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Synthesis of nucleoside 5′-triphosphates labelled with radioactive phosphorus isotopes

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Abstract. The review presents an analysis of the chemical chemicoenzymic, and enzymic methods of synthesis of nucleoside 5′-triphosphates labelled with phosphorus isotopes (^{32}P and ^{33}P) in the α- and γ-positions. The major part of the review is devoted to enzymic methods of synthesis, because they have undoubted advantages. The enzymic reactions are described in detail, the specificity and availability of the enzymes are considered, and data on the radiation stability of certain enzymes are presented. The bibliography includes 29 references.

I. Introduction

Radioactive isotopes have long been used in the study of the structures and functions of nucleic acids. Mainly the ^{14}C carbon atom was used initially. As the technique for the measurement of radioactivity developed and liquid scintillators began to be employed, there was a sharp growth of interest by investigators in tritium-labelled compounds. This was due to the much higher molar radioactivity of such compounds, which made it possible to reduce by two orders of magnitude the amount of specimen required for an adequate test of its radioactive emission.

The phosphorus-32 isotope has also long been used. Compounds labelled with ^{32}P were initially obtained by the ^{31}P$(n,\gamma)^{32}$P reaction involving the irradiation of an inorganic compound, usually an orthophosphate salt, with neutrons, whereupon the molar radioactivity of the product was not higher than that of the tritium-labelled compounds.

Instead of the whole nucleic acid molecules, only their small fragments, obtained by genetic-engineering methods, have become the objects of study during the last few years. This required a transition to the use of picomolar amounts of material, whereas a much higher specific radioactivity is needed for a reliable test of the specimen. From this point of view, the most suitable isotope proved to be phosphorus-32 obtained by the ^{32}S$(n,p)^{32}$P reaction involving the neutron irradiation of elemental sulfur. The specific radioactivity of phosphorus-32 without a carrier (for 100% isotope enrichment) is 9131 Ci mAt^{-1}, i.e. is 300 times greater than for tritium. Apart from this isotope, the phosphorus-33 and sulfur-35 isotopes, which also have a fairly high specific radioactivity (see Table 1), have been used successfully to label nucleic acids.

Nucleic acids labelled with radioactive isotopes of phosphorus (^{32}P and ^{33}P) as well as sulfur (^{35}S) are widely used in molecular-biological research and constitute the cornerstone of the principal methods such as hybridisation on filters, hybridisation in situ, sequencing of DNA, and many others. The choice of the radioactive label is determined mainly by two parameters: the sensitivity of the autoradiographic detection and spatial resolution.[1] These parameters depend in their turn on the β-emission energy of the radioactive isotope and its half-life, which determine the maximum specific radioactivity of the corresponding isotope. Table 1 presents the physical characteristics of the above isotopes.

Table 1. Physical characteristics of the radionuclides.

Physical characteristic	Isotope		
	^{32}P	^{33}P	^{35}S
Half-life/days	14.3	25.4	87.4
Maximum β-emission energy/MeV	1.71	0.20	0.167
Theoretical specific radioactivity of the radionuclide/Ci mAt^{-1}	9120	5200	1488
Maximum specific radioactivity (virtually attainable) of the labelled nucleotides/Ci mmol^{-1})	7000	4500	1500

In the determination of single animal genes by Southern's blot-hybridisation method,[2] the decisive factor is the requirement for the maximum sensitivity of the autoradiographic detection needed for subpicogramme amounts, which may be ensured only by use of the phosphorus-32 isotope, which possess the highest specific radioactivity among the isotopes considered here. On the other hand, in the method for the dideoxynucleotide sequencing of DNA, the requirement for sensitivity may be abandoned in favour of the high resolution of the bands on the autoradiogram,[3] which can best be ensured by labelling nucleic acids with sulfur-35

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and also with phosphorus-33, which have a low energy and therefore the path traversed by their β-particles in the gel is short. This applies equally to the method of hybridisation in situ, for which spatial resolution is decisive.

In many instances, phosphorus-33 has an advantage over sulfur-35, because it makes it possible to increase greatly detection sensitivity, whilst only slightly weakening spatial resolution. This has proved particularly important for the method based on the polymerase chain reaction ('cyclic sequencing') which makes it possible to sequence relatively small amounts of the matrix at high temperatures in order to reduce the amount of artefacts in the determination of the structure.[4] The ^{33}P-labelled primer ensures an adequate sensitivity and permits an autoradiographic exposure in the course of one night. This applies also to the method used in the analysis of the polymorphism of single-stranded configurations, in which the requirements for both spatial resolution and for detection sensitivity are fairly stringent.[5] A more detailed justification of the choice of the radioactive isotope for the above research methods has been given in the review by Evans and Read.[1]

One may note yet another advantage of nucleoside 5'-phosphates labelled with phosphorus-33 over the same compounds, labelled with sulfur-35, namely their complete chemical identity with the natural products, which eliminates any artefacts in the enzymic reactions in which labelled nucleoside 5'-triphosphates are employed as substrates. One should note that the majority of the methods impose stringent requirements on the quality of the nucleoside 5'-triphosphates labelled with radioactive phosphorus atoms, especially those with a high molar radioactivity, but it is not always possible to supply these compounds to remote regions in the course of 2–3 days at the appropriate temperatures using dry ice. We therefore thought it useful to survey the known methods of synthesis of these compounds.

The literature on this specific topic is extremely limited, since mainly the outline synthetic schemes are published in the open press without the technological details, which are mostly part of the [classified] know-how. It must be emphasised that even the most thorough and scrupulous copying of the published methods does not always lead to the expected results. In the present review, we attempted to present not only the published synthetic schemes but also to assess their reliability and reproducibility on the basis of the authors' personal experience.

Nucleoside 5'-triphosphates containing the radioactive isotope in the α- or γ-positions in the phosphate groups are usually employed to obtain labelled nucleic acids in vitro. Deoxynucleoside triphosphates with a phosphorus label in the α-positions are used to introduce the label into the 3'-end of DNA with the aid of various DNA polymerases and for the synthesis of a radioactive DNA molecule containing the label in one, two, or all four nucleotides. Adenosine triphosphate with a phosphorus label in the α-position is used to introduce the label into the 5'-ends of DNA and RNA. Scheme 1 presents the formulae of all the nucleoside triphosphates used in molecular-biological research, the synthesis of which is described in the present review.

The synthesis of nucleoside 5'-triphosphates with a phosphorus label in the α- and γ-positions and having a high molar radioactivity (3000–6000 Ci mmol^{-1}) involves a series of specific difficulties.

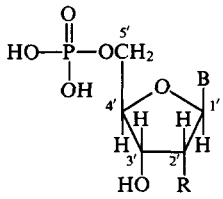
1. The reactions used to obtain the target compounds must not lead to an uncontrolled isotopic dilution of the radioactive phosphorus by the nonradioactive isotope – phosphorus-31.

2. The location of the radioactive atom must be rigorously fixed. The use of methods which might alter the location of the radioactive atom or introduce the label in different positions in an uncontrolled manner is not permissible.

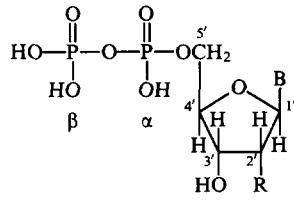
3. The high molar radioactivity of the nucleoside 5'-triphosphates used in molecular biology makes it necessary to carry out the syntheses with amounts of the radioactive isotope of 10^{-9}–10^{-8} mol, which corresponds to a radioactivity of 10–100 mCi (for phosphorus-32).

4. The low concentration of the radioactive substance requires the use of a large excess of the nonradioactive

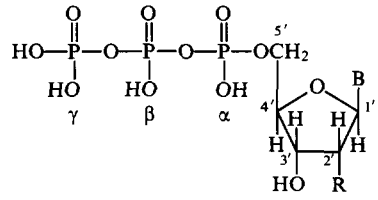
Scheme 1



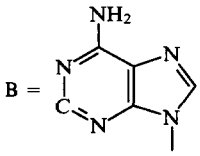
Nucleoside 5'-monophosphates



Nucleoside 5'-diphosphates



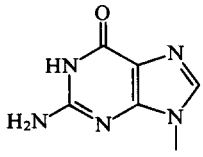
Nucleoside 5'-triphosphates



Adenine

R = OH (AMP, ADP, ATP)

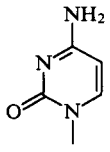
R = H (dAMP, dADP, dATP)



Guanine

R = OH (GMP, GDP, GTP)

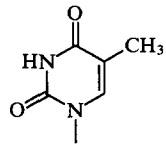
R = H (dGMP, dGDP, dGTP)



Cytosine

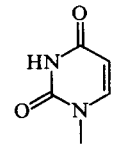
R = OH (CMP, CDP, CTP)

R = H (dCMP, dCDP, dCTP)



Thymine

R = H (TMP, TDP, TTP)



Uracil

R = OH (UMP, UDP, UTP)

components in order to increase the rate and yield of the reaction, which frequently leads to the appearance or side products.

5. For a high bulk-phase radioactivity of the reaction mixture (1 – 10 Ci ml^{-1}), the reaction time must be reduced to a minimum, since considerable amounts of mainly unidentified products, induced by the ionising radiation, accumulate (radiolysis products as well as the products of radical reactions).

6. The purification and especially the analysis of the products are hindered by the small amount of the substance and virtually preclude the use of the classical purification methods applied in organic chemistry (crystallisation, extraction, distillation, filtration, etc.) and analytical methods such as NMR, EPR, and IR spectroscopy, mass spectrometry, and X-ray diffraction.

7. Experiments with radioactive substances make it necessary to ensure the personnel safety, so that all manipulations in the syntheses must be performed observing radiation safety standards, preferably at a distance, in isolated compartments. The available descriptions of the methods of synthesis of nucleotides and nucleoside 5'-triphosphates labelled with radioactive phosphorus atoms are extremely scanty. The starting material in these syntheses is orthophosphoric acid. All the methods can be divided into the following groups:

(a) chemical methods in which chemical reagents are used in all the stages;

(b) chemicoenzymic methods in which enzymic reactions are employed together with chemical ones;

(c) enzymic methods in which all the stages of the synthesis are carried out with the aid of enzymes.

Nucleoside 5'-triphosphates labelled with radioactive phosphorus atoms in the α- or γ-positions are of greatest interest for molecular biology and the present review is devoted to the analysis of the methods of synthesis of these compounds. Most attention has been devoted to enzymic methods of synthesis because at present these procedures are used on an increasingly wide scale for the synthesis of nucleic acid precursors labelled with radioactive isotopes of not only phosphorus and sulfur but also of carbon, as well as tritium-labelled ones.[6]

The majority of studies considered in the present review were carried out using [^{32}P]orthophosphoric acid, but all the methodological procedures presented are valid also for syntheses in which [^{33}P]orthophosphoric acid is used. This is also confirmed by the personal experience of the authors of the review.

II. Synthesis of nucleoside 5'-[γ-$^{32(33)}$P]-triphosphates

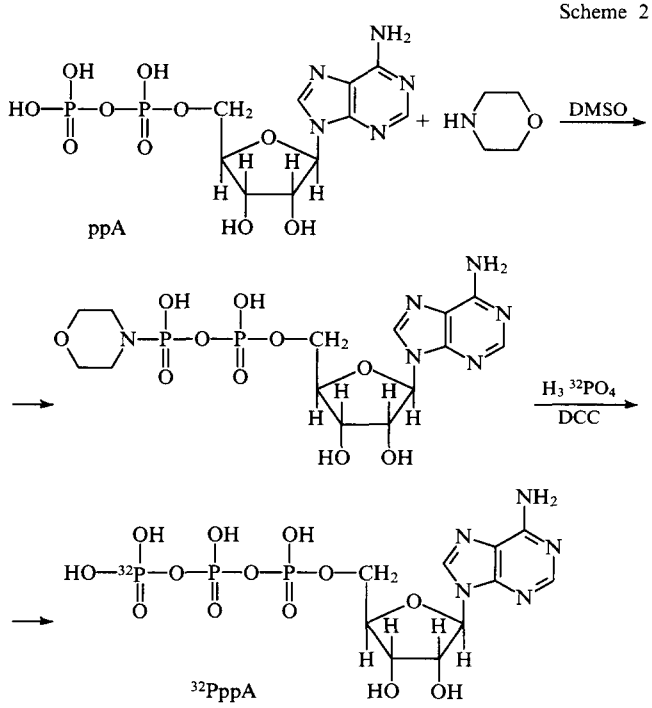
1. Chemical methods

The synthesis of nucleoside 5'-triphosphates labelled with radioactive phosphorus in the γ-position is not a very complex task. Moffat[7] was the first to propose a method of synthesis of [γ-^{32}P]ATP (^{32}PppA) from ADP (ppA) and [^{32}P]orthophosphoric acid ($^{32}P_i$). The reaction, involving the formation of an intermediate morpholide derivative, was used to activate the β-phosphate of ADP (Scheme 2). The reaction with $H_3\,^{32}PO_4$ was carried out in anhydrous dimethyl sulfoxide (DMSO) in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC).

[γ-^{32}P]ATP with a molar radioactivity of 10 mCi $mmol^{-1}$ was obtained by this method in 65% yield. Such a low molar radioactivity of the product was explained by the lack at the time of [^{32}P]orthophosphoric acid with a high molar radioactivity. At the present time the method makes it possible to obtain a product with a high molar radioactivity (up to 1000 Ci $mmol^{-1}$). The main disadvantage of the proposed method is the instability of the intermediate morpholide derivative (it decomposes at room temperature in several hours).

This method was improved by a group of investigators at the All-Union Institute of Molecular Biology (VNII MB) in Novosibirsk,[8] who were able to select conditions for the stable isolation and storage of the intermediate activated ADP

Scheme 2



derivative. It was suggested that 4-(*N,N*-dimethylamino)-pyridine be used to accelerate the second stage — the synthesis of [γ-^{32}P]ATP proper. With the aid of this modification, it is possible to obtain comparatively simply [γ-^{32}P]ATP with any specified molar radioactivity up to the limiting value. The method can be applied successfully also to the synthesis of other nucleoside 5'-triphosphates by employing the corresponding nucleoside 5'-diphosphates.

The reaction is also carried out in anhydrous dimethyl sulfoxide and proceeds fairly rapidly (10 – 20 min), but is frequently accompanied by the accumulation of side products of the type AppppA, AppppA, and other unidentified compounds. This factor not only lowers the yield of the target product but also greatly complicates its isolation. On the other hand, the presence of such impurities in the target product is extremely undesirable, because they possess pronounced sorption properties. For this reason, this method has not come to be widely used in laboratory practice or in industrial manufacture.

2. The chemicoenzymic method

The only chemicoenzymic method of synthesis of [γ-^{33}P]ATP described in the literature involves the formation of acetyl [^{33}P]phosphate from acetic anhydride and [^{33}P]orthophosphoric acid and subsequent phosphorylation of ADP by this reagent with the aid of acetyl kinase.[9]

Bearing in mind the wide variety and reversibility of the enzymic reactions with participation of ATP, several versions similar to that already described could be proposed — for example, the synthesis of [γ-^{32}P]ATP from the ^{32}P-labelled phosphoenol pyruvate with the aid of pyruvate kinase or from labelled creatine phosphate with the aid of creatine kinase. Such reactions would proceed rapidly without the formation of side products and with a satisfactory yield. Nevertheless, the cost of the preparation of labelled precursors from [^{32}P]orthophosphoric acid, i.e. acetyl [^{32}P]phosphate, [^{32}P]phosphoenol pyruvate, or creatine [^{32}P]phosphate, and of their isolation from the reaction mixture before the enzymic stage, renders the synthesis laborious and uncompetitive compared with enzymic methods and it has therefore not come to be widely used.

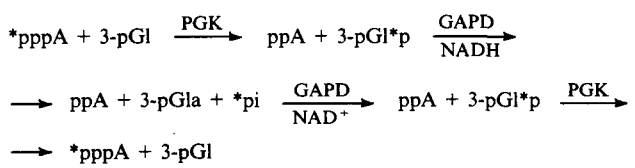
Overall, the chemicoenzymic methods of synthesis of [γ-^{32}P]ATP are nowadays of purely theoretical interest and are not used for preparative purposes.

3. Enzymic methods

The simplest in execution and most effective method of synthesis of nucleoside 5'-triphosphates labelled with ^{32}P (or ^{33}P) in the γ-position proved to be the enzymic method. The initial idea of the method utilised the reversibility of the glycolytic reactions in the reconstructed fragment of the nucleoside chain (Scheme 3).

With the aid of phosphoglycerate kinase (PGK) and glyceraldehydephosphate dehydrogenase (GAPD), ATP was converted into ADP and orthophosphate via the intermediate 1,3-diphosphoglycerate (pGlp) in the presence of 3-phosphoglycerate (3-pGl). The addition of [^{32}P]orthophosphoric acid to the reaction mixture in the last stage inevitably leads to the formation of [γ-^{32}P]ATP owing to the reversibility of the enzymic reaction during the attainment of equilibrium in the system (Scheme 3a).

Scheme 3a



*p = the phosphate group whose exchange is catalysed by the enzymes PGK and GAPD

This method, proposed by Glynn and Chappell,[10] was rapidly incorporated into laboratory practice. The method acquired particular popularity by virtue of the simple and readily reproducible procedure proposed by Maxam and Gilbert[11] for the determination of the DNA sequence. The entire synthesis of [γ-^{32}P]ATP by this method took less than 2 h and the product could be used without isolation for the introduction of the label in the 5'-end of DNA. However, the method does not allow, in principle, the synthesis of [γ-^{32}P]ATP without dilution by the phosphorus-31 isotope and in reality affords the final product in 60%–70% yield and with a molar radioactivity of 1000–1500 Ci mmol^{-1}.

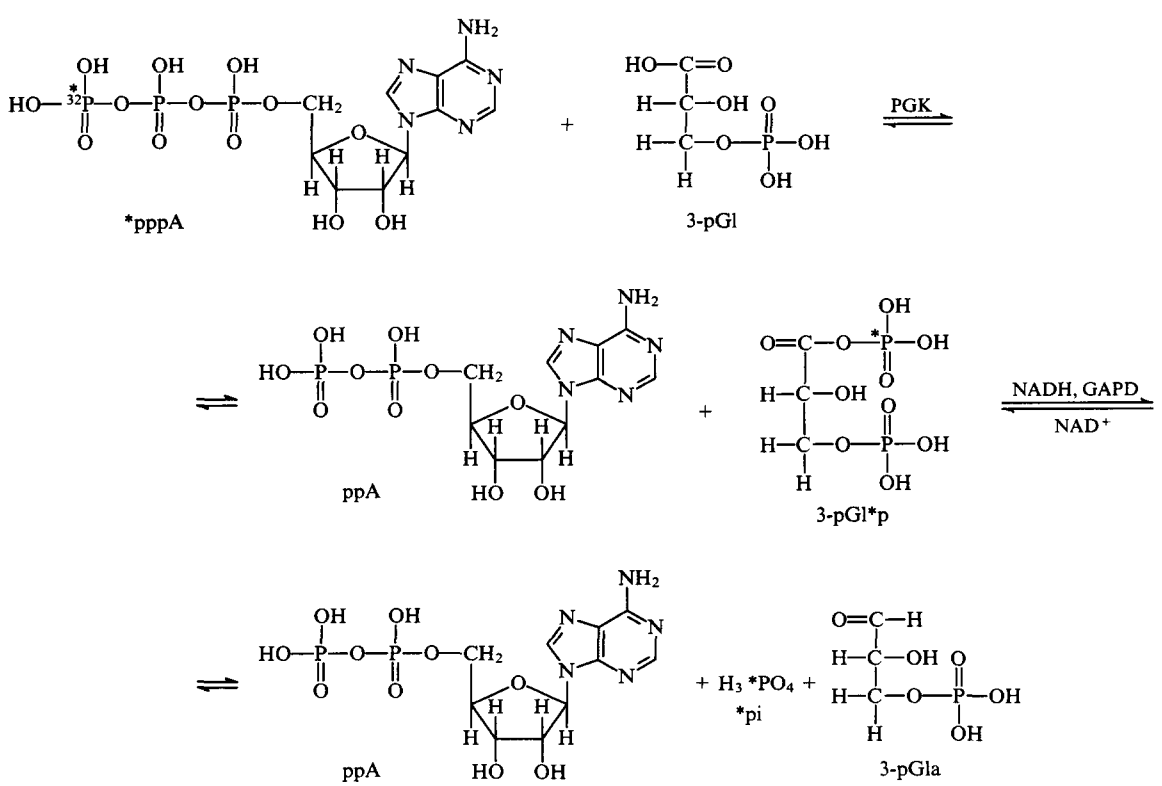
Later Stupar and Leskovac[12] analysed the method of Glynn and Chappell from the standpoint of the optimisation of the reaction yield and the molar radioactivity of the [γ-^{32}P]ATP obtained.

In 1973, Schendel and Wells[13] published a study in which they suggested that the scheme underlying the Glynn–Chappell method be 'inverted' and that the direct synthesis of [γ-^{32}P]ATP from glyceraldehyde-3-phosphate and [^{32}P]orthophosphoric acid be employed. Theoretically, this would make it possible to obtain [γ-^{32}]ATP with the maximum possible molar radioactivity, equal to that of the initial [^{32}P]orthophosphoric acid, i.e. without isotopic dilution. Bearing in mind the instability of glyceraldehyde-3-phosphate, the authors suggested that a stable and commercially available preparation be employed — the diethyl acetal of glyceraldehyde-3-phosphate. Free glyceraldehyde-3-phosphate was then formed in the first stage — the chemical hydrolysis of the diethyl acetal — and this was followed by the enzymic synthesis proper. However, the admixture of inorganic phosphates formed from the unstable glyceraldehyde-3-phosphate in the first stage of the hydrolysis of the diethyl acetal lowers the radioactivity of [γ-^{32}P]ATP sharply and in an uncontrolled manner owing to isotopic dilution. This method has not therefore found extensive application either in laboratory practice or in industry.

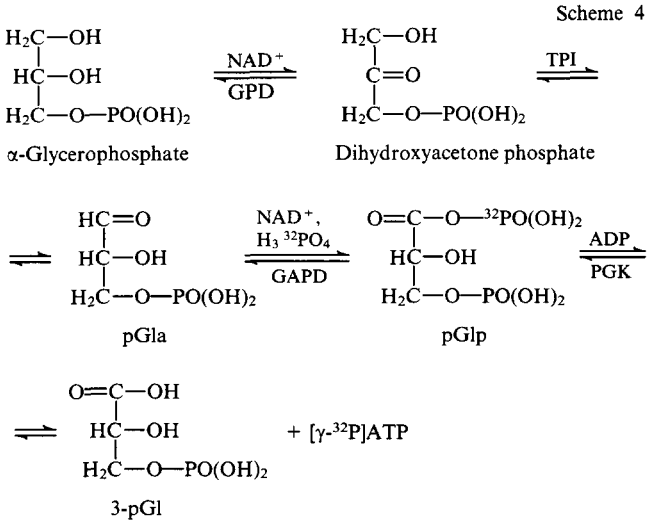
In order to increase the molar radioactivity of the preparation, Walseth and Johnson[14] improved the method further — a fragment of the glycolysis chain was supplemented by three more fragments: glycerophosphate dehydrogenase (GPD), triosephosphate isomerase (TPI), and lactate dehydrogenase (LDH). Glyceraldehyde-3-phosphate was then synthesised from α-glycerophosphate, while LDH was added to the system in order to regenerate the NAD^+ (Scheme 4). Since LDH does not participate directly in the synthesis of [γ-^{32}P]ATP, it is not shown in Scheme 4.

The yield of ATP increased to 90%–98% and, which is most important, the molar radioactivity rose to the limiting values, which were determined by the molar radioactivity of the initial [^{32}P]orthophosphoric acid and also by the content of the 'cold' orthophosphate in the reagents employed.

Scheme 3



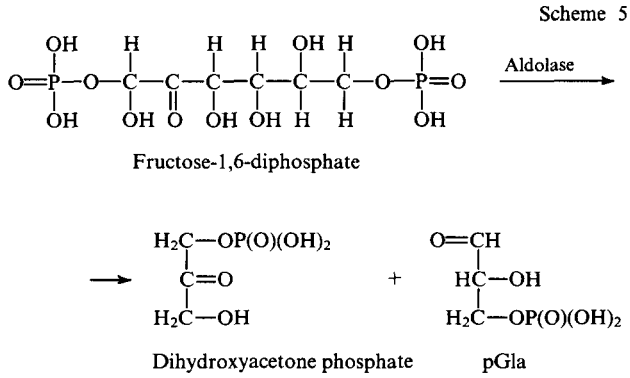
Scheme 4



Instead of ADP, one can use other nucleoside 5′-diphosphates: GPD, IDP, CDP, UDP, dADP, dGDP, which afford the corresponding triphosphates with the phosphorus label in the γ-position. The method is simple and readily reproducible and has found extensive application in various laboratories. Judging from the extremely scanty and fragmentary literature data, all the principal producer companies obtain [γ-^{32}P]ATP precisely by this method (possibly with some modifications).

Banschke and coworkers[15] proposed yet another method of synthesis of glyceraldehyde phosphate. They used the cleavage of fructose-1,6-diphosphate by aldolase (Scheme 5).

Scheme 5



In our view, this path suffers from at least two disadvantages compared with the synthesis in accordance with Scheme 4. Firstly, fructose-1,6-diphosphate is not very stable in solution and there is therefore a real possibility of isotopic dilution by 'cold' phosphorus. Secondly, in order to increase the yield, the accumulated dihydroxyacetone phosphate must be removed, for example with the aid of the same TPI. Thus the method has no special advantages, since the replacement of GPD by aldolase and hence of α-glycerophosphate by fructose-1,6-diphosphate is not justified. Admittedly, the authors indicate the greater resistance of aldolase to the effect of ionising radiation, which may be important for industrial synthesis. There is no doubt that the thoroughly tested synthesis presented in Scheme 4 is preferable for laboratory use in the range 1 – 10 mCi.

Quite recently, ICN Biomedical, Inc. patented[16] yet another method of synthesis of [γ-^{32}P]ATP — a kind of intermediate variant between the Johnson – Walseth and Schendel – Wells methods. Lynn, the author of this scheme, suggested that α-glycerophosphate and glycerophosphate dehydrogenase be abandoned and that dihydroxyacetone phosphate be used immediately as the initial nonradioactive starting material. In fact, he proposed that the Johnson – Walseth scheme 'be shortened' by one (the first) stage (Scheme 4). At the same time, Lynn suggested that the usual NAD-regenerating system (lactate dehydrogenase – pyruvate) be replaced by a more exotic system (malate dehydrogenase – oxaloacetate). According to the authors' communication, these two changes make it possible to scale up the synthesis to a charge up to 500 mCi of [^{32}P]orthophosphate and an unchanged high yield (~95%) over a period of 15 – 20 min. Bearing in mind existing literature data[17] indicating the low stability of glycerophosphate dehydrogenase towards ionising radiation from phosphorus-32 this is extremely likely. Indeed, the direct application of the Johnson – Walseth method makes it possible to obtain satisfactory results for the synthesis involving 1 – 10 mCi of phosphorus-32 (the usual laboratory synthesis). An increase in the amount of phosphorus-32 to 100 mCi or further to 1 Ci reduces sharply the yield of the reaction.

It must be emphasised that in Lynn's method all the ingredients of the reaction mixture, including the enzymes, are taken in logical and justified amounts, which can be in no way said of the original study of Johnson and Walseth. Nevertheless, the 'ICN' patent appears more like an attempt to 'get round' the Johnson – Walseth patent rather than a serious attempt to improve the technology and is hardly likely to be incorporated widely into practice.

We proposed a fairly simple and convenient modification of the Johnson – Walseth method for the large-scale synthesis (100 – 1000 mCi of phosphorus-32). Its laboratory version has been described in Karamyshev's dissertation.[18] The essential feature of the modification consisted in the preliminary formation of glyceraldehyde-3-phosphate directly in the reaction vessel: after centrifugation, the ammonium sulfate residue of the enzyme mixture was dissolved in a buffer containing dithiothreitol and all the necessary ingredients for the synthesis of [γ-^{32}P]ATP, with the exception of [^{32}P]orthophosphoric acid and ADP, were introduced into the solution. After preincubation for 20 min, the above remaining ingredients were added and the synthesis was carried out. Such changes in the order of the introduction of the reagents into the reaction mixture do not entail any fundamental alterations, but, according to our observations, they increase and stabilise the yield of the product of the synthesis by virtue of the effective generation of glyceraldehyde-3-triphosphate (in accordance with Scheme 4) before the start of the synthesis of [γ-^{32}P]ATP proper.

III. Synthesis of nucleoside 5′-[α-$^{32(33)}$P]-triphosphates

The scheme designed for the synthesis of nucleoside 5′-triphosphates (NTP) labelled with phosphorus-32(33) in the α-position is much more complex than those described above and requires the solution of virtually two independent problems. The first involves the synthesis of nucleoside 5′-monophosphate (NMP) with a phosphorus label by introducing [$^{32(33)}$P]orthophosphate rigorously in the 5′-position in the nucleoside. The second problem involves the synthesis of the nucleoside 5′-[α-$^{32(33)}$P]triphosphates themselves from the corresponding 5′-[$^{32(33)}$P]NMP.

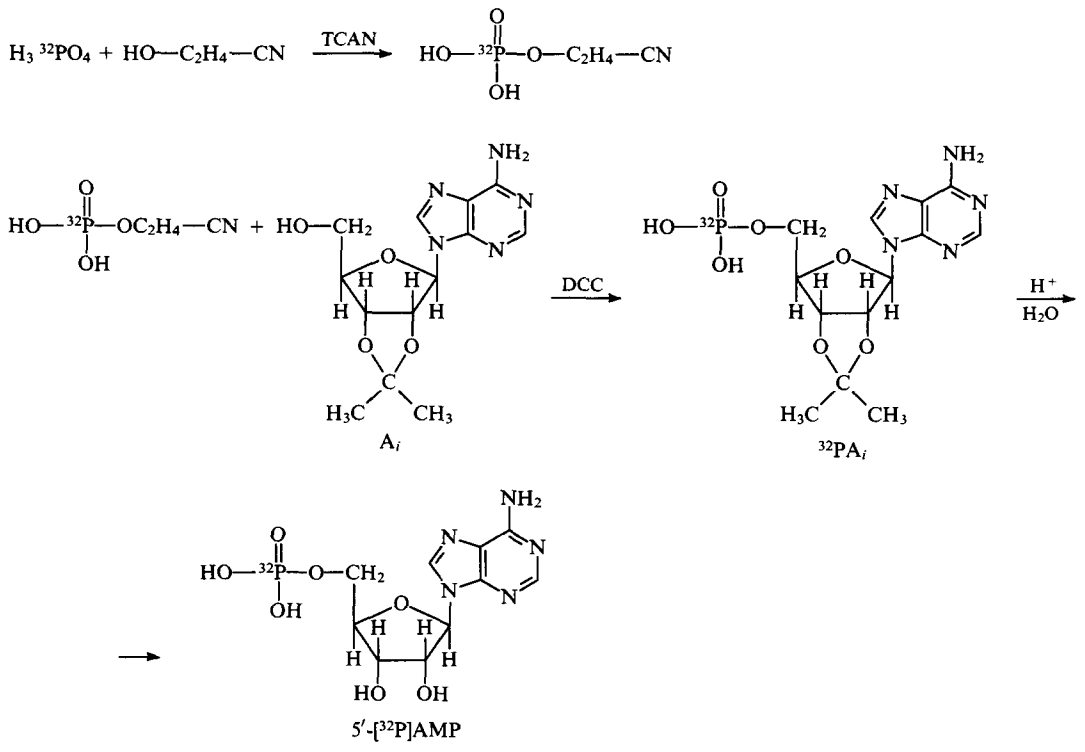
1. Synthesis of nucleoside 5′-[$^{32(33)}$P]monophosphates

a. Chemical methods

Since the majority of the chemical methods for the introduction of labelled orthophosphate into nucleosides are nonspecific, nucleosides with protected 2′- and 3′-hydroxy-groups are employed for the synthesis of 5′-[$^{32(33)}$P]NMP. After the synthesis, the protecting group (the isopropylidene, acetyl, or benzoyl groups) are removed by acid or alkaline hydrolysis.

The first nucleoside 5′-monophosphate labelled by phosphorus-32 was synthesised by Moffat,[7] who employed ^{32}P-labelled β-cyanoethyl phosphate in the presence of DCC in order to phosphorylate 2′,3′-*o*-isopropylideneadenosine (A_i)

Scheme 6



(Scheme 6). The labelled β-cyanoethyl phosphate was obtained by condensing [^{32}P]orthophosphoric acid with β-cyanoethanol in the presence of trichloroacetonitrile (TCAN). $^{32}PA_i$ was hydrolysed in an acid medium to give the ^{32}P-labelled adenosine 5′-monophosphate — 5′-[^{32}P]AMP.

Symons[19] simplified the synthesis of the nucleoside 5′-[^{32}P]monophosphate by employing TCAN in the synthesis of 5′-[^{32}P]AMP directly from the protected adenosine and the triethylammonium salt of [^{32}P]orthophosphoric acid. Such simplification immediately rendered this method extremely popular both in laboratories and in industry. It is noteworthy that, despite its apparent simplicity, the method suffers from a series of disadvantages, the main ones of which — the formation of a considerable amount of side products and the low yield were noted by Maslova et al.[20] The rigorous requirements as regards the quality of the reagents and solvents and also the extremely complex mixture of reaction products forced investigators to continue the search for alternative methods of synthesis.

An improvement of Symons' method was proposed by Ammosov and coworkers.[21] It involved the use of nucleosides with an acetyl protecting group instead of nucleosides with isopropylidene or benzoyl protecting groups. The conditions used in the removal of the acetyl protecting group after the condensation of the protected nucleoside with [^{32}P]orthophosphoric acid are fairly mild (the removal usually involved treatment with an ammonia solution), which makes it possible to obtain the final product in a yield up to 70%–80%. On the other hand, in the case of the isopropylidene protecting group, which is removed under significantly more severe conditions (by treatment with hydrochloric acid), the yield of the pyrimidine nucleosides in the reaction carried out by the Symons procedure does not exceed 40%, while in the case of derivatives of 2′-deoxynucleosides of the purine series the preparation of the nucleoside 5′-[^{32}P]monophosphate becomes altogether problematical.

The attempt by Biebricher[22] to use urea as the condensing agent was interesting. A solution of the corresponding nucleoside, the ammonium salt of [^{32}P]orthophosphoric acid, and urea was evaporated to dryness and the dry residue was heated for several hours at 110–120 °C, cooled, and dissolved in water. Nucleoside 5′-[^{32}P]monophosphates were identified in the solution in yields of 15%–40%, depending on the nature of the heterocyclic base of the nucleoside. Unfortunately, the very complex mixture of unidentified side products of the reaction hinders the use of this method.

The attempt by Hurlbert and Furlong[23] to use a biosynthetic procedure based on *E. coli* to obtain [^{32}P]nucleotides is also of purely historical interest. The main disadvantage of this method is the very low molar radioactivity (10–20 mCi mmol^{-1}). The uncontrolled isotopic dilution and the high requirement by *E. coli* for inorganic phosphate preclude in principle the formation of radioactive phosphorus-labelled nucleotides with a high molar radioactivity.

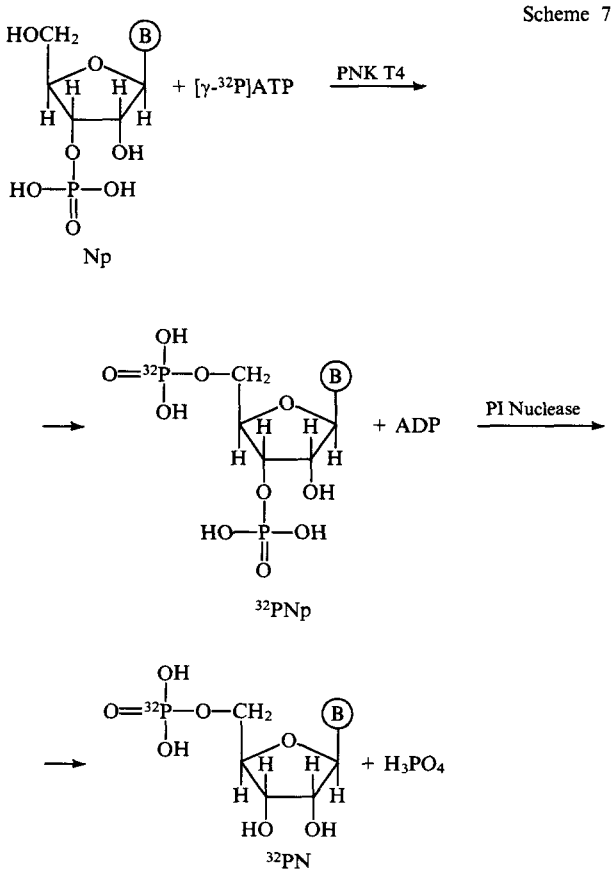
b. Enzymic methods of synthesis of nucleoside 5′-[$^{32(33)}$P]monophosphates

Johnson and Walseth[24] proposed an enzymic method of synthesis of nucleoside 5′-[^{32}P]monophosphates with the aid of a T4 phage polynucleotide kinase (PNK T4) and the PI nuclease (Scheme 7). The nucleoside 3′-monophosphate (Np) is the acceptor and [γ-^{32}P]ATP is the donor of radioactive phosphate.

The use of [γ-^{32}P]ATP for the synthesis of radioactive phosphorus-labelled nucleoside 5′-monophosphates proved to be entirely justified, since the enzymic method of synthesis of [γ-^{32}P]ATP makes it possible not only to synthesise this product rapidly and in a high yield but also to carry out the subsequent synthesis of ^{32}P-labelled nucleoside triphosphates with the aid of PNK T4 in the same reaction mixture without isolating [γ-^{32}P]ATP. This method is universal because PNK T4 phosphorylates all natural nucleoside 3′-monophosphates. The high rate and specificity of the enzymic reactions ensure a minimal amount of side products. All the advantages listed have attracted attention because of their technological applicability. The only significant disadvantage of the method is the need for a number of extremely expensive and very pure enzymes.

The lack of pure and stable enzymes — nucleoside kinases — precluded hitherto a test of yet another possibility of obtaining radioactive phosphorus-labelled nucleotides. This is the procedure based on the direct transphosphorylation of nucleosides

Scheme 7



with the aid of nucleoside kinases and [γ-^{32}P]ATP. The simplicity of this approach is evident, but nucleoside kinases are as a rule rigorously specific to the nature of the heterocyclic base of the nucleoside and it is therefore necessary to have 4–5 highly purified nucleoside kinases in order to synthesise all the natural nucleoside 5'-[$^{32(33)}$P]monophosphates. Attempts to isolate these enzymes from various sources have been frequently undertaken, but so far as we are aware stable preparations suitable for the preparative synthesis of nucleotides have not been obtained hitherto.

2. Methods for the phosphorylation of nucleoside 5'-[$^{32(33)}$P]monophosphates to nucleoside 5'-[α-$^{32(33)}$P]-triphosphates

a. Chemical methods

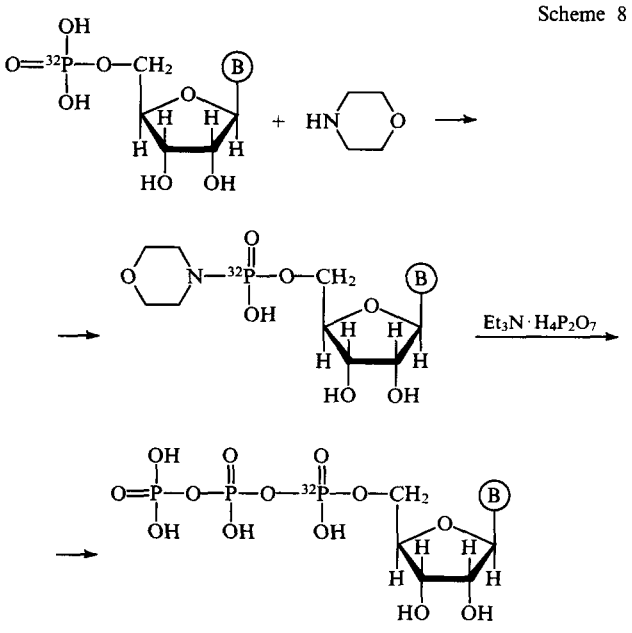
Moffat[7] synthesised nucleoside 5'-[α-^{32}P]triphosphates via the morpholide activation of nucleoside 5'-[^{32}P]monophosphate. The morpholide formed reacts with the triethylammonium salt of pyrophosphoric acid, affording a satisfactory yield (Scheme 8).

The [α-^{32}P]ATP obtained according to Scheme 8 had a very low molar radioactivity — about 10 mCi mmol^{-1}. Despite the satisfactory yield (65%), the method has not received recognition, since attempts to increase the molar radioactivity of the product led to a sharp fall in yield and to the accumulation of side products.

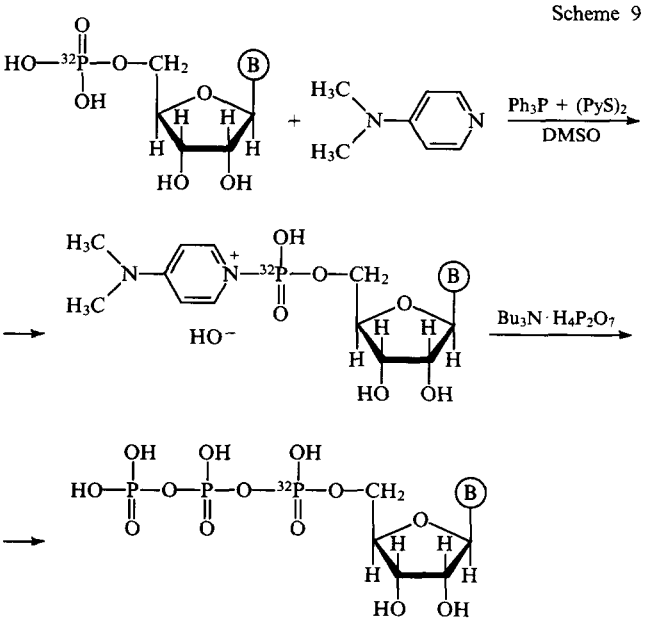
Siberian investigators proposed another more successful method for the activation of the nucleoside 5'-[^{32}P]monophosphate[25] involving the synthesis of an intermediate — a derivative of 4-(*N*,*N*-dimethylamino)pyridine. An oxidation-reduction couple was used as the catalyst: triphenylphosphine and di(4-pyridyl) disulfide. The intermediate formed is extremely unstable — it reacts even with the tributylammonium salt of pyrophosphoric acid directly in the same reaction vessel (Scheme 9).

All the natural nucleoside 5'-[α-^{32}P]triphosphates with a molar radioactivity of 2000–3000 Ci mmol^{-1} and above have

Scheme 8



Scheme 9

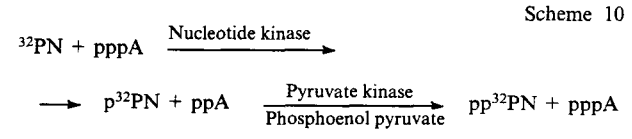


been synthesised in accordance with Scheme 9. The yield was 15%–40% relative to the initial [^{32}P]orthophosphoric acid. The method proved very useful in syntheses involving 1–10 mCi of phosphorus-32. An increase in the activity of the radioactive starting material to 100–200 mCi leads to the formation of a very complex mixture and reduces the yield of the target product, apparently as a consequence of the radiolysis of the reaction components.

b. Enzymic methods

The variants of the enzymic phosphorylation of nucleoside 5'-[$^{32(33)}$P]monophosphates to the triphosphates with the aid of the corresponding nucleotide kinases are more promising for the solution of this problem. The phosphorylation reaction can be represented by Scheme 10.

Scheme 10



The kinase fraction of proteins isolated from the bacterial biomass by means of ammonium sulfate fractionation, and not the individual enzymes, was used initially to phosphorylate nucleoside 5′-[^{32}P]monophosphates.[26] Such an enzyme preparation undoubtedly contained a large amount of ballast protein, including undesirable impurities: phosphatases and nonspecific ATP-ases. In order to suppress the degradation of the labelled nucleotides and nucleoside triphosphates and to maintain a constant level of ATP, the reaction mixture was treated with an ATP-regenerating system: creatine phosphate – creatine kinase or phosphoenol pyruvate – pyruvate kinase.[23,26] Acetyl phosphate was sometimes used, whereupon the addition of an exogenic acetate kinase was not required.[27] Nevertheless, the reproducibility of the results was very poor.

This led Biebricher[22] to develop a simple method for the simultaneous isolation of all the required nucleoside monophosphate kinases from *E. coli*. Although the enzymes were not purified to homogeneity, nevertheless the level of the impurity enzyme activities in the preparations employed by Biebricher was many times lower and the reproducibility of the syntheses was significantly increased.

A method for the isolation from *E. coli* of all the necessary nucleoside monophosphate kinases with a fairly high purity especially for the synthesis of all the nucleoside 5′-[α-^{32}P]triphosphates was developed later.[28]

Johnson and Walseth[24] used in the synthesis commercial preparations of pyruvate kinase and nucleoside monophosphate kinases: adenylate kinase (myokinase), guanylate kinase, and nucleoside monophosphate kinase (all the enzymes were produced by the Boehringer – Mannheim Company). Here attention should be drawn to a number of characteristic features of these preparations.

1. Adenylate kinase (myokinase) and guanylate kinase are individual enzymes and although the preparations of these enzymes produced by the Boehringer – Mannheim Company contain a lot of ballast protein and sometimes also undesirable impurities, nevertheless the synthesis of [α-^{32}P]ATP and [α-^{32}P]-dATP and hence of [α-^{32}P]GTP and [α-^{32}P]dGTP proceeds in an entirely satisfactory manner.

2. The nucleoside monophosphate kinase preparation is not an individual enzyme but a protein fraction containing, apart from the ballast protein, at least two of the required enzymes — thymidine monophosphate kinase and cytidine monophosphate kinase. The names of these enzymes in a pure form are missing from the catalogues of the relevant companies.

3. It has come to be assumed that cytidine monophosphate kinase is capable of phosphorylating also UMP (and conversely), i.e. that cytidine monophosphate kinase and uridine monophosphate kinase are the same enzyme.

4. The ratios of the enzyme activities of the 'nucleoside monophosphate kinase' preparation in relation to TMP, CMP, and UMP differ appreciably from batch to batch. The level of the activity of the TMP kinase and to a somewhat lesser extent the UMP kinase is particularly variable. The effectiveness of the use of the nucleoside monophosphate kinase can therefore be different for different substrates — the synthesis of CTP or dCTP usually proceeds most effectively, that of UTP is less satisfactory, and that of TTP is still less effective.

5. Pyruvate kinase — the enzyme catalysing the phosphorylation of NDP to NTP by phosphoenol pyruvate — leads to more ready phosphorylation of purine nucleotides than pyrimidine nucleotides. The phosphorylation of TDP is especially difficult.

Despite the above features of these Boehringer – Mannheim preparations, the enzymic phosphorylation of 5′-[^{32}P]NMP to give 5′-[α-^{32}P]NTP is greatly superior to all other procedures for the phosphorylation of nucleoside monophosphates as regards the effectiveness and technological applicability of the process.

The overall enzymic synthesis of [α-^{32}P]NTP can be represented by Scheme 11.[29]

Scheme 11

$$ppA + {}^{32}P_i \xrightarrow{GPD,\ TPI,\ GAPD,\ PGK,\ LDH} {}^{32}PppA \quad (1)$$

$$^{32}PppA + Np \xrightarrow{PNK\ T4} {}^{32}PNp \quad (2)$$

$$^{32}PNp \xrightarrow{PI\ nuclease} {}^{32}PN + P_i \quad (3)$$

$$^{32}PpN + pppA \xrightarrow{Nucleotide\ kinase} p^{32}PN + ppA \quad (4)$$

$$p^{32}PN \xrightarrow[Phosphoenol\ pyruvate]{Pyruvate\ kinase} pp^{32}PN \quad (5)$$

$^{32}P_i$ — inorganic orthophosphate ion labelled with the radioactive phosphorus-32 (or phosphorus-33) isotope

All the reactions take place in a single reaction vessel and the intermediates do not have to be isolated. Scheme 11 does not include yet another reaction which the authors recommend should be carried out, but this does not affect the principles of the method. This concerns the decomposition of the residual unreacted [γ-^{32}P]ATP after the reaction with PNK T4. In order to avoid the introduction of a label in the β-position in the nucleoside 5′-diphosphate, the authors suggested that, in the stages following the second stage of the phosphorylation reaction, hexokinase should be used to decompose the residual [γ-^{32}P]ATP after the dephosphorylation of the diphosphate by the PI nuclease. After treatment with hexokinase, the mixture was heated for 2 min on a water bath in order to thermally inactivate the enzymes and the synthesis was continued in the same vessel in accordance with Scheme 11.

The method of synthesis of nucleoside 5′-[α-^{32}P]triphosphates proposed by Johnson and Walseth has incontrovertible advantages over all the methods described above and has found extensive application both in laboratory practice and in the corresponding industrial processes.

Analysis of the trends in the development of the methods of synthesis of nucleoside 5′-[$^{32(33)}$P]triphosphates labelled with radioactive phosphorus in the α- or γ-positions shows that the enzymic methods are preferable and are gradually displacing the chemical and chemicoenzymic methods of synthesis from laboratory practice and from industrial manufacture.

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