25 M glycyl-glycine buffer pH 7.4, 0.2 ml.; 2.0 M nicotinamide, 0.1 ml.; 0.01 M NaF, 0.1 ml.; H_2O to 1.0 ml. Enough enzyme was added to give approximately 40% removal of the NMN. Incubation 45 minutes at 37°C. 1 ml. 10% trichloracetic acid was added to the incubation mixture. Following centrifugation, the filtrate was neutralized and DPN was determined by the alcohol dehydrogenase method (see methods). DPN synthesis expressed as the total amount of DPN in the 1.0 ml. of reaction mixture.

cytoplasmic activity was confirmed in another way (Table II). A portion of the cytoplasmic fraction (SFH), which by itself showed no synthetic activity, when combined with some of the particulate fraction considerably enhanced the activity of the latter. In the presence of sodium fluoride, however, the activity of the combined fractions was reduced to that obtained with the particulate fraction alone.

To demonstrate the reversibility of the synthesis, that is, the pyrophosphorolysis of DPN, PP and DPN were used as the substrates (Table III) and the amount of DPN broken down was determined at the end of the reaction period. In the control specimen, DPN was incubated at 37°C, without the addition of PP but in the presence of the enzyme, to determine any breakdown of DPN by a DPN nucleosidase which might be present. Unless otherwise stated, nicotinamide was always added, to inhibit the DPNase (114). In the presence of sodium fluoride, the hemolysate showed a much greater activity than in its absence. This enhancement again could be attributable only to inhibition of the inorganic pyrophosphatase, in which circumstance inorganic pyrophosphate tends to accumulate and thus drive the equilibrium to the left (reaction 14). Sodium fluoride had no effect on the pyrophosphorolytic activity of the particulate fraction, nor did the SFH shown any activity. When the SFH was combined with the particulate fraction, it caused a diminution in the activity of the latter, but in the presence of sodium fluoride,

TABLE II

DPN Synthesis by Fractions Prepared from Chicken Erythrocytes.

Preparation	NaF	DPN	Activation
		(μ Moles)	(per cent)
Particulate	-	0.059	-
SFH	-	0	-
Particulate + SFH	-	0.101	67%
Particulate + SFH	+	0.055	-6%

Same conditions as in Table I, except that 0.1 ml. NMN was used as substrate. Incubation: 2 hours at 37°C.

TABLE III

Pyrophosphorolysis of DPN in the Chicken Erythrocyte.

Preparation	NaF	DPN	ΔDPN	DPN split
		(μM)	(μM)	(per cent)
Hemolysate	-	0.126	-0.018	12.5%
Hemolysate	+	0.0198	-0.124	86.0
Particulate fraction	-	0.025	-0.155	86.0
Particulate fraction	+	0.022	-0.158	88.0
SFH	-	0.150	0	0
Particulate + SFH	-	0.108	-0.072	40
Particulate + SFH	+	0.011	-0.169	94

0.004 M DPN, 0.1 ml.; 0.01 M PP, 0.1 ml.; 0.15 M $MgCl_2$, 0.1 ml.; 0.25 M glycyl-glycine buffer, pH 7.4, 0.2 ml.; 2.0 M nicotinamide, 0.1 ml.; 0.01 M NaF, 0.1 ml. Sufficient enzyme was added to achieve almost total pyrophosphorolysis of DPN. Incubation: 45 minutes. DPN was determined as in Table I, and expressed as total amount in the reaction mixture.

the activity was restored to the usual value. Thus again, as in the forward reaction, a metabolic interrelationship between the activity of the nuclear and cytoplasmic fractions was demonstrated.

With reference to the maturation of the erythrocyte, it was important to determine whether any pyrophosphorolytic activity could be elicited in the cytoplasmic fraction of the chicken erythrocyte. In the assay for enzyme activity in the SFH, this fraction is usually diluted with sodium chloride to make the volume up to that of the original whole hemolysate (as described under Methods). The experiment was carried out for the same length of time, and with the same substrate concentrations, as with the whole hemolysate and the particulate material. Erythrocyte fractions, therefore, were prepared, but this time the SFH was not made up to the original volume of the hemolysate. The period of the experiment was extended, and the extent to which pyrophosphorolysis of DPN had occurred was expressed as the percent of DPN that had undergone breakdown (Table IV). The undiluted SFH showed some activity, but after centrifugation at 25,000 x g. for 2 hours, the activity was found to be diminished by 28%. The lost activity was recovered in the gelatinous precipitate that had been thrown down during the centrifugation. One may conclude, therefore, that any activity of the SFH (cytoplasmic fraction) of the chicken erythrocyte probably may be attributable to the presence of

finely divided nuclear material. Chicken erythrocytes do not contain mitochondria, and the activity which in most somatic cells, is usually attributed to the mitochondria, in the chicken erythrocyte is confined to the nucleus (137). At the speed of centrifugation used, only nuclear fragments of the cells would be thrown down. Hogeboom et al. (125) observed in their studies with mouse liver homogenates, that although DPN pyrophosphorylase activity was concentrated in the nuclei, a variable amount of activity was always present in the cytoplasm. By exercising greater care in the preparation of their samples, they found that the activity in the cytoplasmic fraction could be diminished. Although the pyrophosphorylase is 'water-soluble', Hogeboom and Schneider (125) found, in testing preparations of mouse liver nuclei, that the nuclear membrane is not permeable to the enzyme. It would appear, therefore, that in our nuclear preparations from chicken erythrocytes, some of the nuclei may have been ruptured and dispersed in colloidal form, thus imparting to the SFH the apparent capacity to synthesize DPN.

A comparison was made of the activity of the whole hemolysate from chicken erythrocytes with that of the cytoplasmic (SFH) and the particulate (nuclear) fractions, the volume of each of these fractions having been made up to that of the original whole hemolysate. In this recovery experiment, the SFH proved to be devoid of activity, while

the particulate fraction contained 80% of the activity of the original whole hemolysate (Table IV).

Throughout our study on the synthesis of DPN from NMN in the chicken erythrocyte, the maximum yield of DPN obtainable under a variety of conditions was equivalent to only 85% of the added NMN. On increasing the amount of enzyme, varying the substrate concentration, incubating for longer or shorter periods of time, the reaction still did not appear to go to completion. In view of the fact that some of the preparations of NMN were contaminated with NR, it appeared possible that NR might be inhibiting the DPN pyrophosphorylase. Using NMN, purified by ion exchange chromatography, the conversion of NMN to DPN still was found to be equivalent to only 85% of the amount of NMN added. It was found further that NR, prepared from NMN by the 5'-nucleotidase and purified, did not inhibit the pyrophosphorylase of DPN at a concentration of 1×10^{-4} M. This concentration was comparable with the quantity of NR present in the impure samples of NMN that were used first in the studies of the synthesis of DPN. Since the extent of the synthesis of DPN from NMN was the same with either purified NMN or NMN contaminated with NR, it seemed clear that NR does not inhibit the DPN pyrophosphorylase (at the concentrations used) and that the failure of the enzyme preparations to convert all of the added NMN to DPN was not due to this factor.

TABLE IV

Distribution of DPN Pyrophosphorylase in Various Fractions Prepared from Chicken Erythrocyte.

Preparation	Pyro-Phosphorolysis	Activity accounted for	Fall in Activity
	(per cent)	(per cent)	(per cent)
Hemolysate	75		
Particulate	60	80	
SFH	0	0	
		Total 80	
SFH (undiluted)	29	-	-
SFH (undiluted, centrifuged)	21	72	28
Residue	5.9	20	-
		Total 92	

Conditions as given in Table III. In the first part, the duration of incubation was 30 minutes. The fractions were prepared as described under methods. In the second part, the SFH was not diluted. An aliquot was centrifuged at 25,000 x g. The supernatant and residue were assayed for DPN pyrophosphorylase; incubation time: 2 hours.

Kaplan et al. (16) recently have demonstrated the presence of an α-isomer of DPN in crude and in purified preparations of DPN. This isomer is not hydrolyzable by the DPN nucleosidase of Neurospora crassa nor can it be reduced by alcohol dehydrogenase in the presence of ethanol. However, like the β-isomer of DPN, it reacts with cyanide. The α-isomer of DPN has an α-linkage between the ribose and the nicotinamide components of the molecule while cozymase has the β-linkage. Kaplan and associates found that even the purified preparations of DPN usually contain from 10-15% of the α-isomer.

To ascertain whether our preparation of NMN (purified) contained the α-isomer along with the normal or β-form, the following experiments were carried out:

NMN was incubated with, and also without, the addition of ATP in the presence of an active particulate preparation from chicken erythrocytes (Table V). Nicotinamide was not added, hence any DPN that might be synthesized would be hydrolyzed by the DPN nucleosidase which is present in the particulate fraction of the erythrocytes. Similarly, any hydrolysis of NMN itself would be detected in the control sample which contained NMN along with the particulate preparation from chicken erythrocytes, but no ATP. The quantity of cyanide-reacting material was found to be the same whether ATP was present or absent. In the presence of ATP, the amount of DPN, as measured by the alcohol

TABLE V

Detection of the α-Isomer of NMN.

NMN	ATP	CN-reacting material	ADHase-reacting	Ratio, $\frac{\text{ADHase-reacting}}{\text{CN-reacting}}$ material
		(μM)	(μM)	(per cent)
+	+	0.243	0.194	80
+	-	0.243	-	

0.003 M NMN, 0.1 ml.; 0.1 M ATP, 0.1 ml.; 0.25 M glycylglycine, pH 7.4, 0.2 ml.; 0.15 M $MgCl_2$, 0.1 ml.; H_2O to 0.6 ml. Enough enzyme added to drive the reaction to completion. Incubation: 3 hours. Added 0.6 ml. 5% trichloroacetic acid and placed in boiling H_2O bath for 30 seconds. Centrifuged. Supernatant neutralized with 2N NaOH and aliquots taken for cyanide-reacting material (see methods) and for DPN assay (see methods).

dehydrogenase method, was found to be only 80% of the total amount of cyanide-reacting material. One interpretation of this finding might be that equilibrium was reached when 80% of the theoretical maximum synthesis of DPN had taken place, the remaining material being unaltered NMN. There are two alternative explanations. The remaining cyanide-reacting material could be the α-isomer of NMN, isolated along with the β-isomer in the preparation of this substance from DPN. This material would react with cyanide, but should not serve as a coenzyme for alcohol dehydrogenase. On the other hand, DPN pyrophosphorylase may be non-specific in the sense that it is capable of catalyzing the synthesis of either the α- or the β-isomers of DPN from ATP and the α- or β-isomers of NMN, respectively. It is reasonable to suppose, therefore, that in this experiment, the remaining cyanide-reacting material could be the α-isomer of DPN since in this case, as with the α- or β-isomers of NMN, the material would react with cyanide but could not serve as a coenzyme for alcohol dehydrogenase. Moreover, neither the α-isomer of NMN nor that of DPN would be hydrolyzed by DPN nucleosidase.

In order to eliminate the possibility that equilibrium was reached when only 80% of the theoretical maximum synthesis of DPN had taken place, a stroma preparation from rabbit erythrocytes was added to the reaction mixture. The stroma of the erythrocyte of this animal contains a very active DPN nucleosidase (145) hence any β-isomer of DPN

synthesized would be removed by hydrolysis as fast as it was formed. In other words, the equilibrium of the reaction would be shifted as far to the right as possible (reaction 14). Under these conditions (Table VI), with NMN and ATP present, no DPN was detectable by the alcohol dehydrogenase method, but there remained a small quantity of cyanide-reacting material, equivalent to 21% of the amount of NMN originally added.

Thus, whereas under the conditions of the former experiment the equilibrium may have been reached when 80% of the theoretical amount of DPN had been synthesized, under the conditions of the latter experiment, the DPN would have been destroyed as rapidly as it was formed and all the NMN should have been used up. Theoretically, therefore, we should expect to find no remaining cyanide-reacting material in the latter case. The fact of the matter was, however, that there remained cyanide-reacting material equivalent to about 20% of the NMN originally present. Since in the latter experiment the material could not have been unaltered β-NMN, it is logical to conclude that it was either an α-isomer of NMN present as a contaminant in the starting material, or the α-isomer of DPN formed from the α-NMN. A mixture of the two isomers of DPN may have been produced in the reaction. In either case, the NMN prepared from DPN by means of the potato nucleotide pyrophosphatase appears to contain about 20% α-NMN. This value is comparable with 10-15% of α-DPN found in purified preparations of DPN by Kaplan et al. (16).

TABLE VI

Detection of the α-isomer of NMN.

NMN	ATP	CN-reacting	ADHase-reacting	Fall in CN-reacting
		(μM)	(μM)	(per cent)
+	-	0.252	0	-
+	+	0.051	0	79%

Same conditions as in Table V, but with the addition of 0.05 ml. of a rabbit erythrocyte stroma preparation containing an active DPN nucleosidase. Total incubation volume 0.65 ml. 0.55 ml. 5% trichloroacetic acid added after incubation.

Rowen and Kornberg (118) demonstrated the synthesis of NMN from NR and ATP using crude fractions from hog liver. The synthesis, however, was found to proceed very slowly. Using the particulate fraction from chicken erythrocytes, or this fraction supplemented with SFH, we were unable to demonstrate the synthesis of DPN from NR and ATP. Indeed, using the particulate fraction from chicken erythrocytes as a source of the synthesizing enzyme, supplemented with rabbit erythrocyte stroma as a source of DPNase, thereby ensuring a complete shift of the equilibrium to the right (reaction 14), the writer was still unable to demonstrate the synthesis of DPN from NR (purified) and ATP. Although the experimental conditions may not have been optimal for the demonstration of nicotinamide nucleoside phosphokinase activity, the writer was obliged to conclude, tentatively, that the chicken erythrocyte does not possess this type of enzyme activity.

Hemolysates prepared from the blood of rabbits with an induced reticulocytosis (50-90% reticulocytes) were tested as a source of DPN pyrophosphorylase. On using different substrate concentrations, increasing the time of the experiment and increasing the quantity of enzyme used, it was not possible to demonstrate the pyrophosphorolysis of DPN with these preparations. There appeared, however, to be a consistent but slow and meagre synthesis of DPN in the presence of added NMN and ATP. The amount of DPN synthesized never was equivalent to more than 3-5% of the NMN added.

This was the maximum yield obtained in spite of numerous attempts, such as those outlined above, to ascertain the optimal conditions. The writer considers these small yields of DPN to be of doubtful significance, particularly since the reverse of the reaction, that is, the pyrophosphorolysis of DPN could not be demonstrated to occur in these cells. It is possible, though hardly likely, that the precursor of the reticulocyte may possess DPN pyrophosphorylase activity and that when this activity is lost, the freedom of the reaction to proceed in one direction is retained, while the reversibility is disturbed.

With human erythrocytes, repeated attempts were made under a variety of conditions, to demonstrate the synthesis or the pyrophosphorolysis of DPN. By increasing the quantity of enzyme used for these experiments and by extending the incubation time up to 5 hours, neither the synthesis nor the pyrophosphorolysis of DPN could be consistently demonstrated. Whenever very slight pyrophosphorolysis of DPN did occur, the rate of breakdown of DPN was erratic with respect to time, and the analytical values obtained were always within experimental error. It occurred to the writer that the sodium chloride solutions in which the erythrocytes were suspended possibly might have an inhibiting influence. Using an hemolysate of packed cells, or intact human red blood cells washed and diluted with potassium chloride or Krebs-Ringer phosphate buffer, then hemolysed, it was still impossible

to demonstrate DPN pyrophosphorylase activity. A purified preparation of NMN was used, on the supposition that the NR, in the impure preparations, might be inhibiting the synthesis of DPN, but still it was not possible to detect any synthesis of DPN. Cysteine was added to some of the incubation mixtures, but without effect. Incubation of NR, ATP and inorganic phosphate did not induce the formation of DPN in the presence of the hemolysate of human erythrocytes. That the human erythrocyte does not contain an inhibiting substance was readily established by showing that the hemolysate of human erythrocytes did not interfere with the conversion of NMN to DPN in the chicken red cellhemolysate. The writer was obliged, therefore, to conclude that under the conditions employed, DPN pyrophosphorylase activity is not present in the human erythrocyte.

DISCUSSION

DPN pyrophosphorylase activity has been demonstrated in the chicken erythrocyte, the enzyme being confined to the particulate fraction. Any activity in the SFH was found to be attributable to the presence of finely divided particulate material which could be precipitated by high-speed centrifugation.

The particulate fraction of chicken erythrocytes consists of residues of nuclei and cell membrane. Rubinstein *et al.* (137) have demonstrated that this fraction possesses

succinoxidase activity and an active tricarboxylic acid system. With material from the erythrocytes, prepared according to the method of Hogeboom et al. (146), Rubinstein found that the succinoxidase activity occurs only in the readily sedimentable fraction. While the succinoxidase and the tricarboxylic acid systems are confined to the mitochondria in most somatic cells, these enzymes in the chicken erythrocyte appear to be confined to the nucleus. In other words, the chicken erythrocytes do not appear to contain mitochondria, but the activities usually attributed to the mitochondria in other cells are associated, in the chicken erythrocyte, with the readily sedimentable fraction. These cells may be analogous to the orthochromatic erythroblast, the most mature of the nucleated forms of the mammalian series of erythrocytes, since Bessis (134) was unable to demonstrate mitochondria in these cells. The more immature cells of the nucleated series, however, do contain mitochondria. It may be, of course, that the mitochondria in the more mature cells are fused in some way with the nuclear material and thus are not distinguishable by biochemical means or on morphological examination.

Since the biochemical evidence appears to support the morphological findings, and conceding that this inference may not be entirely correct, one may conclude from the foregoing considerations that the DPN pyrophosphorylase in the chicken erythrocyte is associated either with the nucleus

or the cell membrane; in any event, it is definitely associated with the readily sedimentable fraction. These two solid cell constituents in tissue homogenates, as Hogeboom et al. (125) have pointed out, are not readily separable. These authors suggest, furthermore, that the DPN pyrophosphorylase activity of their preparations of mouse liver nuclei more likely is associated with nuclear rather than with the membrane material, since the membrane residue constitutes a relatively small fraction of the total sedimentable material. Similarly, in our nuclear preparations from chicken erythrocytes, the membrane residues comprise only a small fraction of the total sedimentable material. It is possible, however, that the enzyme activity may be highly concentrated in the membrane of the cell. A clarification of the matter probably will not be achieved until the complete separation of the nuclear from the membranous fraction of the readily sedimentable material has been accomplished and a study of the DPN pyrophosphorylase in the isolated nuclei has been carried out. Stern et al. (123) employing organic solvents and differential centrifugation, have succeeded in separating the nuclear from the membrane material in a variety of tissues including the fowl erythrocyte. They demonstrated, furthermore, the morphological purity of the nuclear material. It still appears to be more likely that the DPN pyrophosphorylase activity is associated with the nuclear rather than the membranous fraction in the chicken

erythrocyte. If the enzyme were to be found in the membrane of the nucleated erythrocyte, one should expect it to occur also in the membrane of the nonnucleated cell. However, the latter was shown not to be so. It is far more reasonable, therefore, to conclude that the enzyme activity is confined to the nucleus of the immature nucleated human erythrocyte, and that after the disappearance of the nucleus, the enzyme activity is no longer detectable in the nonnucleated red blood cell.

As mentioned previously, the writer was able to demonstrate neither the synthesis nor the pyrophosphorolysis of DPN in the human or the rabbit erythrocyte, or the rabbit reticulocyte. With some of the preparations containing both erythrocytes and reticulocytes from the rabbit (only 50-90% reticulocytosis), a slight amount of DPN appeared to have been formed. The amount was so small that it is doubtful whether any synthesis occurred. It is difficult to resolve this finding with the observation of Rubinstein et al. (138) that the rabbit reticulocyte retains succinoxidase activity and a few of the enzymes of the tricarboxylic acid system, since as pointed out before, these activities are generally associated with the nuclei in the nucleated erythrocyte. It would appear, therefore, that in the loss of the nucleus from the nucleated precursor, the activity which is generally associated with the mitochondria is retained in the reticulocyte while that which is strictly confined to the nucleus is

lost. Later, during the transition of the reticulocyte into a mature nonnucleated erythrocyte, the "mitochondrial" activity also disappears.

The observation that the nuclear material from chicken erythrocytes contains an active DPN pyrophosphorylase raises the question whether, in the maturation of the mammalian erythrocyte, the nucleus undergoes fragmentation or dissolution (130,131). If fragmentation takes place, one would expect to find some residual enzymatic activity in the mature nonnucleated cell since water-soluble components such as DPN pyrophosphorylase in particular (125) would tend to be dispersed in the cytoplasm. It may be recalled that in the maturation of the mammalian red blood cell, according to one hypothesis, the nucleus is extruded from the cell (129), while according to another, the mature erythrocyte represents a cytoplasmic 'bud' arising from a primitive precursor and thus is liberated in the nonnucleated form (132). Notwithstanding the class difference between the bird and the mammal, it is reasonable to suppose that there is a similarity at least in the enzyme components of the mature red blood cell of the bird and thoseof the nucleated precursors of mammalian erythrocytes.

Another mechanism for the synthesis of DPN has been demonstrated by Leder and Handler (114) who showed that on incubating intact human erythrocytes with nicotinamide over a long period there was an increase in the cellular content

of pyridine nucleotides. Between 75 and 95% of this material appeared to be NMN, and the remainder DPN. A synthesis of DPN similarly was noted with hemolysates from human erythrocytes, supplemented with nicotinamide, ATP, glucose and fructose diphosphate (115). In this case, the amount of DPN synthesized accounted for less than 10% of the total increment in the content of pyridine nucleotides. Whether the DPN was formed directly from nicotinamide or by way of a synthesis of NMN, is not known. The former process appears to be the more likely, since in our studies with human erythrocytes there was no evidence of the formation of DPN from NMN and ATP, nor was there any evidence of the pyrophosphorolysis of DPN. In other words, the human erythrocyte apparently contains no active DPN pyrophosphorylase. Recently, Handler (147) has expressed the opinion that the human erythrocyte, in vitro, cannot synthesize DPN. The increase in the total amount of pyridine nucleotides observed in their previous studies apparently, therefore, must have been attributable to NMN or this nucleotide and another unidentified substance. The question arises as to whether the human erythrocyte can synthesize DPN "in vivo". Previous studies on the effect of administered nicotinic acid on humans and animals, and the effect of deprivation of the vitamin are of little value in elucidating this question, since the substance measured was pyridine nucleotide and not DPN. Even if it were definitely established that DPN is synthesized, one could not tell

whether the coenzyme has been synthesized in the nucleated precursor of the erythrocyte in the bone marrow and was retained in the circulating mature erythrocyte. Only by comparing the extent of DPN synthesis with the normal rate of generation of fresh erythrocytes could one determine whether the mature mammalian erythrocyte can synthesize DPN.

Hogeboom et al. (125) pointed out that the DPN synthesized in the nuclei of liver cells must traverse the nuclear membrane in order to participate in the glycolytic activity of the cytoplasm of the cell. Inorganic pyrophosphate, the other product of DPN synthesis, must also traverse the nuclear membrane. The writer has found (Chapter II) that in the chicken erythrocyte, the inorganic pyrophosphatase is confined entirely to the soluble or cytoplasmic fraction of the cell. If inorganic pyrophosphate were to accumulate in the nucleus, and be unable to participate in other chemical reactions within the nucleus, its high concentration would tend to oppose the synthesis of DPN. But by being able to pass through the nuclear membrane, its destruction by hydrolysis in the cytoplasm is ensured. Furthermore, since DPN pyrophosphorylase appears to be confined to the nucleus, it is unlikely that DPN will be broken down by pyrophosphorolysis, at least, in the cytoplasm. Thus, the nucleated erythrocyte affords an interesting example of the metabolic integration between the nucleus and the cytoplasm, involving enzymes which apparently are confined to their

respective cellular compartments and substrates which can traverse the membranes separating the compartments. The picture is one of facilitated cellular function.

Whether the gradual decrease in the concentration of DPN in the erythrocyte during storage, as observed by Pappius et al (1), contributes significantly to the progressive failure of the glycolytic system of the cell during storage is still an open question. The present study has indicated that the mature human erythrocyte cannot replenish DPN at least by synthesis from NMN and ATP.

CHAPTER II

The Inorganic Pyrophosphatase of the Erythrocyte

INTRODUCTION

As shown in the previous chapter, the synthesis of DPN from the substrates NMN and ATP (reaction 14) in the isolated nuclei of chicken erythrocytes is enhanced by the addition of the cytoplasmic fraction (SFH) of these cells to the medium. The activation was attributed to the presence of an inorganic pyrophosphatase in the cytoplasm. With the cleavage of inorganic pyrophosphate, which is one of the products of the reaction, a shift in the equilibrium toward the synthesis of DPN occurs. An interrelationship was shown to occur between the enzymatic activities of the nucleus and the cytoplasm of the nucleated erythrocyte. To obtain a more complete picture of the mechanism involved in the synthesis of DPN in the red blood cell, the writer undertook to study the enzyme inorganic pyrophosphatase in greater detail.

Several biochemical reactions have recently been discovered, of which inorganic pyrophosphate is a by-product of the reactions. The following are some examples:

(i) Lipmann et al. (156) demonstrated the synthesis of acetyl-CoA by purified enzyme preparations from pigeon liver and yeast and found pyrophosphate to be among the products of the over-all reaction, as follows:

(17) ATP + acetate + CoA $\rightleftharpoons$ AMP + PP + Acetyl-CoA.

(ii) Kalckar (81) showed that the synthesis of uridine diphosphate glucose (UDPG) proceeded in the following manner:

(18) UTP + glucose-1-phosphate $\rightleftharpoons$ UDPG + PP.

(iii) The preliminary stage of the synthesis of protein by rat liver was shown by Hoagland (157) to occur in much the same way as the synthesis of acetyl-CoA (reaction 17). The over-all reaction may be represented as follows, with hydroxylamine as the amino-acid acceptor:

(19) ATP + amino acid + NH_2OH $\longrightarrow$ AMP + PP + amino acid NHOH.

(iv) Kornberg et al. (87) have recently purified an enzyme from yeast that catalyzes the synthesis of AMP in the following way:

(20) Adenine + PRPP $\rightleftharpoons$ AMP + PP.

The same enzyme appears to be capable of catalyzing the synthesis of inosinic acid and guanylic acid from hypoxanthine and guanine respectively; while a different enzyme, purified from yeast as well, can catalyze the synthesis of orotidine-5'-phosphate from orotic acid (86). The mechanism of the

synthesis of this compound is the same as for the synthesis of AMP.

The first two and the last reactions shown above are completely reversible. In the third reaction shown here (reaction 19) the intermediate steps leading to the formation of the hydroxamic acid - amino acid (not illustrated) are reversible, only the final steps of the reaction being irreversible.

The accumulation of inorganic pyrophosphate in the cell in which any of the foregoing reactions can occur, would tend to shift the equilibrium of the reaction to the left. The removal of inorganic pyrophosphate appears to be ensured, however, by the presence of the enzyme inorganic pyrophosphatase, which catalyzes the hydrolysis of the inorganic pyrophosphate to inorganic phosphate. This enzyme has been found in a wide variety of plant and animal tissues. The isolation of inorganic pyrophosphate, the distribution of the enzyme catalyzing its hydrolysis, the characteristics of the enzyme with respect to the pH optimum of activity, its activation and inhibition by various substances and the isolation and purification of the enzyme will be discussed. Most of the characteristics of the enzyme will be described in chronological order of their discovery. The presentation of each discovery illustrates a point, and in this way lends coherence to the introductory remarks. The work relating to the erythrocyte will be described in a separate section since the writer's work has been concerned particularly with this cell.

(a) Inorganic Pyrophosphatase; its Distribution in Nature and the Mechanism of its Action.

In 1928, Lohmann (158) isolated and identified inorganic pyrophosphate from a muscle brei of the frog. In addition, he demonstrated the cleavage of the inorganic pyrophosphate in this system. Mann (159) in 1944, isolated in pure form pyrophosphoric and metaphosphoric acids from the mycelia of the microorganism Aspergillus niger. Lindahl et al. (160) described a method for the isolation and purification of inorganic pyrophosphate from baker's yeast. On hydrolysis of this material with normal hydrochloric acid for 7 minutes at 100°C, the total content of phosphate appeared as orthophosphate. In all of these reports, the authors did not state whence the inorganic pyrophosphate arose. Inorganic pyrophosphate does not apparently participate in biochemical reactions other than the reversible reactions already described. The heat of hydrolysis of inorganic pyrophosphate is about 9000 calories per mole (161). If the inorganic pyrophosphate liberated as a by-product by various biosynthetic mechanisms were not utilized, then its destruction would be wasteful of energy which would be dissipated as heat. Notwithstanding this loss, the removal of the inorganic pyrophosphate in this manner would favor the synthesis of important compounds which could be used in a variety of ways by the intact cell (vide supra). Furthermore, the hydrolysis of inorganic pyrophosphate would liberate orthophosphate

which could be utilized for the synthesis of other high-energy phosphate compounds.

Neuberg et al. (162) synthesized potassium diphenyl-pyrophosphate and showed that a mushroom phosphatase was able to hydrolyze this aromatic pyrophosphate. Later, (163) he prepared potassium diorthocresol pyrophosphate and demonstrated the hydrolysis of this substrate by a taka-diastase phosphatase, and by preparations of horse liver, kidney and muscle. In 1928, Kay (164) reported the presence of inorganic pyrophosphatase activity in extracts of kidney cortex, duodenal mucosa, bone and lung of the cat, kidney of the pig and growing bones of the young rat. Muscle extract, blood plasma and an extract of gastric mucosa showed only weak pyrophosphatase activity. The optimum pH at which the hydrolysis occurred was 7.2 - 7.8. Fleury and Courtois (165,166) studied the inorganic pyrophosphatase and the glycerophosphatase activities of various cereal grains, almond preparations and taka-diastase. The optimum pH for the pyrophosphatase activity was about 5.6 - 5.8. They showed that there was a parallellism between the two enzymes with respect to their activity and distribution, but that they differed in some of their characteristics and could be separated from one another. Earlier, Pett and Wynne (167), using sodium-β-glycerophosphate, sodium hexosediphosphate and pyrophosphate as substrates, showed that the microorganism Clostridium acetobutylicum Weizmann exhibited

phosphatase activity. Magnesium ions enhanced the extent of hydrolysis of these substrates, the optimal concentration for the pyrophosphatase being 1 x $10^{-4.5}$ M. Higher concentrations of magnesium resulted in an inhibition of the enzyme activity. Similarly, the presence of calcium, fluoride and heavy metals reduced the activity of these preparations.

Lohmann (168) reported also that the hydrolysis of pyrophosphate by liver extract depended on the presence of magnesium ions, and that the activation by magnesium was maximal when the ratio of magnesium to pyrophosphate was equal to or greater than 2. He found that calcium ions inhibited the pyrophosphatase activity. Bauer (169), who purified a pyrophosphatase from bottom yeast, pointed out that the relationship between the magnesium and pyrophosphate concentrations, with respect to the optimal enzyme activity, depended on the pH of the medium; for example, the more acid the medium, the higher the concentration of magnesium required to activate the enzyme. Bauer observed also that the inhibition produced by calcium could be reversed by the addition of magnesium ions. While magnesium ions had little effect in overcoming the inhibition by sodium fluoride, in this instance higher concentrations of pyrophosphate tended to overcome the fluoride effect. In 1943, Roche et al. (170) showed that the intestinal alkaline pyrophosphatase lost the greater part of its activity when dialyzed (for 27 hours at 37°C) against substances like α, α'-dipyridyl or sodium

diethylcarbonate which form complexes with metals. The enzyme could be reactivated by the addition of manganese, magnesium, calcium, ferrous or zinc ions. Zinc ions exerted a synergistic effect when used in conjunction with the manganese, magnesium or calcium ions.

An inorganic pyrophosphatase of yeast was purified and studied by Bailey and Webb (171). The enzyme was activated specifically by magnesium ions, the optimal concentration being 0.002 M. The enzyme was inhibited by alloxan, but this inhibition could be reversed with cysteine. A fresh preparation of the enzyme was not activated by cysteine, but when the enzyme was stored for 2 weeks at 0°C and the activity of the enzyme was allowed to fall about 40%, cysteine effected a partial reactivation of the enzyme activity. These authors pointed out that with substrate concentrations greater than 0.03 M, inhibition of the enzyme occurred. Furthermore, the observed inhibition by calcium ions was found to depend on the ratio of calcium to magnesium ions present in the incubation medium. The inhibition by calcium was of a non-competitive nature. Sodium fluoride, copper ions and iodoacetate all inhibited the enzyme activity. Gordon (172, 173,) studied the inorganic pyrophosphatase activity of rat brain. As with the aforementioned yeast enzyme, the enzyme preparation of rat brain was inhibited by alloxan, cuprous ions and iodoacetate. These facts suggested to the author, that the brain enzyme depended on sulfhydryl groups for its

activity. The enzyme was practically inactive in the absence of magnesium ions, the optimal concentration of magnesium for the maximum activity being 0.02 M. Calcium ions and sodium fluoride also inhibited this inorganic pyrophosphatase. Binkley and Olson (174) used brain tissue as a source of the enzyme, and found that, in addition to magnesium, glutathione or cysteine were essential for the demonstration of full activity. With high levels of magnesium, inhibition of the enzyme activity was observed.

The pyrophosphatase activity of rat sarcoma (induced by benzpyrene) was studied (175). The administration of colchicine to the rats produced tumor regression, but in addition, the pyrophosphatase activity of the tumors was diminished. Colchicine did not exert an inhibitory effect on the enzyme activity of liver, kidney, muscle or skin. When administered intravenously, the pyrophosphatase activity of the erythrocytes remained unaltered.

McElroy *et al.* (176) succeeded in partially purifying the enzyme from extracts of firefly 'lanterns'. Magnesium was found to be essential for the enzyme activity, but cysteine produced no enhancement of the hydrolysis of the pyrophosphate. Neither alloxan nor iodoacetate affected the pyrophosphatase. Unlike the enzyme from yeast and brain, this particular pyrophosphatase did not seem to require sulfhydryl-containing compounds for its activation. The pH optimum was 8.3. Calcium and fluoride inhibited the enzyme,

and the extent of the inhibition by calcium depended on the calcium : magnesium ratio. Heppel et al. (177) showed that metaphosphate formed a complex with magnesium, but was not hydrolyzed by a yeast pyrophosphatase. But metaphosphate inhibited the activity of the enzyme when pyrophosphate was used as a substrate. These authors then suggested that the inhibitory effect observed with high concentrations of substrate could be attributed to binding of magnesium ions. Using bull semen as a source of pyrophosphatase, Heppel et al. (177) showed that when the molar ratio of magnesium to pyrophosphate exceeded 1, there was a diminution in the rate of hydrolysis of the substrate. But in this preparation, no inhibition was observed when the substrate concentration was increased. The yeast enzyme was specific for inorganic pyrophosphate and had no effect on ATP, ADP, AMP, DPN, thiamin pyrophosphate, sodium metaphosphate, sodium triphosphate or sodium β-glycerophosphate.

Swanson (178) investigated the inorganic pyrophosphatase in rat liver homogenate, and found that the optimum pH for activity lay between 7 and 8. The exact position of the peak shifted when the magnesium concentration was varied. The highest activity was obtained with a magnesium concentration of 0.02 M at pH 7.4. Swanson suggested that the inhibition observed with calcium and fluoride was due to the formation of inactive complexes which compete for the enzyme with magnesium pyrophosphate, the "true substrate".

With calcium, the complex was presumed to be calcium pyrophosphate, and in the case of fluoride, magnesium-fluoropyrophosphate. The pyrophosphatase was found to be concentrated in the soluble (cytoplasmic) fraction of the liver preparation. Gilmour et al. (179) studied the pyrophosphatase activity of locust muscle. While magnesium activated the enzyme, inhibition of the enzyme was observed when substrate concentrations higher than maximal were used. These authors suggested that the effect here was due to the binding of magnesium by the additional substrate, in which circumstance the magnesium was no longer available for activation at the enzyme surface.

Naganna and coworkers (180-184) studied the characteristics of the inorganic pyrophosphatase in guinea pig tissues and in a number of plants. The enzyme from various tissues of the guinea pig was found to be activated by magnesium, and inhibited by calcium, fluoride, mapharside, copper or formaldehyde. Cysteine reversed the mapharside effect. No activity was found in the blood plasma of these animals. Using potatoes, these authors found two inorganic pyrophosphatases, one with a pH optimum of activity on the alkaline side, and the other on the acid. The "alkaline enzyme" was active only in the presence of high concentrations of magnesium (0.01 - 0.02 M). Calcium and fluoride exhibited an inhibiting effect on this enzyme, but mapharside produced no inhibition of the activity, nor did cysteine

enhance the activity. The alkaline enzyme was found to be destroyed by acid, and the "acid enzyme" was destroyed by alkali. While calcium exerted no effect on the acid enzyme, fluoride inhibited its activity. These workers then studied the pyrophosphatase activity of germinating wheat and French beans. These plant tissues, unlike animal tissues, showed a high acid pyrophosphatase activity, and as with the potato enzymes, the alkaline pyrophosphatase, like the acid phosphatase, was active only in the presence of added magnesium. With the same substrate concentration, the alkaline pyrophosphatase activity was greater than the acid pyrophosphatase in the green beans, whereas in the seeds, the reverse was true.

Boyer et al. (185) studied the inorganic pyrophosphatase of rat brain. The activity of the enzyme was determined at various concentrations of magnesium and pyrophosphate. Their data show that inorganic pyrophosphate itself, and to a lesser extent magnesium pyrophosphate, have an inhibiting effect on the enzyme. With this information, the authors were able to plot theoretical curves showing the enzyme behavior at various magnesium and pyrophosphate concentrations, on the assumption that magnesium pyrophosphate was the "true substrate" for the enzyme. Since these curves coincided well with the experimental observations, the authors concluded that their assumption was correct and that the true substrate for the enzyme was magnesium pyrophosphate. In this way,

they were able to explain the inhibiting effects of pyrophosphate and excess of magnesium pyrophosphate. The requirement of the enzyme for a much higher magnesium than pyrophosphate concentration was apparently attributable to the removal by magnesium of the potent inhibiting effect of free pyrophosphate. Free magnesium had no such effect.

Recently, Oginksy et al. (186) prepared cell-free extracts of the microorganism Streptococcus faecalis, strain F2H, and demonstrated the presence of two enzymes capable of hydrolyzing inorganic pyrophosphate, one with a pH optimum of about 8.5 activated primarily by magnesium and the other enzyme with a pH optimum of about 5.3 activated primarily by cobaltous ions. The latter enzyme was inhibited by heavy metals, but not by sodium fluoride. The activation by cobalt was further stimulated by the addition of histidine, but the latter could be replaced by cysteine, lysine or histamine. The cobalt-activated acid pyrophosphatase was found also in a wide variety of other microorganisms.

Kunitz (187) isolated the inorganic pyrophosphatase from baker's yeast. By purification of the enzyme they succeeded in obtaining a crystalline material. The enzyme was found to be a protein of the albumin type with a pI around 4.8. The enzyme was most stable at a pH of 6.8. Like the impure preparation, the enzyme was activated by magnesium and inhibited by calcium. Using this crystalline

enzyme, Schachman (188) conducted studies on the physical properties of the material. From the viscosity, diffusion and ultracentrifuge data, he suggested that the molecular weight is around 60,000.

From the foregoing remarks, a general picture of the behavior of the enzyme inorganic pyrophosphatase may be obtained. The enzyme is indeed very widely distributed in nature. In almost all cases, it is inactive in the absence of magnesium, while calcium ions produce an inhibition of the activity. When fluoride was tested as an inhibitor, it was found to have an adverse effect. In only one instance (186), sodium fluoride did not influence the enzyme activity, and in this case, the enzyme was activated by cobaltous ions. The enzyme appears to require free sulfhydryl groups for maximal activity, since cysteine and glutathione enhanced the activity. Similarly, "-SH inhibitors" produced a diminution in the enzyme activity. The evidence presented, and particularly that of Boyer (185), points to the fact that magnesium pyrophosphate is the true substrate for the enzyme.

(b) <u>The Inorganic Pyrophosphatase of the Erythrocyte</u>.

In 1940, Sjoberg (189) found inorganic pyrophosphatase activity in the blood and plasma of man, dog and the horse. The optimum pH for activity was found to be 7.4. When blood cells were maintained in an isotonic medium, no activity was

observed, and only when the cells were hemolysed was activity apparent. The stroma of the red blood cells proved to be inactive. Roche et al. (190) showed that the hemolysate of mammalian erythrocytes contained two "acid pyrophosphatases", which were activated by magnesium and ascorbic acid. These enzymes could be separated from the phospho-monoesterases, also present in blood, by adsorption on kaolin at pH 5. Naganna et al. (191,192), using hemolysates of human erythrocytes, studied some of the characteristics of the inorganic pyrophosphatase. Magnesium, of course, activated the enzyme while calcium and fluoride (2×10^{-4} M) were inhibitory. Iodoacetate, alloxan, dehydroascorbic acid and ascorbic acid inhibited the enzyme, while glutathione and cysteine enhanced its activity. These authors suggested, therefore, that free sulfhydryl groups were essential for the action of the enzyme. While this is probably true, it is difficult to explain the adverse effect of ascorbic acid. Naganna pointed out that the activating effect of cysteine was more apparent with the aged enzyme than with a fresh enzyme preparation. In their studies on the distribution of phosphatases in human erythrocytes, Maizels et al. (193) reported that the inorganic pyrophosphatase activity was confined to the cytoplasmic fraction (SFH) and that none was observed in the stroma of these cells. As mentioned previously, Swanson (178) observed that the inorganic pyrophosphatase of homogenates of rat liver was concentrated also in the soluble or cytoplasmic

fraction of these preparations. Bloch-Frankenthal (194), in her studies on the pyrophosphatase of the rat erythrocyte, pointed out that the rate of hydrolysis of pyrophosphate was a function of the concentration of magnesium. Frankenthal plotted the rate of the hydrolysis of the substrate against the magnesium chloride concentration, and similarly, the change in pH against magnesium concentration in a pyrophosphate solution without the enzyme. Because of the distinct similarity between the two graphic representations, she suggested that the actual substrate for the inorganic pyrophosphatase was magnesium pyrophosphate. She postulated that the complex was formed in the following way:

(21) $Mg^{2+} + (HP_2O_7)^{3-} \rightleftharpoons H^+ (Mg\ P_2O_7)^{2-}$

In more acidic solutions, therefore, a higher magnesium concentration was required for activation of the enzyme, or formation of a complex with the pyrophosphate (169). By varying the magnesium concentration along with that of pyrophosphate so that the ratio of magnesium to pyrophosphate remained constant, Frankenthal obtained a Km of 5.4×10^{-4} for the inorganic pyrophosphatase of the erythrocyte of the rat. Boyer (185), however, claimed that this result was invalid. Since pyrophosphate was known to inhibit the enzyme, the true "substrate" : velocity ratio could not be determined. The inhibiting effect of pyrophosphate could be overcome only by adding an excess of magnesium to the

reaction mixture. Since magnesium pyrophosphate itself, in high concentration, inhibited the enzyme, this method of determining the Km similarly could not be considered as being strictly correct. However, it had greater merit than the other methods. Sjoberg (195) showed that the inorganic pyrophosphatase activity of the erythrocyte of the horse was inhibited by calcium and that the inhibition became more pronounced as the ratio of calcium to magnesium increased. High concentrations of pyrophosphate inhibited the enzyme, the optimal concentration being 0.003 M. Inorganic pyrophosphatase had no effect on the substrate as long as the red blood cells remained intact.

From the foregoing discussion on the inorganic pyrophosphatase in the erythrocyte, it is clear that the characteristics of the enzyme in this cell are similar to that of the same enzyme in other tissues. In the writer's studies on the inorganic pyrophosphatase in the erythrocyte, he was concerned only with certain aspects of its behavior. It was necessary to know something about the distribution of the enzyme in the nucleated and the nonnucleated erythrocytes because of our previous observations on the apparent interrelationship between nuclear and cytoplasmic activities with respect to the synthesis of DPN. In addition, it was important to ascertain the mechanism by which calcium ions and sodium fluoride exert their inhibiting effect on the enzyme and to know more about the nature of the substrate for the inorganic pyrophosphatase.

MATERIALS AND METHODS

In the studies on the inorganic pyrophosphatase of the red blood cell, human blood was used in most of the experiments. Blood was collected in heparin, centrifuged, and the plasma and 'buffy layer' removed. The red cells were washed three times with 0.9% sodium chloride, then diluted to 10 times the sedimented volume with the saline. The erythrocytes were then hemolysed by freezing in a dry-ice-alcohol mixture, then thawing in a water bath kept at 37°C, the procedure being repeated twice. The hemolysate was dialyzed against running tap water for 2-4 hours, then stored at 5°C until required for the experiment. To ascertain the distribution of the enzyme within the erythrocyte, the hemolysate was centrifuged at 3000 rpm., and the stroma-free supernatant was removed. The stroma residue was washed 5 or 6 times with saline, and made up to the volume of the original hemolysate.

For the studies on the distribution of the pyrophosphatase in the nucleated erythrocyte, chicken blood was collected in heparin, centrifuged, hemolysed and fractionated into the SFH and particulate material as previously described.

Similar studies were carried out with the rabbit reticulocytes and erythrocytes. The reticulocytes were obtained from animals with an induced reticulocytosis (50%). Blood was collected in heparin, the reticulocytes were washed, hemolysed and fractionated into SFH and particulate material as previously described.

The erythrocytes were obtained from normal rabbits and fractionated. The pyrophosphatase activity was compared with that of the reticulocyte preparations.

Inorganic pyrophosphatase activity was determined by measuring the amount of inorganic phosphate liberated during the incubation of sodium pyrophosphate in the presence of the enzyme and magnesium chloride. The optimal concentration of magnesium chloride was found to be 0.02 M. The inorganic phosphate was measured by the method of Fiske and SubbaRow (142). The volume and molar concentration of the ingredients used for the assays are described under Table VIII. The same conditions applied for all of the other experiments, unless otherwise stated.

The inorganic pyrophosphatase activity was found to be linear with time for about 60 minutes after the first 15 minutes of incubation (Figure 4). An increase in the enzyme concentration resulted in a proportional increase in the velocity of the enzyme-catalyzed reaction (Figure 4).

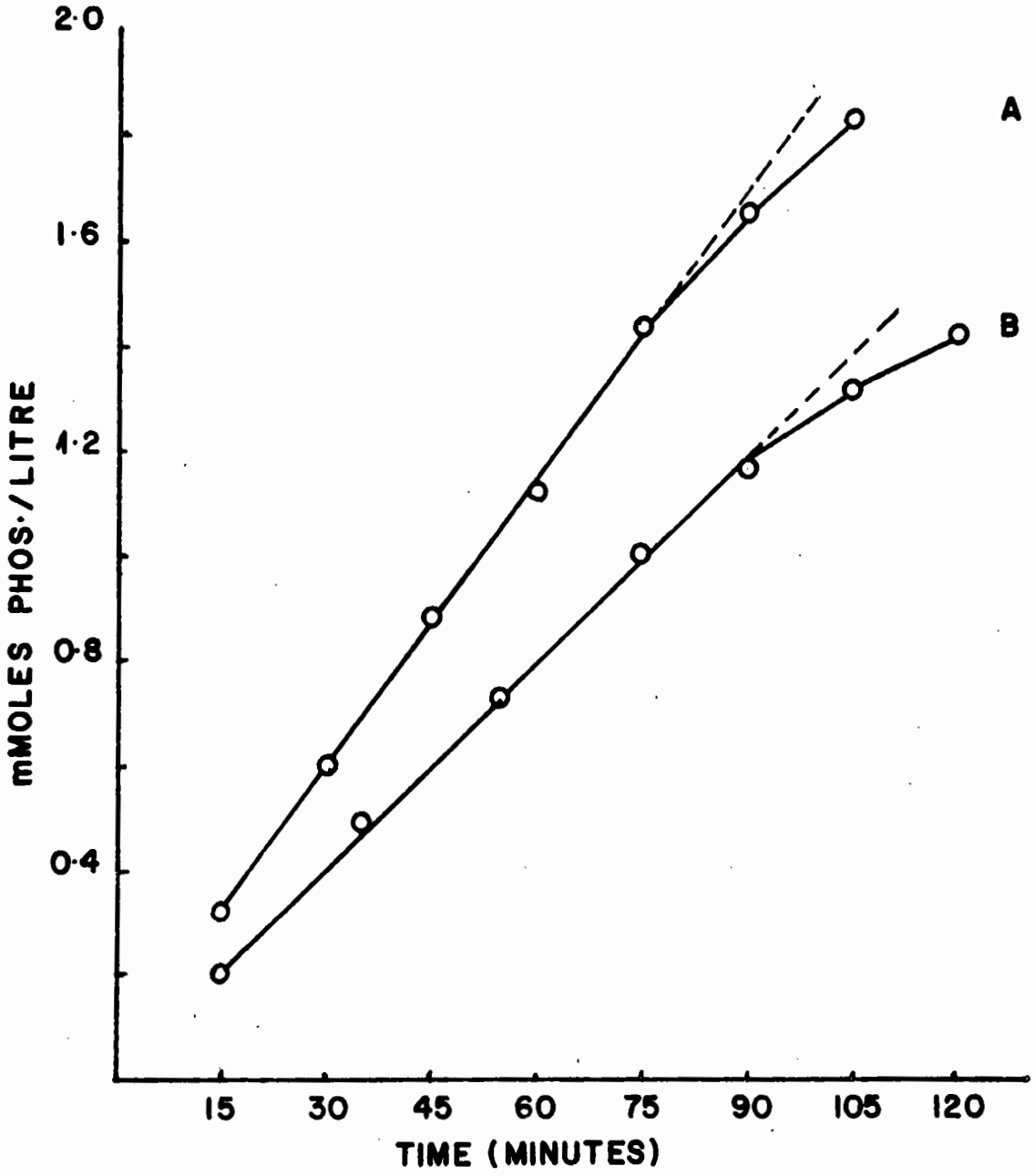
EXPERIMENTAL

As mentioned previously, Maizels *et al.* (193), in a study on the distribution of phosphatases in human erythrocytes, observed that the inorganic pyrophosphatase activity was confined to the SFH and that no activity was demonstrable in the stroma. Swanson (178) showed that in homogenates of

FIGURE 4

The Pyrophosphatase Activity of the Human Erythrocyte.

In curve A, the concentration of the enzyme is 3/2 times that of curve B.


2·0
1·6
1·2
0·8
0·4
mMOLES PHOS·/LITRE
A
B
15
30
45
60
75
90
105
120
TIME (MINUTES)

rat liver, the pyrophosphatase is concentrated in the soluble portion. In the human erythrocyte, the rabbit reticulocyte and the chicken erythrocyte, the pyrophosphatase activity is confined entirely to the soluble or cytoplasmic fraction of the cells (Table VII). In addition, the activity in the rabbit reticulocyte was found to be about 15 times as great as that of the rabbit erythrocyte.

The pyrophosphatase activity of specimens of human erythrocytes tends to fall off significantly within a few days (at 5°C) following collection, but the activity in the aliquots used for the assay can be restored, at first, by adding cysteine or glutathione to the reaction mixture (Table VIII). Similarly, pre-incubation of the reaction mixture resulted in a diminution of the enzyme activity, while the addition of cysteine or glutathione beforehand was found not only to protect the enzyme but restore the activity to normal or further increase it. These sulfhydryl compounds, therefore, protected the enzyme from denaturation (as in pre-incubation), and also enhanced the activity of the denatured enzyme (see day 3, Table VIII, no pre-incubation). By the fifth or sixth day following collection of the blood, the inorganic pyrophosphatase activity of the red blood cells fell to almost zero, and the addition of either cysteine or glutathione no longer was effective in restoring or increasing the activity. These findings strongly suggest that the pyrophosphatase contains free sulfhydryl groups which are required by the enzyme for the attainment of full activity.

TABLE VII

Inorganic Pyrophosphatase Activity of the Rabbit Reticulocyte and the Rabbit, Human and Chicken Erythrocyte.

Fraction	Phos. liberated (μM/cc.fraction/hr.)	Recovery (per cent)
Rabbit erythrocyte hemolysate	30	
Rabbit reticulocyte hemolysate	444	
Rabbit reticulocyte SFH	347	79
Rabbit reticulocyte particulate fraction	2.9	1
		Total 80
Human erythrocyte hemolysate	212	
Human erythrocyte SFH	214	101
Human erythrocyte particulate fraction	5.5	2.5
		Total 103.5
Chicken erythrocyte hemolysate	201	
Chicken erythrocyte SFH	158	78
Chicken erythrocyte particulate fraction	3.3	2
		Total 80

The conditions employed for the experiment are the same as those described in Table VIII.

TABLE VIII

The Effect of Cysteine and Glutathione on the Inorganic Pyrophosphatase Activity of the Human Erythrocyte.

Day of Collection	Pre-incubation	Cysteine	Glutathione	Phos. liberated
				(mMoles/litre)
Day 0	-	-	-	0.34
	-	+	-	0.39
	-	-	+	0.35
	+	-	-	0.14
	+	+	-	0.48
	+	-	+	0.45
Day 3	-	-	-	0.14
	-	+	-	0.26
	-	-	+	0.23
	+	-	-	0.07
	+	+	-	0.27
	+	-	+	0.13
Day 6	-	-	-	0
	-	+	-	0.07
	-	-	+	0.01
	+	-	-	0
	+	+	-	0.04
	+	-	+	0

0.01 M PP, 0.2 ml.; 0.4 M $MgCl_2$, 0.1 ml.; 0.2 M cysteine, 0.1 ml.; 0.2 M glutathione, 0.1 ml.; erythrocyte hemolysate 0.1 ml.; 0.1 M Tris buffer pH 7.5 to 2.0 ml. Pre-incubation for 10 minutes at 37°C where indicated. Incubation 15 minutes at 37°C. Precipitation with 8.0 ml. of 10% trichloroacetic acid. Inorganic phosphate determined on aliquots of the filtrate.

By reducing the magnesium concentration to suboptimal levels, a diminution or "apparent inhibition" of the enzyme activity was observed (Figure 5). This could be explained by assuming that pyrophosphate is the substrate for the enzyme, and that magnesium ions act principally as metallic activators. If the concentration of magnesium were reduced, less of the "active" enzyme would be available for the substrate, and an increase in the pyrophosphate concentration would produce no increase in the apparent inhibition provided the substrate itself was not an inhibitor of the enzyme. This, in fact, proved to be the case. At suboptimal magnesium concentrations, the degree of the apparent inhibition produced by the substrate was the same at all levels of pyrophosphate concentration (Table IX). But there is another possible explanation. If one assumes that the true substrate for the enzyme is magnesium pyrophosphate, and that pyrophosphate is an inhibitor of the enzyme (185), then the removal of magnesium would result in the accumulation of excess pyrophosphate with a resultant inhibition of the enzyme. Now, if the pyrophosphate concentration were increased, the degree of inhibition similarly would be increased. However, if pyrophosphate were a "non-competitive" inhibitor of the enzyme, the extent of inhibition would be the same over a range of substrate concentrations, as the writer has observed. It was impossible to say from these observations, therefore, whether magnesium pyrophosphate is the true substrate for the inorganic

FIGURE 5

The Effect of Magnesium on the Inorganic Pyrophosphatase Activity of the Human Erythrocyte.

A. Magnesium Chloride, 2×10^{-3} M.

B. Magnesium Chloride, 4×10^{-3} M.

C. Magnesium Chloride, 1×10^{-2} M.

D. Magnesium Chloride, 2×10^{-2} M.

All the concentrations are expressed as the final concentrations.

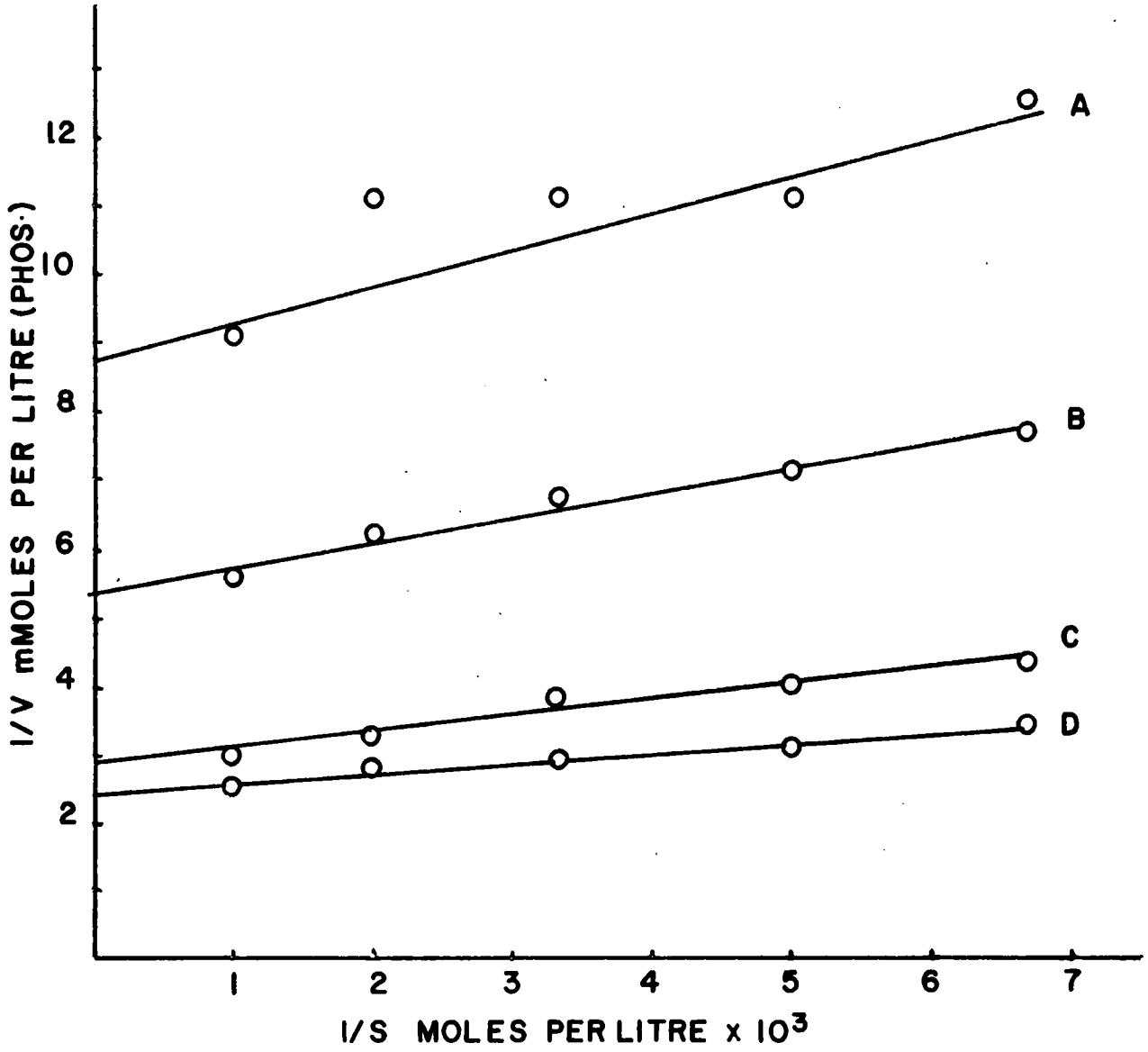

12
10
8
6
4
2
1/V mMOLES PER LITRE (PHOS·)
A
B
C
D
1
2
3
4
5
6
7
1/S MOLES PER LITRE x 10³

TABLE IX

The Effect of Magnesium on the Inorganic Pyrophosphatase Activity of the Human Erythrocyte.

Molarity of Pyrophosphate	"Inhibitory" influence of less than optimal concentrations of Magnesium Chloride.		
	1×10^{-2} M	4×10^{-3} M	2×10^{-3} M
	per cent "inhibition"		
1.5×10^{-4}	21	55	72
2×10^{-4}	22	56	72
3×10^{-4}	23	55	79
5×10^{-4}	16	55	75
1×10^{-3}	18	55	72

All the concentrations are expressed as the final concentrations.

pyrophosphatase. Boyer (185), however, has noted, on other grounds, that pyrophosphate can act as an inhibitor of the enzyme. Under these circumstances, the writer's results confirm Boyer's observations, and support the view that the true substrate for the inorganic pyrophosphatase is magnesium pyrophosphate.

Calcium ions inhibit the pyrophosphatase activity of the human erythrocyte, the inhibition being of the non-competitive type (Figure 6). The value of Ki was found to be 4×10^{-4} M, and for Km 1.2×10^{-4} M. This was a valid determination, since the incubation media contained an excess of magnesium, hence there could be no question of inhibition by the pyrophosphate. The ratio of "substrate" to velocity could therefore be applied, using the value of the concentration of pyrophosphate as the substrate concentration in the calculation. In determining the nature of the inhibition produced by calcium ions, first the pyrophosphate concentration was varied, while the magnesium concentration was kept constant at a final concentration of 0.02 M. As previously mentioned, the inhibition by calcium was of the non-competitive type. When the magnesium ion concentration was varied along with that of the inorganic pyrophosphate, while the ratio of the concentration of magnesium to pyrophosphate was kept constant at 20:1 (an excess of magnesium), the inhibition of the enzyme by calcium ions still was of a non-competitive character.

FIGURE 6

The Effect of Calcium on the Inorganic Pyrophosphatase Activity of the Human Erythrocyte.

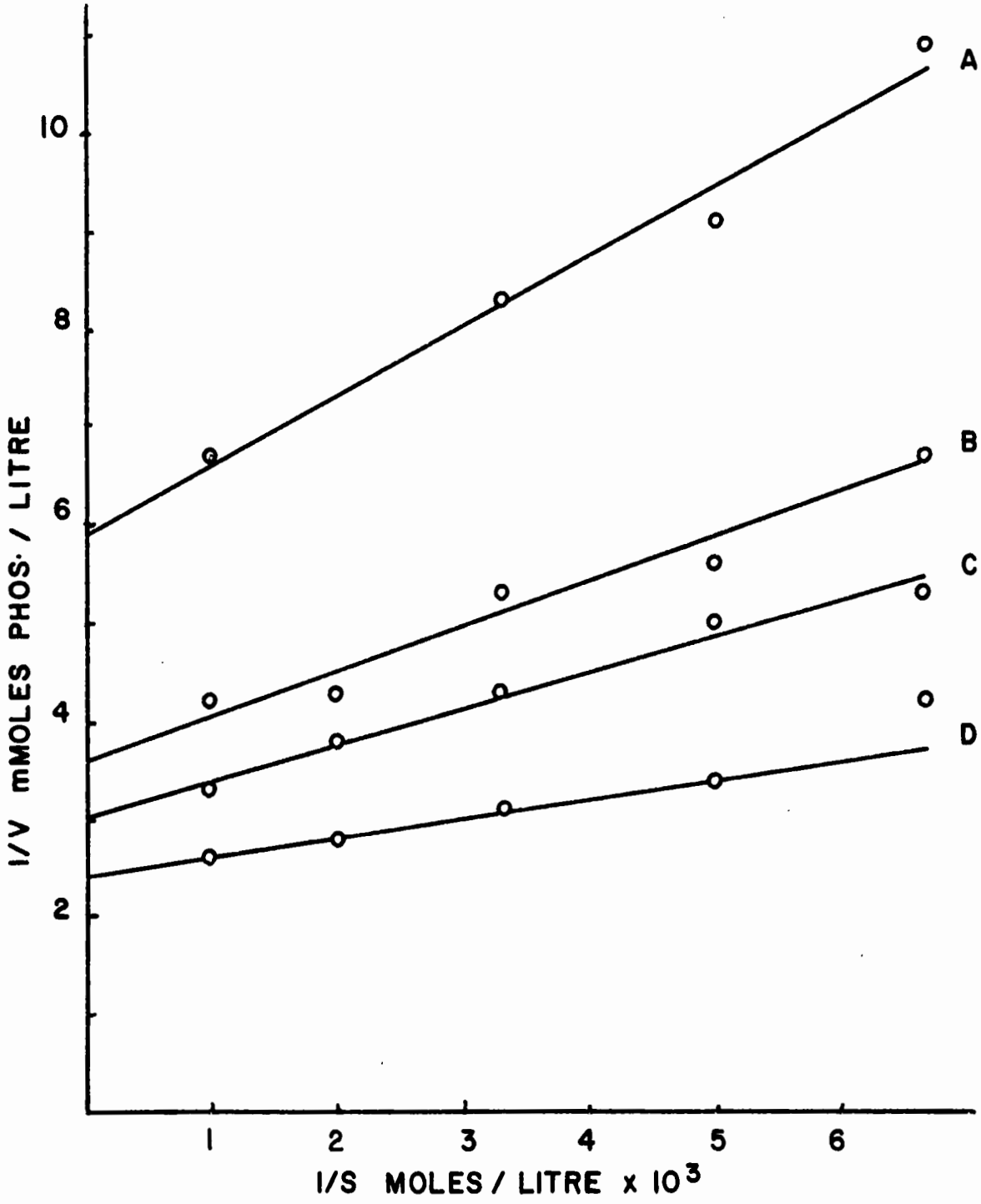
A. Calcium Chloride, 5×10^{-4} M.

B. Calcium Chloride, 2×10^{-4} M.

C. Calcium Chloride, 1×10^{-4} M.

D. No calcium chloride added.

All the concentrations are expressed as the final concentrations.


A
B
C
D
10
8
6
4
2
1/V mMOLES PHOS. / LITRE
1
2
3
4
5
6
1/S MOLES / LITRE x 10³

An effort was made to determine the significance of the calcium inhibition with respect to the nature of the true substrate for the inorganic pyrophosphatase. If magnesium does not form a complex with pyrophosphate, then one might expect calcium to compete with magnesium as a metal-activator of the enzyme. Sjoberg (195) did in fact show that a competition existed between magnesium and calcium with respect to the activation of the enzyme. Once the calcium replaced magnesium as the activator, the number of active enzyme sites would be diminished. The affinity of the enzyme for the substrate would remain unaltered (non-competitive inhibition) and the extent of the inhibition of the enzyme would be unchanged over a range of substrate concentrations in the presence of a given concentration of calcium (non-competitive inhibition, Table X). The experimental results can be explained equally well on the assumption that magnesium pyrophosphate is the substrate for the enzyme. In this case, calcium would be expected to compete with magnesium for the pyrophosphate. This would result in a "reduction" of the amount of magnesium available for combination with the pyrophosphate, or would give rise to a relative excess of calcium pyrophosphate. The latter would then compete with the magnesium pyrophosphate for the enzyme and the effect observed would be the same as if the concentration of magnesium were reduced or the enzyme were inhibited by pyrophosphate. Calcium, in this case, would be

TABLE X

The Effect of Calcium on the Inorganic Pyrophosphatase Activity of the Human Erythrocyte.

Molarity of Pyrophosphate	Inhibitory influence of Calcium Chloride		
	5×10^{-4} M	2×10^{-4} M	1×10^{-4} M
	per cent inhibition		
1.5×10^{-4}	60	37	21
2×10^{-4}	62	38	31
3×10^{-4}	64	42	30
5×10^{-4}	53	36	28
1×10^{-3}	62	38	23

All the concentrations are expressed as the final concentrations.

acting merely as an agent for the removal of available pyrophosphate, and although magnesium would be present in the free state, the pyrophosphate would be unavailable to it. Swanson (178) pointed out that calcium ions inhibit the inorganic pyrophosphatase of liver by forming a calcium pyrophosphate complex which competes with magnesium pyrophosphate for the enzyme. It is probably not a strict competition between the two complexes, but more likely an inhibition of the enzyme by calcium pyrophosphate acting through the pyrophosphate component, the calcium merely competing with magnesium in the formation of the pyrophosphate complex. These results still do not tell us whether or not magnesium pyrophosphate is the true substrate for the inorganic pyrophosphatase. But at least they afford evidence in support of this interpretation.

In the writer's earlier experiments on the effect of fluoride on the inorganic pyrophosphatase, the inorganic pyrophosphate concentration was varied while the magnesium concentration was maintained constant at 0.02 M; that is, at the concentration that was found to be optimal for the maximum amount of pyrophosphate used. The observed effect of fluoride was somewhat unusual, giving rise to a 'quadratic' type of inhibition. A word of explanation regarding the nature of this obscure type of inhibition might be in order here.

Ebersole et al. (196) suggested that many of the enzyme-catalyzed reactions in biological systems cannot be represented by the Michaelis-Menten formulation, since the enzyme-substrate complex undergoes dissociation in several steps. They postulated that one of the products, which ordinarily would be involved eventually in the reaction, instead, combines with the inhibitor. According to Ebersole et al., this rather unusual type of inhibition can be represented in the following way. By plotting the reciprocal of the substrate concentration against the reciprocal of the velocity, the straight lines obtained for various values of the inhibitor have different intercepts on the axis. They begin with a slope of nearly zero, then change to a common slope equal to that obtained when the concentration of the inhibitor is zero. This effect is clearly evident in the figure (figure 7) in which the lines diverge towards the ordinate. It is important also to note that in this type of inhibition, in contrast with the non-competitive type, the extent of inhibition is increased as the substrate concentration is increased (Table XI). In non-competitive inhibition, the degree of inhibition remains the same irrespective of the substrate concentration (Table X).

Quadratic inhibition may be related to another uncommon variety, namely, "uncompetitive" inhibition. The foregoing experiment was repeated, this time the magnesium concentration being varied along with that of pyrophosphate,

FIGURE 7

The Effect of Sodium Fluoride on the Inorganic Pyrophosphatase Activity of the Human Erythrocyte.

A. Sodium Fluoride, 5×10^{-5} M.

B. Sodium Fluoride, 2.5×10^{-5} M.

C. Sodium Fluoride, 1×10^{-5} M.

D. No sodium fluoride added.

All the concentrations are expressed as the final concentrations.

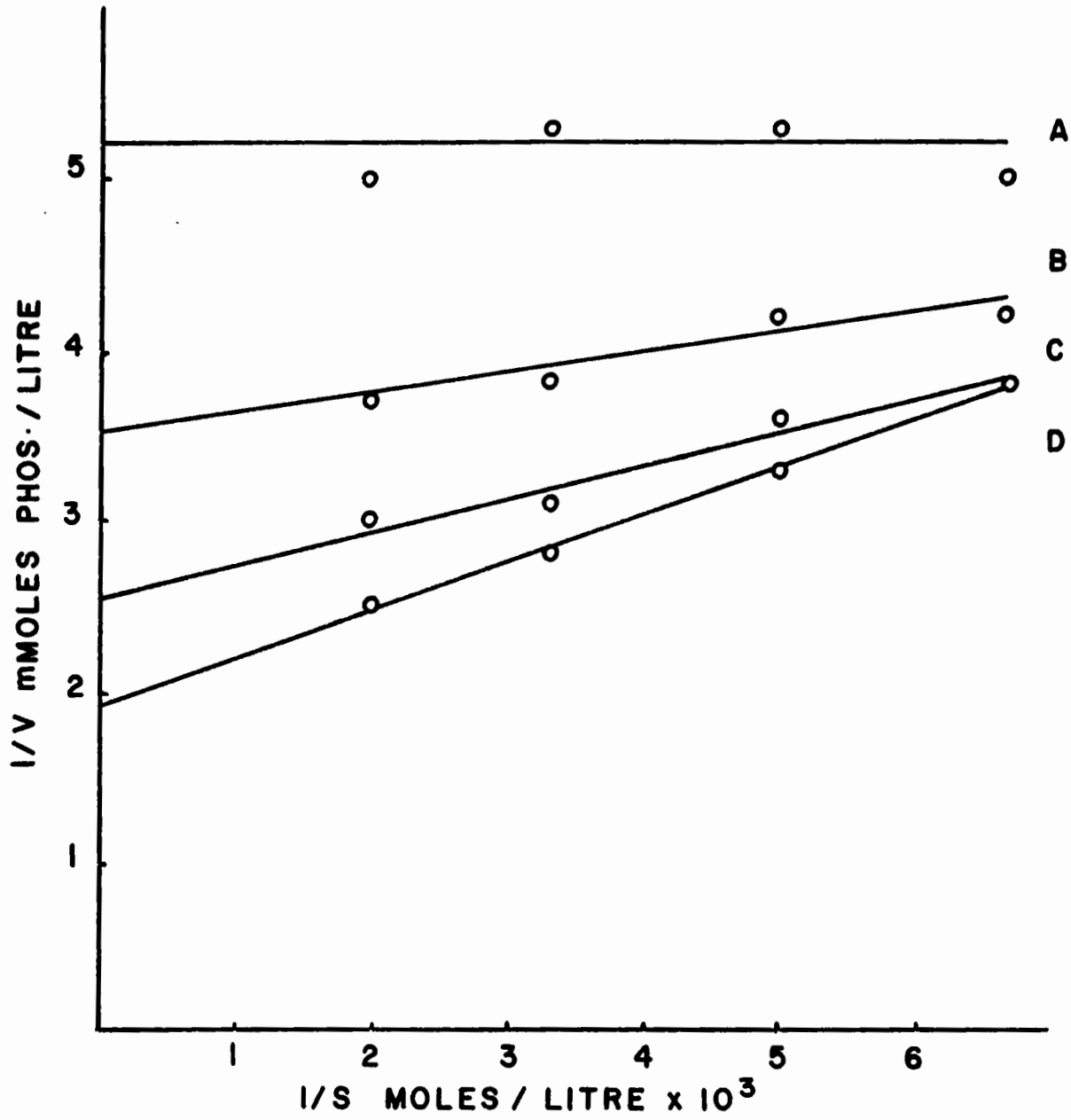

A
B
C
D
5
4
3
2
1
1/V mMOLES PHOS. / LITRE
1
2
3
4
5
6
1/S MOLES / LITRE x 10³

TABLE XI

The Effect of Fluoride on the Inorganic Pyrophosphatase Activity of the Human Erythrocyte.

Molarity of Pyrophosphate	Inhibitory influence of Sodium Fluoride		
	5×10^{-5} M	2.5×10^{-5} M	1×10^{-5} M
	per cent inhibition		
1.5×10^{-4}	23	7.7	0
2.0×10^{-4}	37	20	6.7
3.0×10^{-4}	47	28	11
5.0×10^{-4}	50	33	18
1.0×10^{-3}	51	35	14

All the concentrations are expressed as the final concentrations.

so that the magnesium : pyrophosphate ratio was maintained at 20:1. Sodium fluoride was used as the inhibitor. In this instance, the inhibition of the enzyme by sodium fluoride was found to be of the "uncompetitive" type (Figure 8). In other words, if the reciprocal of the substrate concentration be plotted against the reciprocal of the velocity of the reaction (197), the intercept on the ordinate increases with increasing concentration of sodium fluoride, but the slope remains unchanged. This type of inhibition arises from the circumstance that the inhibitor can combine with the enzyme-substrate complex, but not with the enzyme alone (196). Since the Ks (Michaelis constant) is equal to the slope divided by the intercept (when plotted according to the method of Lineweaver and Burk (197)), in uncompetitive inhibition the apparent Ks is diminished (the intercept increases but the slope remains unchanged). Thus, with an increase in the concentration of the inhibitor, the enzyme-substrate dissociation constant is diminished or the affinity of the enzyme for the substrate is increased. In the previous case, illustrating quadratic inhibition, the writer observed that a slope of nearly zero was obtained with the limiting concentration of the inhibitor (Figure 7). In such cases, then, the enzyme-substrate dissociation constant approaches zero, and the affinity of the enzyme for the substrate approaches infinity.

Further evidence suggesting that there may be a

FIGURE 8

The Effect of Sodium Fluoride on the Inorganic Pyrophosphatase Activity of the Human Erythrocyte.

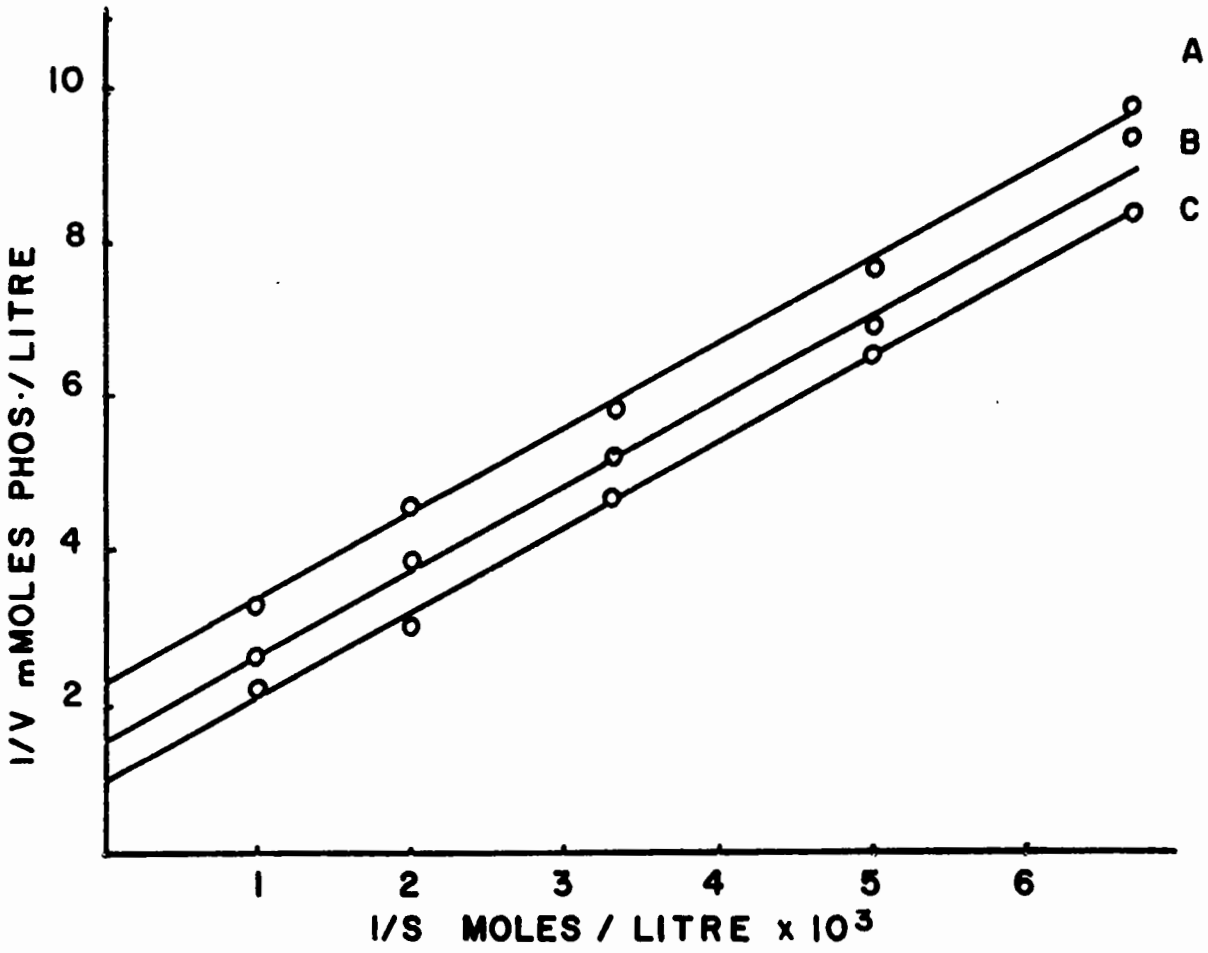
The concentration of magnesium was varied along with that of sodium pyrophosphate, so that the magnesium : pyrophosphate ratio was always 20:1.

A. Sodium Fluoride, 5×10^{-5} M.

B. Sodium Fluoride, 2.5×10^{-5} M.

C. No Fluoride added.

All the concentrations are expressed as the final concentrations.


A
B
C
10
8
6
4
2
1
2
3
4
5
6
1/V mMOLES PHOS./LITRE
1/S MOLES / LITRE x 10³

relationship between these two rather uncommon forms of inhibition is apparent from the figure 7, which indicates quadratic inhibition. It is evident that, with the decrease in the inhibitor concentration, there is a tendency for the line to become parallel to that observed in the absence of the inhibitor (uncompetitive inhibition). Thus it would seem possible that the quadratic and uncompetitive types of inhibition are but different manifestations of the same type of inhibition, produced by varying the concentration of either the activator (such as magnesium) or of the inhibitor.

Quadratic inhibition has been observed with phosphoglucomutase (198), enolase (199), and lecithinase (200). In all of these cases, sodium fluoride was the inhibitor, and magnesium was the metallic activator although with lecithinase, either calcium or magnesium ions could serve equally well as the activator. In the case of phosphoglucomutase, Najjar (198) considered that the "true inhibitor" is either a magnesium-fluoro-glucose-1-phosphate complex or a magnesium-fluoro-glucose-6-phosphate complex, that is, either a complex of the substrate or of the product. For enolase, Warburg (199) suggested that the inhibitor was a magnesium-fluoro-phosphate complex. The phosphate here is neither a substrate nor a product of the reaction, but serves as a complexing agent of the magnesium and fluoride. Arsenate could replace phosphate, but pyrophosphoric acid could not replace orthophosphoric acid. With lecithinase, Najjar (200)

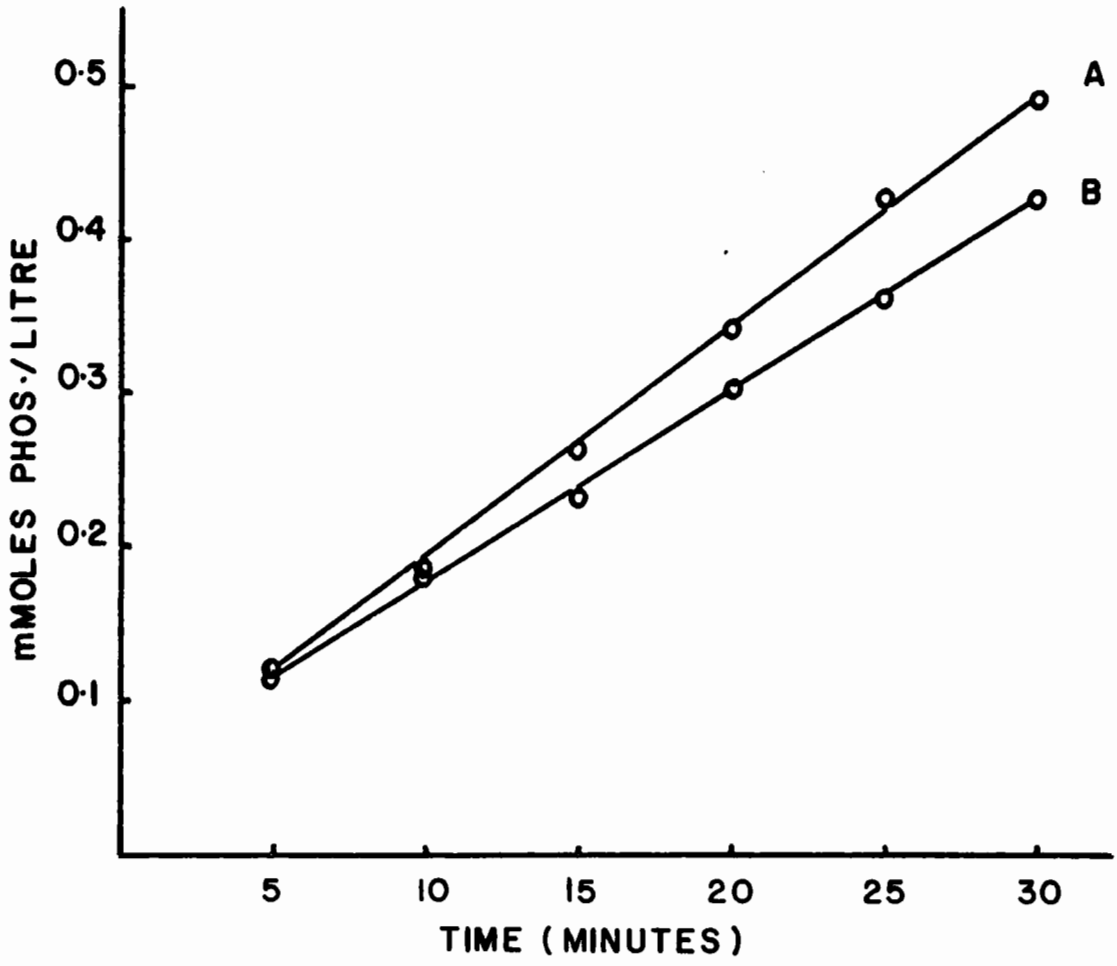
FIGURE 9

The Effect of Sodium Fluoride on the Inorganic Pyrophosphatase Activity of the Human Erythrocyte.

A. No Sodium Fluoride.

B. Sodium Fluoride, 1×10^{-5} M.

All the concentrations are expressed as the final concentrations.


0·5
0·4
0·3
0·2
0·1
mMOLES PHOS·/LITRE
A
B
5
10
15
20
25
30
TIME (MINUTES)

considered that the true inhibitor of this enzyme was a magnesium-fluoro-lecithin complex, that is, a complex of the substrate.

In the case of the inorganic pyrophosphatase, the inhibitor appears to be a magnesium-fluoro-phosphate complex, rather than a magnesium-fluoro-substrate complex. When the enzyme activity was followed with respect to time, both in the presence and in the absence of sodium fluoride, a progressive inhibition was observed with fluoride as the reaction proceeded (Figure 9). This could mean that with the progressive hydrolysis of the substrate, there was an increasing formation of the inhibitor complex whose accumulation tends to retard the reaction. In the case of inorganic pyrophosphatase, therefore, the true inhibitor, in the presence of fluoride, appears to be a magnesium-fluoro-phosphate complex, that is, a complex of the product of the reaction, rather than of the substrate.

DISCUSSION

One of the difficulties encountered in the study of the quadratic inhibition is that the concentration of the true inhibitor is not known, nor can it be determined. The concentration changes continuously, whether it is a complex of the substrate or of the product. Nevertheless, from a graphical analysis of the data the type of inhibition becomes

evident. The problem then is to determine the nature of the inhibitor complex and the manner in which this complex affects the enzyme-catalyzed reaction. With inorganic pyrophosphatase, the problem is complicated further, since the concentrations of the true substrate are not known, assuming that magnesium pyrophosphate is the true substrate for the enzyme.

In the quadratic type of inhibition, the true inhibitor apparently may be a complex of the substrate or of the product depending on the enzyme system in question. With inorganic pyrophosphatase, the true inhibitor appears to be a complex of the product of the reaction. In the lecithinase system (200), the inhibitor is evidently a substrate complex, while with phosphoglucomutase (198), the inhibitor may be either a substrate complex or a complex of the product. With a given enzyme system, and under a given set of conditions, quadratic inhibition appears to be produced by the formation of an inhibitor complex, one component of which is either the substrate, the product, or as in the case of enolase, simply inorganic phosphate.

With respect to the manner in which the inhibitor complex exerts its effect, Najjar (200), has pointed out that with the lecithin-lecithinase system, in the presence of fluoride, a magnesium-fluoro-lecithin complex is formed. The fluoride ions, apparently, have an affinity for the "active" sites on the lecithin molecule. In the form of the

inhibitor complex, lecithin cannot undergo cleavage and thus the enzyme-catalyzed reaction is inhibited. In the case of the pyrophosphatase, Boyer (185) has suggested that magnesium may act on the pyrophosphate by increasing the electron deficiency in the phosphorous atom. The P-O bond would then be more susceptible to cleavage by the oxygen of water. The enzyme might have an affinity for the magnesium, so that the magnesium combines with only one phosphorous atom, with consequent labilization of only one P-O bond. Now if fluoride were to combine with magnesium pyrophosphate, the effect of magnesium on the phosphorous might be altered, and the extent of the cleavage of the pyrophosphate be reduced. But the writer's results indicate that the inhibitor is a magnesium-fluoro-phosphate complex, that is, a complex of the product of the reaction. The question then arises: what is the mechanism of action of this type of inhibitor?

First, in the writer's experiments with the inorganic pyrophosphatase of the human erythrocyte, the quadratic inhibition could be made to assume the characteristics of the uncompetitive type, apparently, by simply altering the concentration of the metallic activator, namely magnesium. As already pointed out, there may be a relation between the quadratic and uncompetitive types of inhibition. Thus it may be that in the quadratic type, the inhibitor complex may exert its effect on the enzyme-substrate complex, as in uncompetitive inhibition, but not on the enzyme alone. The

observed differences between the two types of inhibition could be attributed to the difference in concentrations of a metallic activator or to an inhibitor such as sodium fluoride, since these substances form part of the inhibitor complex.

Thus, with the inorganic pyrophosphatase it would appear that magnesium-fluoro-phosphate forms a complex with a magnesium pyrophosphate-enzyme complex. As the concentration of pyrophosphate is increased, there is an increase in the amount of phosphate liberated, with a consequent increase in the amount of inhibitor formed. Thus, as the pyrophosphate is increased, the extent of the inhibition of the enzyme-catalyzed reaction tends to be increased.

It is important to note that in all the aforementioned examples of quadratic inhibition, the metallic activator forms part of the inhibitor complex. Whether simple removal of magnesium would produce inhibition to the extent observed in these enzyme systems is hardly likely since, with inorganic pyrophosphatase, the amount of magnesium present is so large compared with that of fluoride and phosphate that the small amount that might be removed by complex formation would not appreciably alter the rate of the reaction. The peculiarity of magnesium (and calcium in the case of lecithinase (200)) in the production of quadratic inhibition should be noted. In this connection, Oginsky (186) has shown that the acid pyrophosphatase of Streptococcus faecalis, which

is activated by cobaltous ions, is not inhibited by sodium fluoride.

The importance of inorganic pyrophosphatase for cellular function has been referred to in the introduction to this chapter. It may be recalled that in the avian and mammalian erythrocyte the enzyme is confined to the cytoplasmic fraction of the cell. DPN appears to be synthesized in the nucleus of the mouse liver cell (125) and in the nucleus of the chicken erythrocyte (Chapter I). Other biosynthetic mechanisms, such as have been outlined in the introduction to this chapter, may occur in other regions of the cell. If inorganic pyrophosphate is liberated as a by-product of these reactions, its removal must be facilitated so that the equilibrium of the synthesizing process will proceed in the direction of synthesis rather than degradation. The removal of inorganic pyrophosphate is ensured by the presence of inorganic pyrophosphatase in the cytoplasm of the cell. In our zeal to determine precisely, within the cell, the location of particular biologic mechanisms, one must not forget the interrelationship of the various cellular components. The synthesis of DPN, for example, takes place in the nucleus of the chicken erythrocyte, while the disposal of one of the by-products of the synthesis, namely inorganic pyrophosphate, takes place in the cytoplasm of the cell.

Thus the avian erythrocyte affords an example of a phenomenon which undoubtedly operates in all somatic cells

but which is practically sometimes very difficult to demonstrate; namely, the functional interrelationship between the nucleus and the cytoplasm.

CHAPTER III

The Diphosphopyridine Nucleotide Nucleosidase of the Erythrocyte

In the previous two chapters some of the mechanisms involved in the synthesis of DPN in the erythrocyte were discussed. Let us now consider the degradation of DPN with particular reference to its hydrolysis by the DPN nucleosidase (DPNase) of the red blood cell. In this connection, the writer's studies were confined to only one aspect of the problem, namely, the mode of attachment of DPN to the hydrolyzing enzyme. Alivisatos *et al.* (145) have shown that a large group of purine and pyrimidine compounds are effective inhibitors of this enzyme. In view of these findings, it seemed possible that a further insight into the nature of the DPN - DPNase complex might be afforded by a study of the effect of various components of the DPN molecule on the DPNase. First, let us review some of the previous investigations concerning the degradation of DPN.

INTRODUCTION

The Biological Degradation of DPN.

The degradation of DPN in the presence of yeast was observed as early as 1906. Harden and Young (3), in their

work on the coferment of yeast juice, discovered that when the juice was allowed to stand (autolyzed) for a few days, the filtrate - which contained the coferment - was no longer able to support the fermentation of sugar in the presence of the residue (apozymase) of the yeast juice. Upon the addition of boiled fresh juice, however, active fermentation occurred. The autolyzed juice itself, therefore, did not have a detrimental effect on the 'ferment'. von Euler et al. (5) observed, similarly, that the cozymase in yeast preparations was hydrolyzed rapidly on standing. Later, Lennerstrand (201,202), pointed out that cozymase, when added to apozymase suspensions in a phosphate buffer containing glucose and hexose diphosphate, activates fermentation at a constant rate for several hours, indicating that the coenzyme remains intact. However, when cozymase is added to a water suspension of apozymase alone, rapid inactivation of the cozymase occurs. The presence of phosphate and the substrate protect the coenzyme, apparently, from inactivation by the disorganized yeast cells. Furthermore, if phosphate and substrate were added to a mixture of apozymase and cozymase after complete inactivation of the latter, a gradual reactivation of the coenzyme occurred within a few hours. Lennerstrand (203) later explained that the inactive cozymase could be reactivated only if the degree of inactivation was not too great. Leloir and Dixon (204), studying the action of cyanide and pyrophosphate on the dehydrogenases, showed that a number of

dehydrogenase preparations from yeast - prepared from baker's yeast by grinding the plasmolyzed yeast in a ball mill and then fractionating the preparation with ammonium sulfate - contained a fraction which could rapidly destroy cozymase.

Other tissues, of course, were shown to contain DPN-degrading properties. von Euler et al. (205,206) showed that cozymase was inactivated by "nucleosidase" preparations from kidney, but not by kidney phosphatase, intestinal erepsin, liver catalase, urease, taka-diastase, trypsin, esterase or lipase. Preparations of macerated liver and kidney, pancreas, stomach and intestine were found to be active with respect to the degradation of cozymase, and the inactivation of this material was attended by the concomitant liberation of inorganic phosphate. Later, von Euler et al. (207) demonstrated the disappearance of the endogenous cozymase present in a brei of rat liver, after incubation of the preparation for a short time at room temperature. When cozymase was added to the liver preparation, (1 mgm. per 100 mgm. liver), one-half of the added cozymase disappeared within 4 hours. Rat liver, rat muscle, dog brain and Jensen sarcoma were found to contain one or more enzymes capable of inactivating DPN (208). von Euler et al. observed that a diminution in the concentration of the reduced form of the coenzyme occurred also. Their preparations of rat muscle inactivated codehydrase II (TPN) as well as codehydrase I (DPN).

Boyland and Boyland (209) showed that extracts from a number of mouse and rat tumors (Jensen rat sarcoma, R 10, Crocker 180, C63, S37) attacked the lactic dehydogenase coenzyme (DPN). With respect to the inactivation of the coenzymes in the erythrocyte, Lennerstrand (203) reported that both coenzyme I and coenzyme II were fairly stable in the whole blood of the dog. When the erythrocytes were washed with saline, however, or hemolysed by laking, there was a considerable diminution in the amount of these coenzymes.

Handler et al. (210) incubated DPN and NR with preparations of rat brain in which the cells had been disintegrated. After 30 minutes, the 'V-factor' activity of DPN (ability to stimulate the growth of H. parainfluenzae) was destroyed, while the NR remained intact. These authors concluded that the DPNase does not hydrolyze the NR. They found also that nicotinamide was a primary product of the action of DPNase on DPN. Leder and Handler (114), observed that the NMN, synthesized by the human erythrocyte, could be hydrolyzed about as rapidly as exogenous DPN by the DPNase from rat brain or from the human erythrocyte.

Myrback (7) reported that if, after sacrificing an animal, a piece of tissue such as a muscle were removed and were allowed to stand at room temperature, the cozymase content of the tissue decreased. However, he was unable to explain the mechanism of the inactivation of the coenzyme. He extracted the inactivating material from liver with cold

water, and found it to be nondialyzable and labile to heat. Likewise, von Euler et al. (211) noted a rapid diminution in the concentration of the cozymase and the reduced cozymase in rat muscle, rat liver, dog kidney, and Jensen sarcoma, after the death of the animal. Jandorf's (212) findings, on the other hand, are not in agreement with that of Myrback and von Euler. Jandorf determined the concentration of DPN in the liver, cerebral cortex, renal cortex and skeletal muscle of the rat at intervals after the death of the animal. The DPN content of all of the tissues except liver remained unchanged over the period investigated (40 minutes for brain, 90 minutes for kidney and muscle). In liver, the initial value of about 1050 µgm. of DPN per gm. of liver decreased sharply at first, but after 20 minutes became stable at a level of 750 ± 20 µgm. of DPN per gm. of liver. Jandorf pointed out that in the intact cell, DPN seems to be protected against the enzymes which hydrolyze it. Inactivation of this coenzyme was found to be favored by procedures such as homogenization, or freezing and thawing, which tend to rupture the cell membrane. Govier (213) studied the change in the DPN content of the heart and deltoid muscles of the dog, immediately following their removal from the animal, and afterward, when the organs were kept for one hour at room temperature. He observed that in the skeletal muscle there was a breakdown of only 10% of the DPN, while in the heart muscle, there was a 25% loss of the coenzyme. Govier

postulated that following the death of the animal, the breakdown products of DPN are not removed, and that the accumulation of the products led to the inhibition of the DPN-hydrolyzing enzymes.

McIlwain and coworkers (214-217) studied some of the properties of the DPNase and demonstrated its potent hydrolytic activity. A culture of Streptococci, for example, was able to inactivate its own dry weight of cozymase within 4 hours. Staphylococci, E. coli, Proteus morganii and H. parainfluenzae all showed DPNase activity. McIlwain noted that nicotinamide was liberated during the inactivation of cozymase by Staphylococci. The same thing was observed when H. parainfluenzae was used as the source of the enzyme. The rapidity of the inactivation of cozymase was not confined to bacteria, however, since with brain tissue a similar rapid inactivation of cozymase was effected. McIlwain pointed out that in the hydrolysis of cozymase, one equivalent of acid was produced for every molecule of nicotinamide liberated. This was apparently due to the transformation of the ionized pyridinium group (strong base) into the much more weakly basic pyridine nitrogen salt. When the reaction was carried out in bicarbonate-carbon dioxide buffers, the hydrolysis of cozymase was attended by the liberation of carbon dioxide, and the velocity of the reaction could be followed manometrically. The DPNase activity was found to be associated with the tissue debris. These workers demonstrated furthermore

that preparations from the central nervous system of mammals which showed DPNase activity, also inactivated coenzyme II (TPN) but at 70-85% of the rate of the inactivation of cozymase. The hydrolysis of TPN, like that of DPN, was found to be accompanied by the liberation of nicotinamide. Since the two coenzymes were found to compete for the degrading system, McIlwain suggested that a single enzyme was responsible for the hydrolysis of both the DPN and TPN. Nicotinamide ribofuranoside and several other nicotinamide derivatives proved to be unaffected by the enzyme. The reduced forms of DPN and TPN were found not to be hydrolyzable by the DPNase. In addition, they did not influence the effect of the DPNase on DPN and TPN.

Concerning the intracellular distribution of the DPNase, Alivisatos and Denstedt (218) showed that the enzyme activity was confined to the stroma of the rabbit erythrocyte. Later, Alivisatos *et al.* (145) reported that the enzyme activity appeared to be situated on the outer surface, or at least in the outer portion, of the membrane of the rabbit erythrocyte. Sung *et al.* (219), in their investigations on the intracellular distribution of DPNase in rat liver, found that the enzyme was about equally distributed between nuclei (their preparation of which contained cellular membrane), microsomes and the supernatant (cytoplasmic) fractions. No DPNase activity was detectable in the mitochondria of these cells. With respect to the distribution of the DPNase in the erythro-

cyte as reported by Alivisatos et al. (218,145), it is of interest to refer back to the work of Lennerstrand (203) who reported that coenzymes I and II, though fairly stable in whole blood, undergo a considerable diminution in amount when the blood is hemolysed by laking with water. Hence, on hemolysis of the cells, the DPN must be exposed to the enzyme. It is not clear, however, why the intracellular DPN content is reduced following washing of the cells with saline. The writer has attempted to point out, albeit indirectly, that in tissues other than blood, prior disruption of the cells is necessary before DPNase activity can be demonstrated.

The degradation of DPN can occur also in other ways. In 1936, Ohlmeyer (220) showed that following the hydrolysis of coenzyme I by yeast maceration juice, adenylic acid appeared among the products. Later, Das and von Euler (221), investigated the effect of the nucleotidase prepared from intestinal mucosa on cozymase. These authors noted that cozymase underwent dephosphorylation, but more slowly than adenylic acid or inosinic acid, when either of these latter compounds was used as substrate. Das and von Euler offered the suggestion that the hydrolysis of cozymase may occur at the pyrophosphate bond. The two mononucleotides which would then have been formed could be dephosphorylated with the aid of the intestinal nucleotidase. The alternative possibility appeared to be that the intestinal enzyme preparation may catalyze the cleavage of the molecule between

the ribose and phosphate moieties of the "nicotinamide half" of the coenzyme. Dephosphorylation of the adenosine diphosphate could then occur, since an adenylpyrophosphatase was known to be present in their enzyme preparation. Chain (222) found that the coenzyme could be inactivated by the venom from the black tiger snake (Notechis scutatus). The author observed further, that for every molecule of cozymase hydrolyzed, 1.7H^+ equivalents were liberated. From this evidence, he suggested that the first step in the cleavage of the cozymase is the cleavage of the pyrophosphate linkage with the formation of two mono-ortho-phosphoric esters. These, in turn, underwent partial hydrolysis with liberation of inorganic phosphate. The enzyme, therefore, was classed as a nucleotidase.

A few years later, Kornberg and coworkers (223,224,139) demonstrated the presence of an enzyme in preparations of rabbit kidney capable of catalyzing the hydrolysis of DPN, TPN and flavin adenine dinucleotide. With all these substrates, the hydrolysis was found to occur at the pyrophosphate bond. In the rabbit brain, the splitting of DPN occurred mainly between the nicotinamide moiety and the remainder of the molecule. Thus the DPNase is of the nature of a nucleosidase. With rabbit kidney preparations, on the other hand, the cleavage of DPN occurred predominantly by virtue of the pyrophosphatase action. Kornberg found it difficult to purify the nucleotide pyrophosphatase from

preparations of rabbit kidney. He used the potato instead, and succeeded in purifying the enzyme several fold. Recently, Kaplan et al. (225), discovered the existence of a DPN pyrophosphatase in extracts of the microorganism Proteus vulgaris X-19 (strain no. 6380). The enzyme was found to be active only after the extracts had been heated. These authors attributed the activation to the inactivation of an inhibitor of the DPN pyrophosphatase by the boiling treatment. The inhibitor of the enzyme proved to be a protein and was non-dialyzable.

The degradation of DPN can occur in any of the following ways. First, the coenzyme can be cleaved at the nicotinamide-ribose bond. This reaction is catalyzed by a DPN nucleosidase. Secondly, the dinucleotide can be hydrolyzed at the pyrophosphate bond, liberating as products adenosine-5'-phosphate and nicotinamide mononucleotide. The enzyme catalyzing this reaction is a DPN pyrophosphatase. Finally, DPN can undergo pyrophosphorolysis, the enzyme catalyzing this reaction being called a DPN pyrophosphorylase. The products of this cleavage are nicotinamide mononucleotide and ATP. This mechanism for the breakdown of DPN has been discussed in Chapter I of this thesis. The literature concerning the DPN pyrophosphatase has been reviewed in the foregoing few paragraphs. Consideration has already been given also to the DPN nucleosidase, its discovery, widespread distribution in nature and its intracellular localization.

Some of the properties of the enzyme have been mentioned. It is pertinent to consider these further, with particular reference to the activation and the inhibition of the enzyme.

In 1942, Elvehjem et al. (226) showed that calcium ions appeared to stimulate the DPNase activity of various tissues of the rat. Spaulding et al. (227) could not confirm these observations, but demonstrated that tocopheryl phosphate could inhibit the DPN nucleosidase activity of washed homogenates of brain, liver, heart, kidney, lung and muscle tissues of the rat. Govier et al. (228), who measured the DPNase activity of the heart tissue of normal guinea pigs, agreed that tocopheryl phosphate was an inhibitor of this enzyme. These authors found, however, that the addition of calcium ions relieved the inhibition produced by α-tocopherol. They suggested that calcium is an activator of the DPNase and that tocopherol is either a direct inhibitor of the enzyme, or alternatively, it elicits its effect by virtue of the removal of calcium.

Mann and Quastel (144) demonstrated DPNase activity in a suspension of triturated rat brain, and showed that nicotinamide inhibited the destruction of the coenzyme. The protective effect is attributable to a competition between nicotinamide and the cozymase for the DPNase. In this connection, Kornberg (224) pointed out that the inhibition of DPNase by nicotinamide is specific for this nucleosidase (DPNase) since the DPN pyrophosphatase, prepared from

rabbit kidney, was found not to be inhibited by nicotinamide. Similarly, Handler and Klein (229), working with a variety of tissues from the rat and the dog, found that the initial step in the inactivation of the pyridine nucleotides by these tissues is the cleavage of nicotinamide from the remainder of the nucleotide molecule. The inhibition of this inactivation by nicotinamide was found to be quite specific and probably of a competitive nature.

Kaplan and coworkers (230-234) contributed a great deal to our knowledge of some of the properties of the DPNase in various tissues. They purified DPNase from the yeast, Neurospora crassa, and showed that the enzyme is located in the soluble fraction of the yeast cells. While the DPNase catalyzed the hydrolysis of DPN and TPN, the enzyme failed to attack NMN, NR or the half or the fully reduced forms of DPN. Nicotinamide had very little protective action against the Neurospora DPNase, concentrations as high as 1.45×10^{-1} M nicotinamide being necessary to reduce the activity of the enzyme by 50%. By comparison, the DPNase purified from beef spleen was inhibited to the same extent by a concentration of nicotinamide of 1.5×10^{-3} M. In other words, the Neurospora enzyme was about 1/100th as sensitive to nicotinamide as the spleen enzyme. With respect to the Neurospora enzyme, Kaplan *et al.* isolated adenosinediphosphate ribose as a product of the cleavage of DPN. While nicotinamide appeared to be a competitive inhibitor of the

Neurospora DPNase, this material evidently was a noncompetitive inhibitor of the spleen DPNase.

Using the DPNase preparation from spleen, Kaplan et al. showed that only the oxidized forms of DPN and TPN were liable to attack, while NMN and NR were not hydrolyzed by the enzyme. They incubated C^{14} labelled nicotinamide with DPN in the presence of the spleen enzyme and isolated the DPN at the end of the experiment. The radioactive carbon was found in the nicotinamide moiety of the DPN. By contrast, when the Neurospora DPNase was used, no labelled DPN was found after the incubation of DPN with labelled nicotinamide and the enzyme. The authors proposed the following series of reactions for the cleavage of DPN:

(22) $\underset{(DPN^+)}{ARPPR\overset{*}{N}} + \underset{(DPNase)}{enzyme} \rightleftharpoons ARPPR^{+}\text{—enzyme} + \text{nicotinamide}$

$$\Big\downarrow H_2O$$

$$ARPPR + enzyme + H^+$$

In this way, Kaplan and associates explained the incorporation of labelled nicotinamide into DPN in the presence of the spleen enzyme. Since the free energy of hydrolysis of the nicotinamide-ribose bond was found to be about -8000 calories at pH 7 and 25°C, it was not surprising that when ARPPR was incubated with nicotinamide in the presence of the enzyme, DPN was not produced. Only when the substrate was present as a complex with the enzyme could the reverse reaction be observed.

Isonicotinic acid hydrazide was found to be ten times more potent as an inhibitor of the beef spleen DPNase than nicotinamide. A curious species specificity was observed, however, since not all the DPNases of animal tissues were found to be sensitive to the effect of the nicotinamide analogue. Using pig brain as a source of the DPNase, the authors isolated the isonicotinic acid hydrazide analogue of DPN following the incubation of DPN in the presence of isonicotinic acid and the enzyme. Similarly, DPN was found to be synthesized from nicotinamide and the INH analogue of DPN. This analogue of DPN exhibited no inhibiting action on any of the several DPN-linked dehydrogenases tested. Analogues of DPN were prepared using pyridine, nicotinyl monomethylamide, monoethylamide and ethyl nicotinate in the presence of DPN and the DPNase obtained from pig brain. In the formation of the DPN analogues and in the inhibition of the enzyme by nicotinamide, the authors concluded that the DPNase exhibited an "ARPPR-transglycosidase" effect.

Alivisatos et al. (235) demonstrated a similar type of phenomenon in the synthesis of 4-amino-5-imidazole carboxamide dinucleotide from the carboxamide and DPN. The reaction was found to be catalyzed by a DPNase prepared from beef spleen. In this case, however, the energy-rich quaternary ammonium group was lost, and protons were liberated. This DPN analogue was not attacked by the DPNase, while the INH analogue of DPN was susceptible to hydrolysis by the enzyme. In the

formation of the INH analogue of DPN, the energy-rich linkage involving the quaternary ammonium is not broken, and protons are not liberated in the reaction. The significance of the carboxamide dinucleotide in the synthesis of the purines is not clear.

In a study previously carried out in our laboratory, Alivisatos et al. (145) had shown that the hydrolysis of DPN by DPNase is inhibited by nicotinamide, adenine and a number of the purine derivatives, notably the dimethyl purines, theobromine and theophylline. The evident affinity of the enzyme for free nicotinamide and adenine suggested to these authors that DPN is capable of assuming a two-point attachment to the enzyme surface through the terminal components nicotinamide and adenine. Furthermore, this dual attachment appears to be essential for the splitting off of the nicotinamide by the enzyme. The writer undertook to investigate the nature of the DPN-DPNase complex by studying the effect of various components of the DPN molecule on the DPNase. The stroma from the rabbit erythrocyte was used as the source of the enzyme.

MATERIALS AND METHODS

DPN, adenine, adenosine, AMP, ADP and ATP were obtained from the Nutritional Biochemical Corporation, and alcohol dehydrogenase, from Sigma Biochemical Company. Lyophilized

snake venom from the species Crotalus adamanteus was purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, and wasused as the source of 5'-nucleotidase for the preparation of NR from NMN. NMN was prepared from DPN according to the method of Kornberg et al. (139) by means of a crude preparation of potato nucleotide pyrophosphatase. This has been described in the section on Materials and Methods in Chapter I. NR was prepared from a mixture of NMN and NR by the method of Rowen and Kornberg (118) using the preparation of snake venom referred to above. This, too, is fully described in Chapter I.

In order to determine the extent of the cleavage of NMN and NR by rabbit erythrocyte stroma, the cyanide method (141) was used throughout. The hydrolysis of DPN and the inhibition of this reaction were studied using the alcohol dehydrogenase method of Racker (140). The precise procedures used are described in Chapter I. An extinction coefficient of 6.3×10^6 cm.^{2} x mole.$^{-1}$ at 325 mμ for the cyanide method and one of 6.3×10^6 cm.^{2} x mole.$^{-1}$ at 340 mμ for DPN were used in the calculation of the results.

The precise conditions for each experiment are outlined under the appropriate tables.

Preparation of Rabbit Erythrocyte stroma.

Rabbit venous blood was collected in heparin. The sample was centrifuged and the plasma and the 'buffy layer' were

removed. The red cells were washed 3 or 4 times with 0.9% sodium chloride and brought to the original volume of the blood specimen with this medium. The cells were then hemolysed by alternately freezing in alcohol-dry-ice and thawing at 37°C, 3 times repeated. The stroma was separated from the stroma-free hemolysate by centrifugation at 3000 rpm. The stroma residue was washed 3 or 4 times with isotonic sodium chloride and the volume was adjusted to that of the original hemolysate with saline.

EXPERIMENTAL

NMN was tested first as a substrate for the DPNase of the rabbit erythrocyte stroma (Table XII). NMN was hydrolyzed at the nicotinamide-ribose bond but the rate of cleavage was very slow compared with that with DPN. The rate of hydrolysis of DPN under comparable conditions is about 200 times as rapid. Leder and Handler (114) observed that NMN, synthesized by the human erythrocyte, was hydrolyzed about as rapidly as exogenous DPN by either the rat brain DPNase or the human erythrocyte DPNase. Kaplan *et al.* (230,231), on the other hand, showed that neither the Neurospora DPNase nor the beef spleen DPNase can catalyze the cleavage of NMN.

Both nicotinamide and adenine inhibit the cleavage of NMN by the DPNase prepared from the stroma of the rabbit erythrocyte (Table XII).

TABLE XII

Inhibition of Rabbit Erythrocyte Stroma DPNase, using NMN as Substrate.

Δconcentration of NMN	Compd. used as inhibitor	Inhibition
(μMoles)		(per cent)
-0.045	-	-
-	nicotinamide	100%
-0.015	adenine	67%

NMN, 0.001 M; 0.03 M acetate buffer pH 4.75; adenine 6.7×10^{-3} M, nicotinamide 3.3×10^{-1} M. Total volume of reaction mixture 0.6 ml. Incubation: 3 hours. Add 0.4 ml. 1 M Ac. buffer pH 4.25. Heat in boiling H_2O bath for 1 minute. Values expressed as μMoles per ml. of reaction mixture.

TABLE XIII

Effect of Various Inhibitors on Rabbit Erythrocyte Stroma DPN-ase, using DPN as Substrate.

Compound used as inhibitor.	Molarity of inhibitor	Initial Conctrn. of DPN	Final Conctrn. of DPN	ΔConctrn. of DPN	Inhibition
		(µMoles)	(µMoles)	(µMoles)	(per cent)
NR	1.67×10^{-4}	0.516	0.306	-0.210	0
Adenine	5×10^{-3}	0.516	0.390	-0.116	40
Adenosine	3×10^{-2}	0.516	0.306	-0.210	0
AMP	1.87×10^{-2}	0.516	0.306	-0.210	0
ADP	1.87×10^{-2}	0.516	0.348	-0.168	20
ATP	1.87×10^{-2}	0.516	0.300	-0.216	0
PP	2.5×10^{-2}	0.516	0.300	-0.216	0
No inhibitor	-	0.516	0.306	-0.210	-

DPN, 6.7×10^{-4} M; 0.03 M acetate buffer pH 4.25. Total volume 0.6 ml. Incubate 10 minutes at 37°C. Add 0.4 ml. 5% trichloroacetic acid. Heat in boiling water bath 30 seconds. Sufficient enzyme was added to give approximately 50% hydrolysis of DPN in 10 minutes. Values expressed as µMoles per ml. of reaction mixture.

Handler and Klein (210) had noted that although DPN was rapidly hydrolyzed following incubation with a broken cell preparation of rat brain, NR under similar conditions, remained intact. We found that NR was not hydrolyzed by an active stroma preparation from rabbit erythrocytes.

Several compounds including ATP and the components of DPN, namely NR, adenine, adenosine, AMP, ADP and PP were tested for their effect on DPNase (Table XIII). As Alivisatos et al. (145) observed, adenine and nicotinamide were found to be effective inhibitors of the enzyme while no inhibition occurred with sodium pyrophosphate. However, whereas these authors demonstrated a slight inhibition with adenosine and AMP, the writer could show no inhibition with these compounds. The discrepancy may have arisen from a difference in the conditions under which the reaction was carried out, or alternatively, from a difference in the assay method. The concentration of adenosine used was about the same as that used by the aforementioned authors, although the concentration of AMP used by this writer was less. In spite of this difference, we would suggest that the alcohol dehydrogenase method used in this study is more specific than the cyanide method used by the previous authors. The inhibition of DPNase activity by ADP, but not by ATP, was a rather unexpected finding. The concentration of ADP used was the same as that of ATP and AMP. The lack of inhibition of the DPNase by NR might possibly be explained by the low

concentration of this compound, but adenosine in 100 times the concentration did not exert an inhibitory effect on the enzyme activity. In the light of these and other observations it would appear that a DPN-DPNase complex is formed according to the mechanism described below.

DISCUSSION

As stated previously, Alivisatos *et al.* (145) showed that the hydrolysis of DPN by the DPNase of the stroma of the rabbit erythrocyte is inhibited by nicotinamide, adenine and a number of purine derivatives, notably the dimethyl purines, theobromine and theophylline. The evident affinity of the enzyme for free nicotinamide and adenine, the presence of an inhibitory effect that is summated when these two compounds are both added in low concentrations to the reaction mixture, the absence of an additive effect when they are both added in higher concentrations, and the circumstance that nicotinamide and adenine occupy terminal positions in the DPN nolecule suggested to these authors that DPN is capable of assuming a two-point attachment to the enzyme surface through the terminal components nicotinamide and adenine.

The writer has made the following additional findings which complicate the picture:

1. DPNase is capable of splitting nicotinamide from nicotinamide mononucleotide which does not contain adenine,

although the rate of hydrolysis of NMN is very slow compared with that of DPN. The hydrolysis of NMN can be inhibited by nicotinamide and also by adenine.

2. Neither adenosine nor nicotinamide riboside inhibits DPNase.

3. DPNase is inhibited by ADP but not by ATP or AMP.

From the first of these observations it would appear that the presence of a terminal adenine component in the molecule is not essential to the action of DPNase in splitting the nicotinamide-ribose linkage. The finding that the hydrolysis of this bond in NMN can be inhibited with adenine as well as with nicotinamide suggests that these compounds may compete for the same site on the enzyme surface rather than for different sites as postulated by Alivisatos et al. (145).

The powerful inhibition of DPNase by adenine and other substituted purines, and the absence of inhibition by adenosine, AMP or ATP suggests that in order for inhibition to occur the purine and particularly the nitrogen in position 9 in the molecule must be free. The only exception to this apparently is the inhibition by ADP. It is improbable that the adenine component of the latter compound plays a part in the inhibition. It is noteworthy that the quaternary nitrogen of nicotinamide must be free since NR does not inhibit the enzyme.

An alternative explanation for the inhibition by ADP is that the pyrophosphate component may be the active group.

If so, this group must possess distinctive properties compared with those of free pyrophosphoric acid, the monophosphate group of AMP or the triphosphate in ATP, all of which compounds cause no inhibition. To test the validity of the hypothesis that organic pyrophosphate as in ADP may constitute a point of attachment to the enzyme, a compound such as nicotinamide ribose pyrophosphate would be useful since this substance should be hydrolyzable by DPNase at about the same rate as DPN. Furthermore, it should be a still more effective inhibitor of DPNase than free nicotinamide, when DPN is used as the substrate.

The flavoproteins, though not strictly comparable, may lend themselves to the same type of inhibitor study for elucidating the nature of the attachment between the flavin adenine dinucleotide and the protein component. Michaelis (236) in his study of Warburg's yellow enzyme, pointed out the probability of a multiple attachment between the flavin prosthetic group and the apoenzyme. He favored the idea that fixation of the flavin moiety occurs at the alloxan and the azine rings. But as Michaelis himself stated: "It is remarkable, however, that without the phosphate group the flavin dye is not bound at all to the apoenzyme". In this sense, an analogy might be drawn between the DPN-DPNase system and the attachment of the flavin prosthetic group to the apoenzyme in the flavoproteins.

The mechanism of the formation of the enzyme-substrate

complex is still poorly understood, and it is certain that the spatial concept of simple physical attachment between chemical groupings on the substrate and sites on the enzyme surface will not adequately explain the phenomena observed. In this connection, Racker (32) stated that the site or sites of attachment between the substrate and the enzyme "are not limited to chemical bonding, but may include electrostatic or van der Waals' forces or the fitting of a substrate group into a 'hole' in the protein by the cohesine forces of water". The use of inhibitors in the investigation of enzyme activity gives us some insight into the nature of the enzyme-substrate complex, with particular reference to the properties of the anchoring sites of the substrate. In this respect, the DPN-DPNase system affords an unusually favorable one for the study of the nature of the enzyme-substrate complex.

In the first two chapters of this thesis, the writer has reported his investigations on the mechanism of the synthesis of DPN in the erythrocyte. The interplay between the nuclear and cytoplasmic activities and some aspects concerning the maturation of the red blood cell have been developed concurrently. Some of the properties of the inorganic pyrophosphatase of the erythrocyte have been discussed. Finally, to complete the picture of the synthesis of DPN in the erythrocyte, some of the properties of one of the degrading enzymes, DPNase, have been investigated.

APPENDIX

The Synthesis of Cholesterol in Blood.

The membrane of the red blood cell is composed of protein, phospholipid, cholesterol, along with some polysaccharide. The participation of these substances in the transport of various inorganic ions and organic materials across the membrane is still poorly understood. It is not known, for example, whether they constitute merely structural components or whether they participate actively in the diffusion and transport mechanisms. The membrane is frequently regarded as a metabolically inert part of the cell and little is known of the turnover of its substituents. The view is gaining support, however, that the membrane exists in a dynamic state and like other parts of the cell requires maintenance. The writer undertook to investigate one aspect of the problem, namely, the biosynthesis of cholesterol in the erythrocyte. In this connection, Parpart (155) pointed out that all of the cholesterol in the erythrocyte is confined to the membrane.

Enormous strides have been made within the last few years toward the elucidation of the mechanisms involved in the biosynthesis of cholesterol. The writer will not attempt to review the literature on this subject completely, but will refer only to some of the more important contributions. For more complete information on this subject, the reader is

referred to excellent reviews by Lieberman (237), Cornforth (238), and Popjak (239), which have appeared recently.

The Biosynthesis of Cholesterol.

In 1937, Rittenberg and Schoenheimer (240) injected heavy water (D_2O) into mice, then by feeding this material raised the heavy water content of the animals to 1.5% of the total body water and maintained this concentration. A group of animals was sacrificed at intervals, and the total body fatty acids and cholesterol were isolated. The deuterium had apparently been incorporated into the cholesterol. In experiments done *in vitro*, these authors showed that the cholesterol does not contain any slowly exchangeable (semi-labile) hydrogen. They postulated, therefore, that the cholesterol was synthesized by means of the coupling of a large number of small molecules. Later, Bloch and Rittenberg (241) fed deuterium-labelled sodium acetate to adult mice and growing rats and after 8 days, killed the animals and determined the deuterium content in the cholesterol and fatty acids isolated from the carcasses. The concentration of deuterium in the cholesterol was found to be many times greater than that of the body water, whilst the deuterium concentration of the fatty acid fraction was about one-half that of the body fluids. These results precluded the possibility that the long chain fatty acids could serve as

intermediates in the biosynthesis of cholesterol. The authors concluded that acetic acid may serve as a precursor in the biological formation of cholesterol. They showed also (242) that the deuterium label was present in the nucleus and the side chain of the degraded cholesterol. Later, Bloch et al. (243) demonstrated the in vitro synthesis of cholesterol by slices of rat liver, incubated aerobically in phosphate buffer in the presence of heavy water (D_2O), deuterium acetate, or acetate containing deuterium in the methyl group and C^{13} in the carboxyl. The cholesterol isolated from the liver slices, incubated with heavy water were found to contain deuterium, and both deuterium and C^{13} after incubation with isotopic acetate. The synthesis of cholesterol did not occur under anaerobic conditions. Little and Bloch (244) investigated the biosynthesis of cholesterol with the aid of acetic acid-1-C^{14}, acetic acid-2-C^{14} and acetic acid labelled with C^{13} and C^{14}. The isolated cholesterol was degraded in part, and, from these studies, the authors showed that carbon atoms 18, 19, 26 and 27, and presumably 17 of cholesterol were derived from the methyl carbon of acetic acid. Likewise, carbon atoms 25 and probably 10 were derived from the carboxyl carbon of acetic acid. In addition, Little and Bloch postulated that the acetate was the sole precursor of the cholesterol and of the 27 carbon atoms of cholesterol, 15 were derived from the methyl carbon of acetate and 12 from the carboxyl. In the ring, the ratio of "methyl" to

"carboxyl" carbons was presumed to be 10:9, while in the isooctyl side chain, the ratio was 5:3. Bloch et al. (245) using a mutant strain of Neurospora which was unable to convert sucrose to acetate, showed that added acetate served as a source for over 90% of the carbon atoms of the ergosterol of this mould. Bloch et al. (246) isolated the cholesterol synthesized in rat liver from either 1-C^{14}-acetate or 2-C^{14}-acetate. The radioactive cholesterol was degraded to permit separate isotopic analysis of the carbon atoms of the isooctyl side chain. As Bloch had postulated previously (244), five of the carbon atoms of the side chain were derived from the methyl groups of acetic acid and three from the carboxyl. Carbon atoms 21, 22, 24, 26 and 27 were derived from the methyl carbon of acetate, and carbon atoms 20, 23 and 26, from the carboxyl. These authors then postulated that units containing five carbon atoms and related to isoprene might serve as intermediates in the biosynthesis of cholesterol from acetic acid. At about this time, Bucher (247) found that homogenates obtained from the liver of the adult rat were capable of incorporating C^{14}-acetate into cholesterol. Care had to be exercized in the homogenization of the tissue, and it was probably because of faulty preparation that the use of disintegrated tissue for the synthesis of cholesterol had been ineffective in the hands of previous investigators. Rabinowitz and Gurin (273) prepared particle-free extracts of rat liver, which

consisted of lysed mitochondria and supernatant (cytoplasmic) material. These water-soluble preparations effected the synthesis of cholesterol from radioactive acetate, in the presence of ATP and DPN.

Langdon and Bloch (248-250) demonstrated that the triterpenoid hydrocarbon, squalene, appeared to be one of the intermediates in the biosynthesis of cholesterol from acetate. These workers fed squalene and 1-C^{14}-acetate to rats, then isolated the nonsaponifiable fraction obtained from the livers and intestine. It was found to contain radioactive material. A portion of this material was diluted with natural squalene and two isomeric hexahydrochlorides were prepared. The specific activity was found to be the same as that obtained in the nonsaponifiable fraction. This indicated that the squalene had been synthesized from the radioactive acetate. Another portion of the radioactive squalene was fed to mice and the cholesterol was isolated from the liver and intestine. The cholesterol so isolated was found to be radioactive. By comparing the results with their previous findings, these authors concluded that squalene was a far more efficient precursor of cholesterol than acetate. In 1953, Woodward and Bloch (251) postulated a mechanism for the cyclization of squalene to form cholesterol. From the evidence on the derivation of the various carbon atoms of cholesterol from acetate, this scheme was entirely tenable. Cornforth (238) has recently suggested an alternative to this

scheme, which again conforms entirely to the facts concerning the synthesis of cholesterol from labelled acetate.

Gurin et al. (252) using a particle-free extract of rat liver, prepared radioactive squalene by adding to this extract squalene and C^{14} acetate or methyl labelled β-hydroxy β-methyl glutaric acid (HMG). The radioactive squalene then gave rise to radioactive cholesterol when incubated in this particle-free, water soluble system. Cornforth and Popjak (253) demonstrated the synthesis of radioactive squalene from methyl labelled acetate by a liver mince. The squalene was isolated and degraded by oxidative ozonolysis to acetone, succinic acid and laevulinic acid. Carbon by carbon degradation of these three fragments was completed. The results showed that the entire carbon skeleton of squalene was formed from acetate, in conformity with the condensation of six isoprenoid units derived from acetate. Bloch (254), using preparations of homogenates from rat liver, centrifuged to remove all but the microsomal and supernatant fractions, showed that the cyclization of squalene to lanosterol and demethylation of the latter sterol to cholesterol took place only when the microsomes were intact. A particle-free preparation, consisting of the supernatant fraction and the microsomes which had been split by sonic vibration, retained the ability to cyclize squalene, but was no longer efficient in catalyzing the demethylation reactions leading to cholesterol. In other words, the reaction --squalene

to lanosterol appeared to take place in the supernatant or cytoplasmic fraction, while the conversion of lanosterol to cholesterol was found in the microsomal component of the cell. Recently, Popjak (255) studied the biosynthesis of squalene and cholesterol from acetate-1-C^{14} catalyzed by rat liver slices and ovarian tissues. The specific activity of the isolated cholesterol was much greater than the isolated squalene. This excluded the possibility that squalene *per se* is the precursor of cholesterol, although it may provide, through some other intermediate, the isoprenoid unit for the cholesterol formation.

In 1949, Bonner and Arreguin (256) showed that β-methyl crotonic acid (β, β-dimethylacrylic, DMA), acetate, acetoacetate, acetone or glycerol were effective precursors of rubber in the guayule plant. This finding stimulated research on the role of short branded-chain fatty acids in the synthesis of cholesterol, since the synthesis of rubber and of squalene appeared to occur in a similar fashion; that is, by the condensation of isoprenoid units. Bloch *et al.* (257) fed doubly-labelled isovalerate to rats. They isolated the cholesterol from the livers of these animals, and found that the isopropyl moiety of the isovalerate was a much more efficient source of carbon atoms for cholesterol than acetate. The carboxyl portion of the isovaleric acid, on the other hand, was less efficiently utilized for cholesterol synthesis than acetate. Gurin *et al.* (258) found, on the

other hand, that methyl-labelled isovalerate is utilized *in vitro* by rat liver slices for the biosynthesis of cholesterol, but less readily than acetate. They noted, furthermore, that acetoacetate was incorporated into cholesterol *in vitro*, but less efficiently than acetate. On the other hand, the addition of nonradioactive acetoacetate depressed the utilization of labelled acetate for the synthesis of cholesterol. That DMA arose from acetate was clearly shown by Rudney (259). He added methyl-labelled acetate and nonradioactive β,β-dimethylacrylate to a homogenate of rat liver, and following incubation of the reaction mixture, he isolated the DMA and showed that it was radioactive. Upon degradation of this fatty acid, the carbon atom 2 was shown to contain 35% of the radioactivity and carbon atoms 4 and 4' contained 63% of the activity. These results were entirely in accordance with the postulated mechanism for the condensation of 3 acetate molecules to form the isoprenoid unit, dimethylacrylic acid. In a similar experiment, Rabinowitz (260), using particle-free extracts of rat liver, demonstrated the conversion of radioactive acetate to radioactive DMA. Cholesterol was isolated as well, and was found to contain $\frac{1}{4}$ to $\frac{1}{2}$ the radioactivity of the aforementioned fatty acid.

Rudney (261) studying the role of β-hydroxy, β-methyl glutaric acid (HMG) in the synthesis of cholesterol, incubated methyl-labelled acetate with a small amount of carrier HMG

in the presence of an homogenate of rat liver. The HMG was isolated, degraded and the distribution of isotope in the HMG was in accordance with the supposition that two acetates condense to form acetoacetate, which in turn condenses with another molecule of acetate to form HMG. The distribution of the isotope in HMG paralleled that found in DMA. About the same time, Rabinowitz and Gurin (262), showed that the incorporation of acetate into HMG (in a particle-free, aqueous extract of rat liver, containing lysed mitochondria) was far more efficient than into cholesterol. Furthermore, these authors showed that pyruvate and acetyl CoA were better precursors of cholesterol than acetate, in this system.

Coon and coworkers (263) investigated the interconversion of some of these short, branched-chain fatty acids. They used extracts of fresh pig heart, heated at 60°C, pH 7.4, which treatment destroyed the crotonase present in the tissue. With this system, they demonstrated the carbon dioxide fixation into β-hydroxy isovaleryl CoA. The radioactive HMG CoA so prepared underwent cleavage, in the presence of cysteine or glutathione, to yield acetoacetate and acetyl CoA. Rabinowitz et al. (264) showed that in their aqueous extract of mitochondria and supernatant material obtained from rat liver, HMG was incorporated into squalene and cholesterol. With the supernatant fraction alone, HMG was readily incorporated into squalene, while DMA and HIV were incorporated less readily. CoA was not required in this system, nor was it

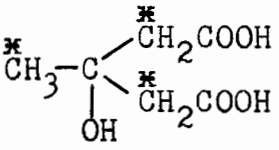
possible to demonstrate the incorporation of the CoA derivatives of the aforementioned acids into squalene.

These results were somewhat different from those obtained by Bloch *et al.* (265,266), who fed labelled HMG, HIV and DMA to rats, and tested the utilization of these fatty acids in the synthesis of cholesterol. The cholesterol isolated from the livers of the animals was radioactive in all cases, but only with DMA was the efficiency of conversion greater than with acetate. If HMG and HIV are supposed to be precursors in the biosynthesis of cholesterol, then they would be expected to be more efficient than acetate in the synthesis of the sterol. The cholesterol which was synthesized following the oral administration of DMA was degraded and the specific radioactivity in carbon atoms 10 and 25 was determined. The activity found indicated that DMA was utilized directly for the synthesis of cholesterol, perhaps by condensation with other isoprenoid units in the manner which Woodward and Bloch (251) had previously postulated for the cyclization of squalene. In any case, the evidence precluded the possibility that the DMA had undergone prior cleavage to form acetate or acetoacetate.

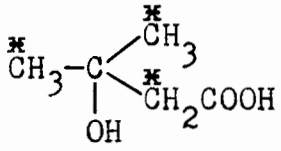
The evidence relating to the synthesis of cholesterol from acetate, and the participation of the branched-chain fatty acid intermediates can be summarized briefly in the following series of reactions. As mentioned above, there are some discrepancies in the literature concerning this

scheme, but this may merely be the manifestation of the difference between studies undertaken <u>in vivo</u> and systems <u>in vitro</u>.

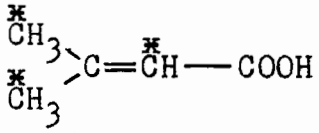
(23) $\overset{*}{CH_3}\ COOH + \overset{*}{CH_3}COOH \rightleftharpoons \overset{*}{CH_3}\ CO\ \overset{*}{CH_2}\ COOH$

(24) $\overset{*}{CH_3}\ CO\ \overset{*}{CH_2}\ COOH + \overset{*}{CH_3}COOH \rightleftharpoons$ 

(HMG)

(25) $HMG \rightleftharpoons$  $+ CO_2$

(HIV)

(26) $HIV \rightleftharpoons$ 

(DMA)

(27) $DMA \longrightarrow X \longrightarrow Cholesterol$

$X \rightleftharpoons squalene$

The methyl carbons in acetate in the foregoing reactions have been marked to demonstrate that the final labelling in the C_5 unit (β , β -dimethylacrylic acid) is in accordance with the theoretical and experimental considerations governing the biosynthesis of squalene and cholesterol.

<u>The Biosynthesis of Cholesterol in Blood</u>.

Popjak and associates (267), studying the metabolism of the circulating erythrocyte, injected radioactive glycine

and radioactive acetate into the rabbit. Cholesterol, protoporphyrin and dinitrophenyl glycine were isolated from hydrolysates of the erythrocytes. The cholesterol was found to be radioactive, the peak of activity occurring 24 hours after the injection of the sodium acetate. The membrane of the erythrocyte appeared to be metabolically active, but the authors were unable to state whether the cholesterol in the erythrocyte arose by a synthetic mechanism or by exchange with the plasma cholesterol. In a similar experiment, Altman et al. (268) injected carboxyl-labelled acetate into the peritonal cavity of rabbits over a period of two days. Blood samples were withdrawn at intervals, and the stroma material of the erythrocytes was isolated. A rapid turnover of the C^{14}-labelled constituents of the stroma had occurred. In splenectomized rabbits, the concentration of C^{14}-labelled constituents in the stroma was greatest. The authors suggested, therefore, that in the absence of the spleen, the catabolic phase of the metabolism of the stroma is altered. Later, Altman (269) incubated rabbit blood in vitro with C^{14}-labelled sodium acetate. The stroma of the erythrocytes was separated following incubation and the radioactivity was found to be distributed among the carbohydrate and lipid fractions, and mostly in the sphingolipids. Altman (270) observed also that the ability of the red blood cell to incorporate acetate into the stroma was diminished when the blood had been stored at 37°C or at 4°C. After

96 hours at 37°C, the incorporation of acetate into the stroma of the erythrocytes exhibited a degree of diminution comparable with that seen after 24 days of storage at 4°C. London and Schwartz (271) investigated the synthesis of cholesterol in the erythrocytes of man. In two human subjects, in whose body water an elevated concentration of deuterium was maintained by the administration of deuterium oxide, these authors observed that the rise in the concentration of deuterium in the cholesterol of the erythrocytes paralleled the rise in the concentration of deuterium of the cholesterol in the plasma. They demonstrated also that there was an insignificant degree of conversion of radioactive acetate to cholesterol in the human erythrocytes when studied *in vitro*. The human erythrocytes are apparently incapable of synthesizing cholesterol from acetate, but the cholesterol of these cells appears to exchange with the cholesterol of the plasma. Schwenk *et al*. (272) demonstrated that human, pig or rat blood, when incubated with labelled acetate, synthesized cholesterol in very small amounts. These authors do not state whether they used the whole blood of these animals, or only the erythrocytes. In this connection, nothing is known of the capacity of the leucocytes to synthesize cholesterol. On the other hand, the ability of the monocytes of the blood and tissues to ingest lipid has been observed repeatedly.

It would appear that the mature erythrocyte is incapable of synthesizing large amounts of cholesterol from sodium acetate. The stroma of the erythrocyte is not metabolically inert, however, since the plasma cholesterol appears to exchange freely with the cholesterol of the stroma of the red cell. The writer undertook to investigate this problem further, using chicken erythrocytes which are nucleated, rabbit reticulocytes and human erythrocytes. We hoped in this way to determine whether the precursors of the erythrocyte possess the capacity to synthesize cholesterol, or whether these cells, like the mature form, derive their structural components from a metabolic pool.

MATERIALS

Acetate-1-C^{14} was purchased from The Radiochemical Centre, Amersham, England, and was diluted with nonradioactive sodium acetate to give a specific activity of 474,890 counts per minute (c.p.m.) per mgm.

EXPERIMENTAL

Experiment I.

Chicken blood was collected in a beaker containing heparin. Sodium acetate-1-C^{14} (10.0 mgm., 4.75×10^6 c.p.m.) was added to 25 ml. of whole blood and the mixture was incubated for 2 hours at 37°C. After the incubation, the

reaction mixture was extracted four times with 200 ml. volumes of 95% ethanol : ether (3:1). Each time, the mixture was broughtto boiling for 5 minutes, then filtered. The combined filtrates were distilled to dryness, then taken up in 150 ml. of hexane. The hexane phase was washed with water (3 x 25 ml.), and the combined water extract was back-extracted with hexane (3 x 50 ml.). The combined hexane back-extraction was washed with water (3 x 25 ml.) and combined with the first hexane back-extract. The total hexane extract was dried over anhydrous sodium sulfate (6 hours), filtered and distilled to dryness to give the total lipid.

The total lipid was weighed, then dissolved in 95% ethanol. Aliquots of this material were plated on copper planchettes and the radioactivity was measured in a windowless flow gas counter. The total radioactivity of the sample is recorded in Table XIV, which contains all the data in this and the succeeding experiments. The total lipid fraction was distilled to dryness, than saponified with a solution of potassium hydroxide in 80% ethanol (10% KOH in aqueous ethanol). In all of the experiments, 10 ml. of the alcoholic potassium hydroxide per gram of total lipid was used. The mixture was treated in a reflux condenser for 6 hours, and after cooling, was diluted with 100 ml. of water and extracted with ether (6 x 50 ml.). The combined ether extracts were washed with water (4 x 25 ml.). The combined water washings were back-extracted with ether (3 x 50 ml.). The combined ether back-extract was washed with

water (4 x 20 ml.). The total ether extract was dried over anhydrous sodium sulfate, filtered and distilled to dryness to yield the nonsaponifiable fraction. This was weighed and the radioactivity determined.

The aqueous phase and water washings were combined, acidified to pH_1 with concentrated hydrochloric acid, and extracted with ether (6 x 25 ml.). The combined ether extract was washed with water (4 x 10 ml.). The water washings were back-extracted with ether (4 x 10 ml.) and the combined ether back-extract was washed with water (4 x 20 ml.). The total ether phase was dried over anhydrous sodium sulfate, filtered and distilled to dryness to yield the fatty acid fraction. This was weighed and the radioactivity determined.

The cholesterol in the nonsaponifiable fraction was precipitated with digitonin (1% digitonin in 80% ethanol, three times the weight of the nonsaponifiable material). The digitonide was centrifuged down and washed with acetone : ether (2:1) two times and then ether, two times. The digitonide was then dried in vacuo at 78°C over phosphorous pentoxide. The digitonide was weighed, then cleaved with pyridine (10-20 times the weight of the digitonide) by heating for one hour. The pyridine was distilled off in vacuo. The cholesterol was extracted with ether, then dried in vacuo at 78°C over phosphorous pentoxide. The cholesterol was recrystallized from 95% ethanol, and the melting point of the crystalline material determined (Kofler block). The cholesterol was taken up in ethanol and the radioactivity determined.

Experiment II

Chicken blood was collected in a beaker containing heparin. The blood (40 ml.) was centrifuged, the 'buffy layer' removed and the erythrocytes washed three times with Krebs-Ringer-phosphate buffer (without the calcium). The buffer, pH 7.4, containing 100 mgm. % of glucose was added to the erythrocytes (20 ml.) to a total volume of 40 ml. Sodium acetate-1-C^{14} (10.15 mgm.) was added, and the mixture was incubated in air for 2 hours at 37°C. After the incubation, 200 ml. of 95% ethanol : ether (3:1) was added, and the total lipid extracted as previously described. The isolation of the fatty acids and cholesterol were accomplished in the same manner as described in Experiment I. The data obtained are listed in Table XIV.

Experiment III

Chicken blood was collected in a beaker containing heparin. The whole blood was centrifuged for 5 minutes at 3000 rpm., and the plasma and 'buffy layer' were removed. The plasma (20 ml.) was added to 20 ml. of the erythrocytes, sodium acetate-1-C^{14} (21.4 mgm.) was added, and the mixture was incubated for 3 hours at 37°C. The incubation mixture was gassed continuously with 95% oxygen-5% carbon dioxide. Following the incubation, 95% ethanol : ether (3:1, 200 ml.) was added, and the total lipid was extracted as previously described. The fatty acids and nonsaponifiable material were isolated and the

cholesterol was isolated from the latter fraction. All fractions were weighed, and the radioactivity determined. The data are listed in Table XIV.

Experiment IV

Chicken blood was collected as before, centrifuged and the plasma and 'buffy layer' removed. Plasma (20 ml.) was added to 20 ml. of erythrocytes. Then nicotinamide (97.6 mgm., final concentration 0.01 M) was added and the mixture was frozen in dry-ice-alcohol and thawed at 37°C, the procedure being repeated twice. Sodium acetate-1-C^{14} (21.9 mgm.), ATP (498.4 mgm., final concentration 0.02 M), DPN (20.8 mgm., final concentration 0.001M) were added to the hemolysate and the mixture was incubated for 3 hours at 37°C. The total lipid, fatty acid, nonsaponifiable and cholesterol fractions were isolated as before, weighed and the radioactivity in each determined. The data are listed in Table XIV.

Experiment V

Human blood was collected in heparin, centrifuged and the plasma and 'buffy layer' removed. The erythrocytes were washed twice with plasma, then the red blood cells (17 ml.) were added to 17 ml. of plasma. A count done on this preparation showed 800 white blood cells per $mm.^3$. Sodium acetate-1-C^{14} (23.90 mgm.) was added to the preparation, and the mixture was incubated for 3 hours at 37°C. The total lipids, fatty acid,

nonsaponifiable fraction and cholesterol were isolated as before, weighed and the radioactivity determined. The data are listed in Table XIV.

Experiment VI

A reticulocytosis was induced in rabbits by the injection of acetylphenylhydrazine. The acetylphenylhydrazine (500 mgm.) was made up in 10 ml. of 95% ethanol, then 10 ml. of water was added. The rabbits were injected with 1 ml. (25 mgm.) of acetylphenylhydrazine daily for six days, then bled on the eighth day. The reticulocyte count was 94% of the total number of red blood cells.

The rabbit blood was centrifuged and the plasma and 'buffy layer' removed. Plasma (19 ml.) was added to an equal volume of the red cells. The final count of white blood cells was 6,000 per $mm.^3$, and the final count of normoblasts in the preparation was 12,000 per $mm.^3$. Sodium acetate-1-C^{14} (20.0 mgm.) was added to the preparation, and the mixture was incubated for 3 hours at 37°C under 95% oxygen-5% carbon dioxide. Following the incubation, the total lipids, fatty acids and nonsaponifiable fractions were isolated as before, weighed and the radioactivity measured. The nonsaponifiable fraction was chromatographed on alumina, and eluted. The eluent consisted of hexane, hexane-benzene in increasing polarity of solvent, benzene, benzene-ether, ether, methanol, chloroform and acetic acid. The cholesterol fraction was removed by benzene-ether 20% and benzene-ether 40%.

These fractions were combined, the solvent distilled and the cholesterol recrystallized from 95% ethanol. The cholesterol was dried, weighed, the melting point determined then taken up in ethanol and the radioactivity measured. The data are listed in Table XIV.

Experiment VII

Chicken blood was collected in heparin. The blood was centrifuged, the plasma and 'buffy layer' removed. The white cell layer was resuspended in Krebs-Ringer-phosphate buffer, pH 7.4 (no calcium added). The residue consisting of red blood cells was recentrifuged four times, and each time the white cell layer was removed and suspended in the buffer. The total white cell fraction in buffer was centrifuged and the buffer removed. The white cells (10 ml.) were suspended in 10 ml. of plasma and an aliquot taken for cell counts. The remainder was washed into an Erlenmeyer flask with 3 ml. of plasma. The preparation contained 200,000 leucocytes per $mm.^3$ and 1,700,000 red cells per $mm.^3$. On a smear (Wright's stain), a large cell resembling a large lymphocyte, with abundant cytoplasm and faintly staining nucleus predominated. This cell exhibited also many of the features of the monocyte, but the characteristic folding of the nucleus was only occasionally seen. Glucose (20 mgm.) and acetate-1-C^{14} (13.25 mgm.) were added to the preparation, and the mixture was incubated for 3 hours at 37°C, under 95% oxygen-5% carbon dioxide. Following the incubation, the total lipids,

fatty acid and nonsaponifiable material were isolated, weighed and the radioactivity determined. The nonsaponifiable fraction was chromatographed on alumina, and the cholesterol isolated and recrystallized as described in the previous experiment. The radioactivity was then determined. The data are listed in Table XIV.

DISCUSSION

It is apparent that the chicken erythrocyte, which resembles the mature nucleated precursor of the mammalian erythrocyte, is incapable of synthesizing cholesterol from acetate. The human erythrocyte is also inactive in this respect. The rabbit reticulocyte, on the other hand, exhibits the capacity to synthesize cholesterol from sodium acetate. However, the preparations used were contaminated with leucocytes and normoblasts. Assuming that the leucocytes of the rabbit possess the same functional capacity as those of the chicken, then the ability of the preparation to synthesize cholesterol cannot be attributed to the relatively small number of these cells present. We know nothing about the metabolism of the normoblasts, however, and it is entirely possible that these cells are capable of synthesizing cholesterol from acetate. In any case, it is evident that the mature nucleated or the mature nonnucleated erythrocyte is inactive in this respect, but that the immature mammalian forms manifest the capacity to synthesize

cholesterol. It is still not clear whether the entire cholesterol content of the erythrocyte is synthesized de novo in the immature forms or whether part, at least, arises from a cholesterol 'pool'. The erythrocyte membrane is not necessarily metabolically inert, since previous work has indicated (271) that the cholesterol in the membrane of the erythrocyte exchanges freely with the plasma cholesterol. The membrane of the erythrocyte, as in other cells, constitutes a "barrier" to the transport of various inorganic ions and organic materials. The role of the lipids in the membrane in facilitating the diffusion of these materials is still not evident.

The leucocytes of avian blood show a considerable degree of activity with respect to the synthesis of cholesterol. Whether this applies only to the avian cells or is generally true for all leucocytes is not known at present. The preparations of leucocytes from the chicken contained large numbers of cells which resemble, morphologically, the large lymphocyte found in the mammal. There were certain features of these cells, however, which were reminiscent of the monocyte. It is well known that the monocyte, in blood and in tissues, displays an enormous capacity to ingest lipid, but the fate of the ingested lipid is still undetermined. It is tempting to suggest that the monocyte may be active in lipid metabolism, both with respect to the catabolism of exogenous lipid and in the synthesis of new lipid material.

The writer wishes to point out that the foregoing experiments are preliminary only, and that work is in progress to clarify the findings and to test the suppositions.

TABLE XIV

The Synthesis of Cholesterol and Fatty Acids in Blood.

Incubation Mixture	Acetate-1-C^{14} added	Total Lipid				Fatty Acids		
		mgm.	c.p.m.	c.p.m per mg.	% incorp.	mgm.	c.p.m.	c.p.m per mg.
Chicken whole blood	10 mgm. 4.75×10^6 c.p.m.	180.10	20,800	115.5	0.44	56.70	5,758	101.6
Chicken Erythrocytes and phosphate buffer	10.15 mgm. 5.46×10^6 c.p.m.	116.05	1,690	14.6	0.031	11.40	171.2	15.0
Chicken Erythrocytes and plasma	21.4 mgm. 10.165×10^6 c.p.m.	233.5	6,964	29.8	0.069	36.80	602	16.4
Hemolysate of Chicken Erythrocytes	21.9 mgm. 10.4025×10^6 c.p.m.	187.05	1,047	5.6	0.010	37.63	121.75	3.2
Human Erythrocytes and plasma	23.9 mgm. 11.3525×10^6 c.p.m.	181.75	1,246	6.9	0.011	15.44	229.6	14.9
Rabbit reticulocytes and plasma	20.0 mgm. 9.50×10^6 c.p.m.	294.84	43,594	147.9	0.459	35.6	6,658	187
Chicken Leucocytes and plasma	13.25 mgm. 6.29375×10^6 c.p.m.	194.07	128,413	661.7	2.04	25.00	16,670	669

TABLE XIV (cont'd.)

Incubation Mixture	Nonsaponifiable			Cholesterol	
	mg.	c.p.m.	c.p.m. per mg.	mgm.	c.p.m. per mgm.
Chicken whole blood	84.85	9,410	111	5.55	
Chicken Erythrocytes and phosphate buffer	40.50	424	10.5	2.40	0
Chicken Erythrocytes and plasma	128.15	2,220	17.3	4.65	0
Hemolysate of Chicken Erythrocytes	71.78	42.1	0.587	13.8	0
Human Erythrocytes and plasma	114.47	381.2	3.33	18.0	0
Rabbit Reticulocytes and plasma	187.08	36,965	198	25.55	59
Chicken leucocytes and plasma	146.55	104,387	712	4.2	502

SUMMARY

The synthesis of DPN from the substrates ATP and NMN has been studied in the erythrocyte. In the chicken erythrocyte, the activity is confined to the readily-sedimentable (nuclear) fraction. No activity could be demonstrated in the reticulocyte of the rabbit or the human erythrocyte. The findings, with respect to the fate of the nucleus in the maturation of the erythrocyte, afford support for the hypothesis that in the maturation of the erythrocyte the nucleus is eliminated by "extrusion", or alternatively, that the mature nonnucleated erythrocyte represents a cytoplasmic bud arising from a primitive nucleated precursor. Evidence is presented that in the preparation of the substrate NMN from DPN, the α-isomer was isolated along with the normal or β-form.

The inorganic pyrophosphatase activity of the erythrocyte has been investigated. In the erythrocyte of the chicken, rabbit and human, and in the rabbit reticulocyte, the enzyme activity is confined to the soluble or cytoplasmic fraction of the cell. The addition of cysteine or glutathione activates the enzyme, after its activity has been diminished by storage or by heating. Added cysteine or glutathione protect, to some extent, the enzyme from inactivation by heat. Calcium ions exert an inhibitory effect on the enzyme activity, the inhibition being non-competitive

in type. Fluoride ions are inhibitory, producing an inhibition which is 'quadratic' in nature. When the magnesium ion concentration is varied, the quadratic inhibition produced by fluoride appears to be converted to an 'uncompetitive' type. Magnesium is required in relatively high concentrations (0.02 M) in order to demonstrate the optimal enzyme activity. The significance of these findings with respect to the nature of the 'true substrate' and the 'true inhibitor' for the enzyme are discussed.

The DPN nucleosidase (DPNase) of the rabbit erythrocyte has been studied. The components of the DPN molecule were tested for their effect on the enzyme activity, and the action of the enzyme on NMN was investigated. From the results, it appears that the DPN is attached to the enzyme at the nicotinamide end of the molecule and probably at the pyrophosphate bond as well.

The synthesis of cholesterol from radioactive acetate has been investigated in blood. The experiments were preliminary only and appear in the Appendix to this thesis. The results indicate, however, that the erythrocyte of the human and the chicken do not possess the capacity to synthesize cholesterol from acetate, but that this activity is demonstrable in the rabbit reticulocyte. The leucocytes of chicken blood, likewise, appear to be able to synthesize cholesterol from acetate.

CLAIMS TO ORIGINALITY

The following observations represent original contributions of the writer:

1. The synthesis of DPN from the substrates ATP and NMN has been demonstrated in the chicken erythrocyte, the activity being confined to the readily-sedimentable (nuclear) fraction. No activity could be demonstrated in the rabbit reticulocyte or the human erythrocyte. The findings afford support for the hypothesis that in the maturation of the erythrocyte the nucleus is eliminated by "extrusion", or alternatively, that the mature nonnucleated erythrocyte represents a cytoplasmic bud arising from a primitive nucleated precursor.

2. In the preparation of NMN from DPN, evidence is presented that the α-isomer was isolated along with the normal or β-form.

3. The inorganic pyrophosphatase activity of the chicken erythrocyte, the rabbit reticulocyte and the human erythrocyte is confined to the cytoplasmic fraction of these cells.

4. Calcium ions were found to inhibit the inorganic pyrophosphatase activity of the human erythrocyte, the inhibition being 'non-competitive' in type.

5. Fluoride ions were found to inhibit the inorganic pyrophosphatase activity of the human erythrocyte, the inhibition being 'quadratic' in nature. This type of inhibition can be converted to an 'uncompetitive' type by

varying the concentration of magnesium ions, which are required in high concentration in order to activate the enzyme.

6. Using the rabbit erythrocyte as the source of the enzyme, evidence is presented which indicates that in the formation of the DPN-DPNase complex, the DPN is attached to the enzyme at the nicotinamide end of the molecule and probably at the pyrophosphate component as well.

7. Neither the human erythrocyte nor the chicken erythrocyte is capable of catalyzing the synthesis of cholesterol from radioactive acetate. Preparations of rabbit reticulocytes or chicken leucocytes are active in this respect.

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