THE BIOLOGICAL INCORPORATION OF PURINES AND PYRIMIDINES INTO NUCLEOSIDES AND NUCLEIC ACID

by

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INTRODUCTION

The fundamental studies by MEYERHOF and his associates on the metabolism of phosphoric esters in muscle extracts marked the beginning of a very fruitful era in which the pathway of breakdown and synthesis of carbohydrates gradually became known. MEYERHOF showed that HARDEN AND YOUNG's discovery of phosphate uptake in cell-free yeast fermentation mixtures could be extended to animal tissues, especially muscle. Later MEYERHOF and his associates and NEEDHAM AND PILLAI in Cambridge showed that esterification of phosphate in muscle was coupled to the oxidation-reduction between phosphotriose and cozymase. This development led to the discovery of the acylphosphates (WARBURG and *coworkers*, LIPMANN). It was known, however, from LUNDSGAARD's studies that muscle, performing alactacid contractions in an oxygen-free atmosphere accumulates large amounts of hexosephosphoric esters. This is further accentuated if dinitrophenol which 'uncouples' oxidative-reductive phosphorylation is added together with iodoacetate. These observations which were made by CORI AND CORI in 1936 indicated that phosphate can also be incorporated into ester linkage by another process which has nothing to do with oxidation-reduction. The phenomenon of phosphate uptake independent of oxidation-reduction was very soon encountered in *in vitro* experiments too. Within the same year PARNAS AND OSTERN reported that the glycogen present in aged and dialysed muscle extracts can react with inorganic phosphate. A few months later CARL AND GERTY CORI isolated α-glucose-1-phosphate from muscle extracts and three years later CORI, CORI, AND SCHMIDT demonstrated the synthesis of a polysaccharide from α-glucose-1-phosphate by means of a muscle enzyme. KIESSLING, a student of MEYERHOF, performed independently in 1939 an analogous *in vitro* synthesis of polysaccharide using a yeast enzyme. During the subsequent years CORI and his associates turned their attention towards the kinetics of starch and glycogen synthesis *in vitro*. A number of important studies on starch, dextran and sucrose formation in enzyme systems from plants and microorganisms appeared during the next three or four years. The studies on the enzymatic synthesis of ribo- and desoxyribonucleosides can also be considered an outgrowth of CORI's fundamental observations on phosphorolysis of glucosidic linkages.

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ENZYMATIC SYNTHESIS OF PURINE RIBO-NUCLEOSIDES

The presence in animal tissues of an enzyme, called nucleosidase which splits of purines from purine nucleosides of the ribose series has been known for many years. KLEIN[1] who made a detailed study of this enzyme found that phosphate and arsenate enhance the enzymatic splitting of purine nucleosides. When I spent some time in 1943–1944 isolating nucleosidases from liver it was done only with the purpose of using these enzymes as analytical tools in an optical micromethod which I was trying to develop at that time. I had no knowledge about KLEIN's work at the time when I came across the observation that nucleosidase subjected to prolonged dialysis loses its activity. In view of observations by MEYERHOF and CORI it was not too far-fetched to try to add inorganic ortho-phosphate to the system and it turned out that this addition completely restored the catalytic activity of the system. Pursuing the analogy to CORI's work on the polysaccharide phosphorylase[2] I attempted to demonstrate the formation of ribose-l-phosphate as a suspected intermediate. These attempts failed quite a few times. Fortunately LOWRY who was my colleague at that time at THE PUBLIC HEALTH RESEARCH INSTITUTE had worked out a new method for phosphate determination which operates at p_H 4. This method, the well-known LOWRY-LOPEZ method[3], permits an estimation of highly labile phosphoric esters such as phosphocreatine and acylphosphates in the presence of inorganic phosphate. With the LOWRY-LOPEZ procedure it became possible to show a clearcut proportionality between liberation of purine and uptake of inorganic phosphate[4]. It was fairly obvious therefore that a new and highly acid-labile phosphoric ester was formed as a product of the enzymatic phosphorolysis of nucleosides. The ester was later obtained as the barium salt. It contained 1 mole pentose for each mole of labile phosphate and for each equivalent of aldose liberated upon mild acid hydrolysis. LOWRY has investigated the lability of ribose-l-phosphate in dilute hydrochloric acid at room temperature and found that 50% of the ester was split after 2.5 minutes incubation in N hydrochloric acid. In view of these properties and the resynthesis experiments described below the new ester was named ribose-l-phosphate.

The next step was an attempt to resynthesize purine nucleosides with ribose-l-phosphate. This was performed by incubating hypoxanthine, ribose-l-phosphate and a fractionated sample of liver nucleosidase about 20 minutes at 25° and subsequently analysing free and incorporated hypoxanthine[5]. It was then found that a large proportion of the hypoxanthine was incorporated in ribosidic linkage and an equimolar amount of labile phosphate was liberated. This enzymatic synthesis of inosine (ribose-l-hypoxanthine) proceeded very far; thus, if equimolar amounts of hypoxanthine and ribose-l-phosphate were incubated with the enzyme about 80% of the phosphoriboside was converted into purine-riboside. If the mixture contained twice as much phosphoriboside as hypoxanthine more than 95% of the latter was incorporated in ribosidic linkage. The equilibrium can be formulated as follows: ribose-l-phosphate + hypoxanthine $\rightleftharpoons$ ribose-l-hypoxanthine + phosphate. The enzyme catalysing this equilibrium was named nucleoside phosphorylase. Nucleoside phosphorylase possesses a certain specificity with regard to the nitrogenous bases added as well as to the pentoses present. Inosine and guanine riboside are the only ribosides which undergo phosphorolysis in the presence of the enzyme used. Adenosine and xanthosine are inert in this system as are pyrimidine ribosides. Likewise hypoxanthine and guanine are the only nitrogenous bases which are incorporated, *i.e.*, which in the presence of the enzyme undergo an exchange with the

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l-phospho group in ribose-l-phosphate. This selective trait with regard to purines will be discussed a little later. With regard to the sugar component the furanoid structure of the sugar seems to be imperative for the reaction. Thus, pyranose-ribose-l-phosphate (synthesized by chemical means by TODD AND LYTHGOE) was practically inactive in the enzyme test as was α-glucose-l-phosphate. Although the furanoid structure of the pentose seems to be essential, other changes in the sugar molecule seem to affect the enzymatic exchange much less. KLEIN had already observed that liver and spleen nucleosidase catalyse the splitting of purine desoxyribosides just as well as purine ribosides. We have found too that nucleoside phosphorylases fractionated by various means catalyse the phosphorolysis of purine desoxyribosides as well as the purine riboside[6,7]. If we assume that the enzymatic catalysis of the two types of nucleosides is due to the same enzyme and there is good evidence for such an assumption, the substitution of an OH group by a H at carbon no. 2 seems to be unessential for the activity of the liver nucleoside phosphorylase.

ENZYMATIC SYNTHESIS OF DESOXYRIBO-NUCLEOSIDES

It was tempting to analyse a little more closely the phosphorolysis of desoxyribosides, and if possible perform an enzymatic synthesis of nucleosides belonging to the desoxyribose series. FRIEDKIN who joined our group here in Copenhagen as a research visitor participated in this project and undertook a closer analysis of some of the components of the system. Guanine desoxyriboside was isolated and subjected to an enzymatic phosphorolysis analogous to that used for ribosides. After removal of the inorganic phosphate the LOWRY-LOPEZ phosphate analysis was performed in order to disclose the the presence of a highly acid-labile ester. The outcome was entirely negative. The failure to detect any ester formation by this method could be due to the fact that the l-ester formed in this case was more stable than ribose-l-phosphate. The other alternative was that the l-ester was even more acid-labile than ribose-l-phosphate. We were inclined towards the latter possibility. This turned out to be correct. If free phosphate and ester phosphate are estimated separately, using precipitation of the true inorganic phosphate by means of ammoniacal ammonium-magnesium sulphate it is possible to detect the formation of a desoxyribose phosphoric ester. This new ester was found to undergo rapid hydrolysis in an acetate buffer of p_H 4 at room temperature. FRIEDKIN found that 50% of the desoxyribose phosphate ester was split in 11 minutes at 25° at p_H 4. This is presumably the most acid-labile phosphoric ester yet described. It has been possible to show that this ester can act as a precursor for desoxynucleoside synthesis *in vitro*. The quantitative assay of the desoxyribose ester is under preparation and it can therefore only be stated that if hypoxanthine is incubated with liver nucleoside phosphorylase in the presence of a moderate excess of the desoxyribose ester (but no inorganic phosphate) more than 50% of the hypoxanthine is incorporated with the desoxysugar. The enzymatic formation of a desoxynucleoside was further substantiated by HOFF-JØRGENSEN using the microbiological technique[6,8]. A proper estimation of the amount of aldose present before and after mild hydrolysis of the new desoxyribose ester is under preparation. It is felt most likely that the new ester is an analogue of ribose-l-phosphate, *i.e.*, a desoxyribose-l-phosphate.

Recently MANSON AND LAMPEN[9] in CORI's department have prepared an enzyme from thymus gland which brings about a splitting of hypoxanthine desoxyriboside

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provided that either phosphate or arsenate is present. The ester formed was isolated and identified as desoxyribose-5-phosphate. The authors have evidence for the presence of an enzyme which catalyses the conversion of a primarily formed 1-ester into the 5-ester. The same two authors have also made recent contributions towards our understanding of the enzymatic splitting of pyrimidine desoxynucleosides, especially thymidine[10]. They have isolated an enzyme from bone marrow and kidney which catalyses a splitting of thymine from thymidine, again provided that either phosphate or arsenate is present. The enzyme preparations contain both purine nucleoside phosphorylase and pyrimidine nucleoside phosphorylase. MANSON AND LAMPEN's observations point also towards a formation of a desoxyribose-1-ester from pyrimidine desoxynucleoside. Thus, addition of hypoxanthine enhances the liberation of thymine from thymidine in the presence of mixed phosphorylases. This effect indicates at least that an enzymatic exchange between hypoxanthine and thymine takes place. However, since the incorporation into ribosidic linkage of hypoxanthine and that of thymine is catalysed by two different enzymes the assumption of a formation of 1-phospho-desoxyribose as a common substrate for both enzymes can explain the above mentioned effect.

THE BIOLOGICAL PATHWAY OF PURINE AND PYRIMIDINE INCORPORATION INTO NUCLEIC ACIDS

The pathway of purine and pyrimidine incorporation into nucleic acids is a problem of major biological importance. The isotope technique has made it possible to make an account of the most significant steps of such a synthesis in the intact organism. In 1941 SCHOENHEIMER and his colleagues initiated some studies on purine incorporation in the intact adult organism. I shall not go into a discussion of the interesting feeding experiments using N^{15} labelled ammonia and C^{13} or C^{14} labelled carbon dioxide which have shed so much light on the synthesis of the purine bases. This discussion is dealing with results of feeding experiments with labelled purines. These studies were initiated by PLENTL AND SCHOENHEIMER[11] and brought into a very successful and fruitful development by the studies performed at the SLOAN-KETTERING INSTITUTE by BROWN and coworkers. It will be recalled that PLENTL AND SCHOENHEIMER found that adult rats fed N^{15} labelled guanine excreted the entire amount of this substance as uric acid and allantoin and correspondingly the guanine of the nucleic acids was found to be devoid of any excess N^{15}. This finding was substantiated 6 to 7 years later by BROWN and coworkers. BROWN and his colleagues synthesized N^{15} adenine and guanine according to recent methods developed by TODD AND LYTHGOE. The most remarkable result of their studies, was the fact that N^{15} labelled adenine was readily incorporated into the ribonucleic acids both as adenine and guanine[12]. If a moderate amount of N^{15} adenine was administered to adult rats about 50% was incorporated as nucleic acid adenine and guanine and the other 50% appeared as allantoin. BENDICH AND BROWN[13] have recently made the interesting observation that 2–6 diamino purine labelled with N^{15} appears in large amounts in the nucleic acid guanine but not in the adenine. Hypoxanthine seems to be converted exclusively into uric acid and allantoin[14].

How are the present results of the studies on liver nucleoside phosphorylase to be interpreted in the light of recent findings gained from isotope experiments performed on intact organisms? It will be recalled that the liver nucleoside phosphorylase catalyses the incorporation of only two purine bases, hypoxanthine and guanine — exactly the

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two purines bases which according to the studies on the intact organism are *not* incorporated into the nucleic acids. We are forced to conclude therefore that the type of incorporation of purines which can be demonstrated in incubates with liver enzymes does not represent the final way by which the intact organism incorporates purines for the maintenance of its protoplasmic nucleic acids. It is even justified to question whether the nucleoside phosphorylase has anything whatever to do with the incorporation of purines into nucleic acids. The nucleoside phosphorylase might for instance play a role in processes other than the incorporation of purines into nucleic acids. This brings us to recall the situation with respect to the amino acid oxidases around 1936. At that time KREBS described a water soluble oxidase which catalysed the oxidation of the d-amino acids and which WARBURG AND CHRISTIAN purified and identified as a flavine enzyme. Six to seven years later GREEN, RATNER, AND NOCITO isolated the oxidase which catalysed the oxidation of l-amino acids and this also proved to be flavoprotein. When we talk about protein metabolism especially combustion of proteins in the animal organism we realize that the oxidation of the amino acids from proteins must be catalysed by the l-amino acid oxidase and not by the d-amino acid oxidase. The physiological function of the latter enzyme still remains obscure. We may apply the same point of view towards the nucleoside phosphorylase. It appears unlikely that the enzyme should simply serve in the breakdown of purine compounds since, as mentioned earlier, in an enzymatic mixture of free purine, phosphate, nucleoside and phospho-riboside the equilibrium is definitely favourable towards nucleoside formation. The possibility should not be overlooked that formation of inosine from ribose-l-phosphate catalysed by liver nucleoside phosphorylase might represent a primary step in the synthesis of purine ribosides prior to the incorporation of adenine. Adenine might then be exchanged directly with the hypoxanthine present in inosine by an enzyme which does not occur in our usual enzyme preparations. The catalytical action of inosine on the deamination of adenine by a bacterial enzyme[15] might be explained on this assumption; *in vitro* studies with labelled carbon or nitrogen in the adenine ring should be able to clarify this problem. As regard to the incorporation of pyrimidine into nucleic acid little is known. The recent team work between BERGSTRØM AND HAMMARSTEN and his group[16] has shed interesting light on this problem. It was found that N^{15} labelled orotic acid can be used as a precursor of the ribonucleic acid pyrimidines of the adult rat. The question regarding incorporation of purines and pyrimidines into desoxyribonucleic acids brings up important new problems regarding the rejuvenation of nuclear components. It is known from the studies by BRUES, TRACY, AND COHN and as well as by HAMMARSTEN AND HEVESY that the phosphorus in the desoxyribonucleic acids is renewed at a much slower rate than that incorporated in ribonucleic acids. In regenerating or growing tissues the renewal of desoxynucleic acid phosphorus is increased markedly. Likewise BROWN and coworkers[17] found that the rate of incorporation of N^{15} adenine into desoxyribonucleic acid in the adult rat is negligible as compared with the corresponding processes taking place in the ribonucleic acid. These observations indicating a very slow turnover of desoxyribonucleic acid components in the adult organism coupled with the knowledge of the existence of a highly active desoxynucleoside phosphorylase poses several new questions. For example the enzymatic system catalysing degradation and synthesis of desoxynucleosides in liver should be taken into account in considering the regulatory mechanisms which control transitions between resting and growing states.

As concluding remarks I should like to add that the two types of approaches, the

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study of enzymatic step reactions *in vitro* and the study with isotope labelled precursors *in vivo* are equally indispensable and exert a mutual and valuable influence on each other. An example is the importance of the EMBDEN-MEYERHOF glycolysis scheme for the interpretation of the distribution of labelled carbon in glycogen from rats fed with labelled carbon dioxide. The ingenious analysis by WOOD and coworkers in this field may well serve as an encouragement for investigators working in allied fields.

SUMMARY

The mechanism of incorporation of purines and pyrimidines into ribosidic linkage has been discussed from various points of view. Results gained from enzymatic studies are not in direct agreement with observations made in intact organism using isotopes. Various ways of interpretations are discussed.

RÉSUMÉ

Le mécanisme de l'incorporation de purines et de pyrimidines dans la liaison ribosidique a été discuté de différents points de vue. Les résultats obtenus par des études enzymatiques ne concordent pas entièrement avec les observations faites dans l'organisme intact au moyen d'isotopes. Différentes possibilités d'interprétation ont été envisagées.

ZUSAMMENFASSUNG

Der Mechanismus der Einverleibung von Purinen und Pyrimidinen in die Ribosid-Bindung ist von verschiedenen Gesichtspunkten aus erörtert worden. Die aus enzymatischen Untersuchungen gewonnenen Ergebnisse stimmen nicht völlig überein mit Beobachtungen welche im unversehrten Organismus mittels Isotopen gemacht wurden. Verschiedene Erklärungsmöglichkeiten werden besprochen.

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