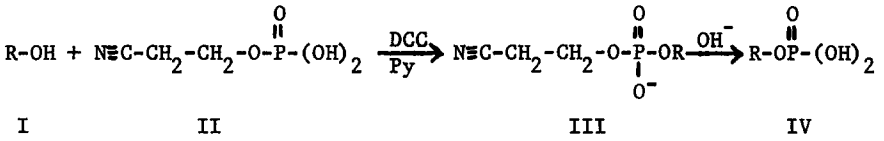
AN IMPROVED SYNTHESIS OF P^{32}-2-CYANOETHYL PHOSPHATE*

K. E. Pfitzner and J. G. Moffatt

Syntex Institute for Molecular Biology,
Palo Alto, California

Received July 17, 1964

The great variety and significance of phosphate esters in biological systems has prompted the development of numerous techniques for the phosphorylation of alcohols (For reviews see: Khorana, 1961; Brown, 1963). For application to the synthesis of nucleotides, undoubtedly the most successful of these methods consists of the reaction of the alcohol (I) with 2-cyanoethyl phosphate (II) and dicyclohexylcarbodiimide (DCC) in pyridine (Tener, 1961). The cyanoethyl residue is then eliminated from the resulting phosphodiester (III) by mild alkaline treatment to give the desired phosphomonoester (IV). Since its introduction this elegant method has found wide application:



I II III IV

In order to adequately study phosphate esters in biological systems, however, it is frequently necessary to use compounds labeled with P^{32}. To this end, Tener has also described a synthesis of P^{32}-2-cyanoethyl phosphate (II) via condensation of 2-cyanoethanol with P^{32}-orthophosphoric acid in the presence of dicyclo-

*Contribution No. 19 from the Syntex Institute for Molecular Biology.

hexylcarbodiimide (Tener, 1961; Tener, 1962). The direct product of this reaction is tetra-(2-cyanoethyl)pyrophosphate (V) which must then be hydrolyzed to di-(2-cyanoethyl)phosphate (VI) from which one cyanoethyl group must be removed by selective elimination giving II. When dealing with radioactive II the difficulties in effecting such a completely selective hydrolysis become apparent and the isolation of the pure product is often only achieved at the expense of a high yield.

$$(NCCH_2CH_2O)_2\overset{O}{\overset{\|}{P}}\text{-O-}\overset{O}{\overset{\|}{P}}\text{-O}(CH_2CH_2CN)_2 \longrightarrow (NC\text{-}CH_2CH_2O)_2\overset{O}{\overset{\|}{P}}\text{-OH} \longrightarrow \text{II}$$

V VI

We have now found that a completely specific, and very efficient, synthesis of P^{32}-labeled 2-cyanoethyl phosphate (II) results from the reaction of triethylammonium P^{32}-orthophosphate with excess 2-cyanoethanol and trichloroacetonitrile (Cramer, 1961). Whereas Cramer and Weimann conduct similar types of reactions at 70° for four hours, we find the present reaction to be almost complete within fifteen minutes at room temperature, the reaction mixture remaining colorless under these conditions. By an extremely simple work-up, the crystalline barium salt of 2-cyanoethyl phosphate is obtained in 85-90% yield, the entire process taking only three hours in all.

Paper chromatography of the product as directly crystallized from the reaction mixture shows the presence of two very minor impurities. One of these (0.7% of the total P^{32}) is orthophosphate and the other (1.7%) is perhaps 2-cyanoethyl diphosphate. Such levels of impurities are of little consequence during subsequent phosphorylation reactions (Wehrli, 1964). The present method is very convenient for the preparation of the crystalline barium salt

of II with specific activities in the range of 1-50 μC per μM. It can also be readily extended to the preparation of II from undiluted P^{32}-orthophosphoric acid (~10 mC per μM) in which case the extracted aqueous reaction mixture is separated by paper chromatography in Solvent I rather than by crystallization (Unpublished experiments by W. E. Wehrli and J. G. Moffatt).

EXPERIMENTAL

Paper chromatography was carried out by the descending technique on Schleicher and Schuell No. 589 orange ribbon paper using the solvent system isopropanol:conc. ammonium hydroxide: water (7:1:2) (Solvent I). Autoradiography was done using Kodak Medical X-Ray Film and determinations of P^{32} were made using a Nuclear Chicago gas flow counter or a Nuclear Chicago scintillation counter.

Free acid P^{32}-orthophosphoric acid[1] (5 mC) was mixed with 0.25 ml. of aqueous non-radioactive 2M orthophosphoric acid (0.5 mmole) and 0.21 ml. of distilled triethylamine (1.5 mmoles) and the mixture was carefully evaporated to dryness on an oil pump. The residue was dissolved in anhydrous pyridine (5 ml.) and rendered anhydrous by three evaporations with 5 ml. portions of pyridine. The final residue was dissolved in 1.0 ml. of distilled 2-cyanoethanol (hydracrylonitrile from Matheson, Coleman and Bell) and to it was added 0.07 ml. of triethylamine (0.5 mmole to replace any of the amine lost during the evaporations) and 1.0 ml. of trichloroacetonitrile[2]. The sealed mixture was kept at room temperature with periodic swirling for one hour and then evacuated

[1]This product, obtained from Abbott Laboratories, was shown to be extremely pure and was used directly.

[2]Obtained from the Aldrich Chemical Co., Milwaukee, Wis.

on the oil pump to remove excess trichloroacetonitrile. The residue was partitioned between 10 ml. of water and 10 ml. of ether and the ether layer was back-extracted with 2-3 ml. of water. The combined aqueous layers were then extracted once more with ether. The aqueous layer (pH~6) was adjusted to pH 8.0 with 0.2M barium hydroxide, and 0.5 ml. of 2M barium acetate was added. A trace of barium phosphate was quickly removed by centrifugation[3] and the precipitate was washed twice with 1-2 ml. of water. The addition of two volumes of ethanol resulted in the crystallization of the barium salt of 2-cyanoethyl phosphate which was washed twice with aqueous ethanol, then with pure ethanol and with ether and dried *in vacuo*. The yield was 137 mg. (85%) and the product, which was chromatographically, electrophoretically and analytically identical to a reference sample of the unlabeled compound, was the dihydrate. Paper chromatography in Solvent I and measurement of the radioactive spots showed the product to be 97.6% pure II (Rf 0.31) contaminated with 0.7% orthophosphate (Rf 0.15) and 1.7% of another compound (Rf 0.20) perhaps 2-cyanoethyl diphosphate. The specific activity was 10 μC per μmole.

REFERENCES

Brown, D. M., Advances in Organic Chemistry: Methods and Results, Vol. 3, Interscience Publishers, New York, p. 75 (1963).

Cramer, F. and Weimann, G., Ber., 94, 996 (1961).

Khorana, H. G., Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest, John Wiley & Sons, Inc., New York (1961).

Tener, G. M., J. Am. Chem. Soc., 83, 159 (1961).

Tener, G. M., Biochemical Preparations, Vol. 9, John Wiley & Sons, New York, p. 7 (1962).

Wehrli, W. E., Verheyden, D. L. M., Pfitzner, K. E. and Moffatt, J. G. J. Am. Chem. Soc., In preparation (1964).

[3] If the aqueous solution is too concentrated or if it is not centrifuged quickly, the barium salt of II may partially crystallize at this point.