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# Hot Atom Chemistry of Phosphorus Compounds. II.[1] Chemical Effects of Neutron Irradiation on Phosphoric Esters of Hexoses*[1]

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Phosphoric esters of hexoses were subjected to neutrons and the resulting $^{32}$P-labeled recoil products were separated by paper chromatography and paper electrophoresis. The main recoil products were phosphorous and phosphoric acids, independent of the chemical form of the target material and the kind of its salt. A small amount of inorganic acids containing a P–P or P–O–P bond was also found. The yield of the retention product was less than a few percents. The presence of organic diphosphorus compound was supposed by paper electrophoretic analysis. An addition of a small amount of water to the target material before irradiation favored formation of $^{32}$P-labeled phosphorus oxyacids in lower oxidation state. The results from irradiation of aqueous solution gave an unexpected chromatographic behavior owing to some unstable complex of recoil $^{32}$P atom with water molecule.

Although the elucidation of the chemical effects of neutron irradiation on biologically important compounds will give a clue to that on cells or tissues in a living body, little is known about the effects on such compounds except a few compounds.[2] The neutron irradiation of ATP (Adeno sine Tri-Phosphate) has been reported.[3] However, the separation of the recoil products was not so good. The same attempt on DNA (Deoxyribonucleic Acid) have been reported by Halmann who studied on the recoil products and on the physical change due to radiolytic damage.[4] In this case, about one half of $^{32}$P-labeled products was found in inorganic phosphorus compounds.

Recently, Yagi and coworkers[5] attempted a phosphorylation of glycerol by irradiating a mixture

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[1] Part 1 of this series: Y. Kiso *et al.*, This Bulletin, **40**, 2779 (1967).

*[1] Presented at the 11th Symposium on Radiochemistry, Tokyo, October, 1967.

[2] M. Halmann, *Chem. Revs.*, **64**, 686 (1964).

[3] a) H. G. Mautner, B. Donnelly, C. M. Lee and G. W. Leddicotte, *J. Am. Chem. Soc.*, **84**, 2021 (1962). b) H. G. Mautner, C. M. Lee and M. H. Krackov, *ibid.*, **85**, 245 (1963).

[4] M. Halmann and I. R. Miller, *Biochim. Biophys. Acta*, **72**, 483 (1963).

[5] T. Yagi, S. A. El-Kinawy and A. A. Benson, *J. Am. Chem. Soc.*, **85**, 3462 (1963).

of glycerol and phosphoric acid. Almost all radioactivities in recoil products identified by paper chromatography were found in inorganic phosphorus oxyacids. The yield of $^{32}$P-labeled organic phosphorus compounds was less than that of inorganic phosphorus compounds.

Thus, systematic study on organic compounds have not yet been made.

As the chemical effects on aromatic phosphorus compounds by bombardment with neutrons have been discussed in the previous paper,¹⁾ we now should like to report the irradiation effects with neutrons on phosphoric esters of hexoses which play an important role in glycolysis. Salts of hexose phosphates chosen were as follows; glucose-1-phosphate dipotassium salt (G-1-P(K)), glucose-6-phosphate barium, potassium and sodium (G-6-P-(Ba, K or Na)), galactose-6-phosphate barium (Ga-6-P(Ba)), fructose-1-phosphate barium (F-1-P(Ba)), fructose-6-phosphate sodium (F-6-P(Na)), and fructose-1,6-diphosphate barium (F-1,6-P(Ba)). After these compounds were irradiated in a reactor, recoil products formed by the $^{31}$P $(n,\gamma)^{32}$P reaction were analyzed by paper chromatography and paper electrophoresis. The effect due to the chemical structure of target material on the production of recoil compounds, and influence by the presence of metallic elements such as barium, sodium and potassium on the recoil reactions are discussed on the basis of the yield of radioactive recoil products, as well as the dilution effcet of the target materials with water on the reaction.

## Experimental

**Materials.** 50% aqueous solution of $H_3PO_2$ was purchased from Nakarai Chemicals Ltd. and stored in a refrigerator. Crystal $H_3PO_3$ (95.3%) was used without further purification. Guaranteed grade of $H_3PO_4$ was used. G-1-P(K) and F-1,6-P(Ba) purchased from Nakarai Chemicals Ltd. and other phosphoric esters of hexoses from Sigma Chemicals were used without further purification. The purity of each sample was checked by activation of chromatographed paper. For preparing phosphoric ester of glucose without containing metallic element, G-6-P(Na) was treated with Dowex 50 ion-exchanger.

**Neutron Irradiation of Solid Materials.** The sample was sealed into a polyethylene tube and put into a larger polyethylene capsule. The capsule was irradiated for 30—60 min in a pneumatic tube of KUR.*²

**Neutron Irradiation of Diluted Solution.** G-6-P(Na) (0.157 g) was dissolved in 1.643 g of water free from any cation. Solutions of 1/3, 1/9, 1/27 and 1/81 for the initial concentration were prepared by diluting with water. An aliquot (about 0.5 *ml*) of each solution was sealed in a quartz tube (dia. 5×50 mm). About twenty quartz tubes were put in a larger polyethylene capsule (dia. 10×15 cm) and irradiated for 10 hr in the slant exposual tube of KUR. The flux of thermal neutron in the slant was $2.5\times10^{11}$ n/cm² sec, cadmium ratio 7.0 and $\gamma$-rays $5.0\times10^{6}$ R/hr.

**Paper Chromatographic Separation of Recoil Products.** After cooling for a week, the irradiated sample was dissolved in water and developed on Toyoroshi No. 50 (2×40 cm) by an assending method for 15 hr, using propanol-ammonia-water (6 : 3 : 1 in volume) as a combined solvent.⁶⁾ Radioactive distribution on the chromatographed strip was examined by autoradiography using Fuji Medical X-ray Film (27.9×35.6 cm) or by an autoradioscanner (Aloka, Model JPC-102). The $R_f$ value of known nonradioactive compound was determined by activation of the chromatographed strip. This values was used for identification of the recoil products.

**Paper Electrophoretic Separation of Recoil Products.** The migration apparatus with multi-compartment cells was used.⁷⁾ Background buffer electrolytes of different pH value were adjusted by mixing 0.1 M HCl, 0.1 M citric acid and 0.1 M $NH_4OH$ (ionic strength $\mu=0.1$ over a range of pH=1.0—11). After a paper strip was uniformly dipped in the background electrolyte solution and then the excess of the solution was removed with another filter paper, about 5 $\mu l$ of a solution (0.05 M) containing irradiated sample was placed on a filter paper (Toyoroshi No. 50, 2×40 cm). The substance was migrated for 1—1.5 hr under a potential gradient of 500 V/34 cm, keeping the temperature of the migration box at 25°C. The strip was taken out, dried by an infrared lamp and followed with autoradioscanning or autoradiography. The mobility of known nonradioactive compound was determined by activation method.

**Neutron Activation of the Chromatographed Paper.** The chromatographed paper strip was enveloped in a polyethylene envelope and rolled to put into a polyethylene capsule. The capsule containing the rolled papers was irradiated for 30 min in the pneumatic tube. After cooling for a week to reduce radioactivities due to impurities, the radioactivities of phosphorus-32 on the strip were located by autoradiography.

## Results and Discussion

Percentage of the radioactivities of recoil products on chromatographed strips are shown in Fig. 1. In general, the yield of hypophosphorous acid in recoil products was small, by taking the fact that the $R_f$ values of $H_3PO_4$, $H_3PO_3$ and $H_3PO_2$ were 0, 0.30 and 0.67, respectively. On the other hand, large amounts of $H_3PO_3$ and $H_3PO_4$ were obtained. Although recoil products from G-6-P(Ba) or Ga-6-P(Ba) deposited mostly at the starting position of the filter paper, all of the activities is not considered to be due to phosphoric acid because the activity of phosphoric acid identified by using electrophoresis is not so much. The inconsistency among results obtained by the paper chromatography and the paper electrophoresis was often found when the target material was insoluble in

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[*2] Kyoto University Reactor (see Ref. 1).

6) T. Weil, *Helv. Chim. Acta*, **38**, 1274 (1955).
7) Y. Kiso, *J. Sci. Hiroshima Univ. Ser. A-II*, **27**, 24 (1963).

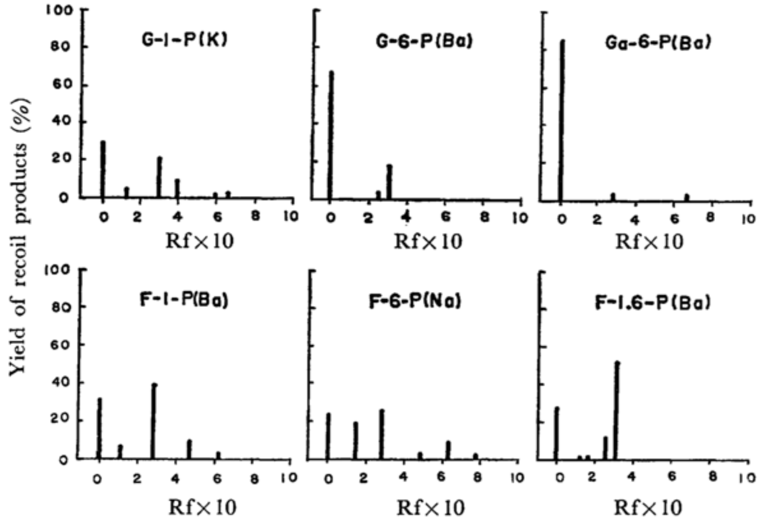

Fig. 1. Distribution of radioactive recoil products separated by paper chromatography.

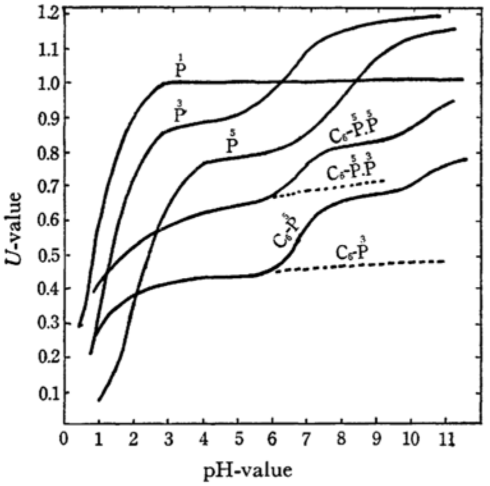

Fig. 2. *U*-pH curves of standard compounds.

$\overset{1}{P}$: $H_3PO_2$, $\overset{3}{P}$: $H_3PO_3$, $\overset{5}{P}$: $H_3PO_4$, $C_6$-$\overset{5}{P}$,$\overset{5}{P}$ : hexose containing two phosphate groups, $C_6$-$\overset{5}{P}$,$\overset{3}{P}$: hexose containing one phoshate and one phosphite groups, $C_6$-$\overset{5}{P}$: hexose containing one phosphate group, $C_6$-$\overset{3}{P}$: hexose containing one phosphite group.

water. In these cases of barium salts, it is considered that the radioactive recoil product formed with recoil atom in a target material was not completely developed with the solvent, but remained in target materials. Thus, paper chromatography was used for only an aid of paper electrophoresis, especially in recognition of recoil products formed from barium salts. The chromatogram patterns by electrophoresis are shown in Figs. 2 and 3.

The *U* value shows the mobility of the compound relative to that of hypophosphorous anion. The curves in figures show the change of the *U* values as a function of pH. The pH values at inflection points in the curves correspond to consecutive dissociation constants of a given compound, respectively.8) Thus, the curve of phosphoric acid has three points of inflection, phosphorus acid two and hypophosphorous acid only one. Hexose esters of phosphoric acids have two points which agree with those determined by a titration method.9)

Another feature of electrophoresis is the fact that the equivalent mobility (mobility/charge number) of the compound is reversely proportional to squear root of the molecular weight,10) providing that the shape and hydration of molecule are similar.[*3] Then, analysis of mobility*U*-pH curve of electrophoresis is useful for identification of the recoil products and to determine the structure of unknown products.11) Almost all inorganic phosphorus compounds show high *U*-values at a range of high pH-values while organic phosphorus compounds do not show high *U* value because of their higher molecular weight. Qualitative distinction between organic and inorganic phosphorus compounds can be easily made at the high pH region unless the organic compound is unstable. The electrophoretic behavior of the standard compounds is shown in Fig. 2. The mobility of the nonradioactive compound was determined by

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8) D. Waldron-Edward, *J. Chromatog.*, **20**, 556 (1965).

9) C. A. Bunton and H. Chaimovich, *J. Am. Chem. Soc.*, **88**, 4082 (1966).

10) V. Jokl, *J. Chromatog.*, **14**, 71 (1964).

11) J. T. Edward and D. Waldron-Edward, *J. Chromatog.*, **20**, 563 (1965).

[*3] Detailed study on the relationship between mobility and chemical form will be reported in *J. Chromatog.*, **36**, 215 (1968).

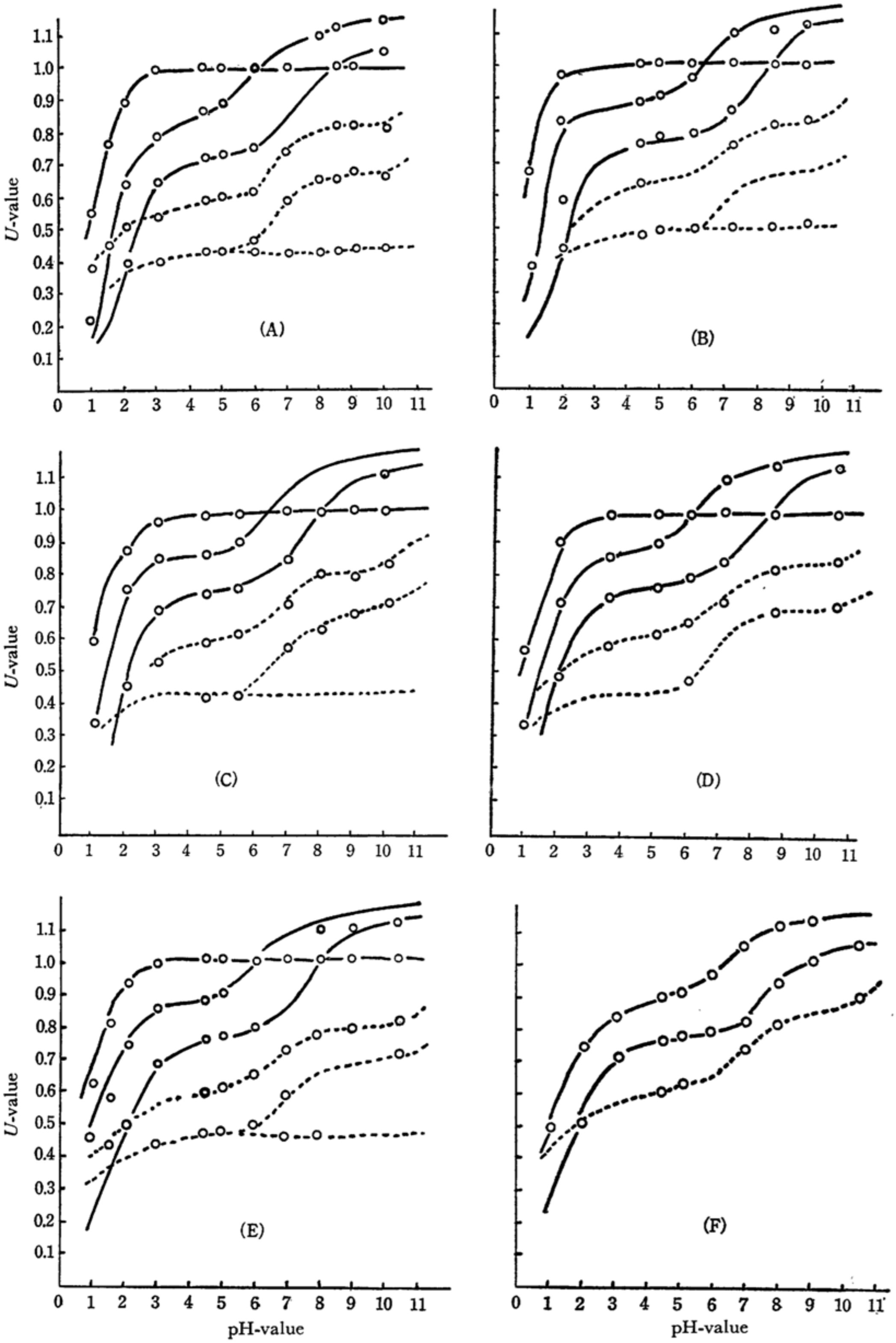

Fig. 3. Distribution of recoil products separated by paper electrophoresis.
A: G-1-P (K), B: G-6-P (Na), C: Ga-6-P (Ba),
D: F-1-P (Ba), E: F-6-P (Na), F: F-1,6-P (Ba).

activating the chromatographed paper with neutrons. The dotted line shows the *U*-pH curve speculated on the basis of the number of dissociable hydrogen in a molecule. The *U*-pH curves of recoil products in irradiated samples are shown in Figs. 3A—F, respectively for the target materials. Main recoil products or products clearly separated without overlapping of different fractions are qualitatively shown in Fig. 3. The dotted lines show the recoil products detected only in autoradiography. The results of quantitative analysis are shown in Fig. 4.

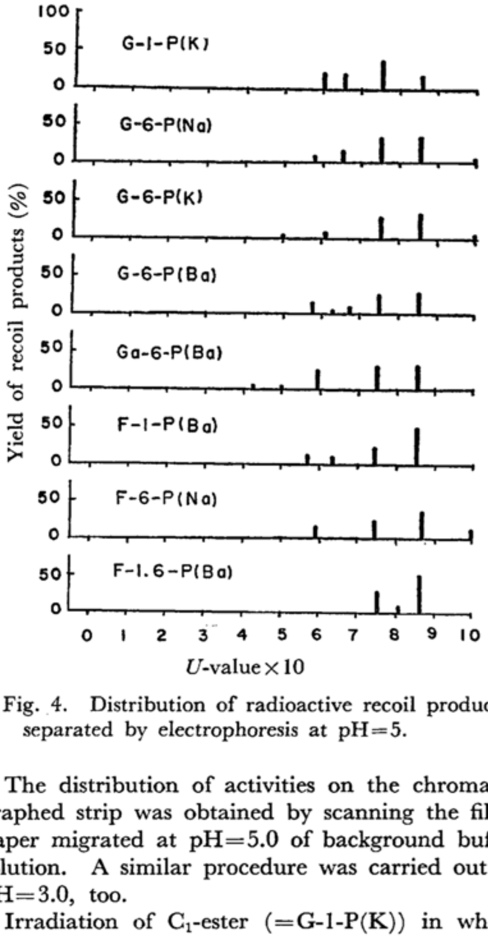

Fig. 4. Distribution of radioactive recoil products separated by electrophoresis at pH=5.

The distribution of activities on the chromatographed strip was obtained by scanning the filter paper migrated at pH=5.0 of background buffer solution. A similar procedure was carried out at pH=3.0, too.

Irradiation of $C_1$-ester (=G-1-P(K)) in which aldehyde group is blocked by phosphate group gave larger amounts of phosphoric acid ($U$=0.75) than that of phosphorous acid ($U$=0.85). On the other hand, irradiation of G-6-P yielded more phosphorous acid than phosphoric acid, independent of the kind of the salts. The tendency found in the case of G-6-P was also observed in irradiation of F-1-P(Ba), F-6-P(Na) or F-1,6-P(Ba). In any case, the yield of hypophosphorous acid was lower, comparing with that of other two inorganic oxyacids. It was concluded from the analysis of the distribution patterns that the yield of hypophosphorous acid is slightly larger in the case of sodium salts than in the case of potassium or barium. In irradiation of F-1,6-P(Ba), hypophosphorous acid was not found even in the autoradiogram.

It was found that P–P or P–O–P bond is included in other inorganic phosphorus compounds formed. As the P–O–P bond is easily formed by heating salts of phosphate,12) the compounds mentioned above can be expected to be formed also by heat evolved in the nuclear reactions of phosphorus or another metals such as sodium, barium or potassium.13) The yield of pyrophosphate ($R_f$= 0.05) estimated from a paper chromatogram was less than a few percents. On the contrary to pyrophosphate, the P–P compound was not formed by heating. When we used the background buffer solution described in Experimental section, the migration zone of $\overset{4}{\mathrm{P}}$–$\overset{4}{\mathrm{P}}$ compound overlapped with that of phosphorous or/and pyrophosphoric acids in a range of pH=4—5. Thus, in the case of identification of P–P compounds, a background buffer solution containing 0.05 M zinc acetate was specially prepared. Since Fenger14) has determined the relative mobiliteis of $\overset{2}{\mathrm{P}}$–$\overset{4}{\mathrm{P}}$ and $\overset{4}{\mathrm{P}}$–$\overset{4}{\mathrm{P}}$ compounds to be 0.55 and 0.45 at pH=4.2, respectively, the radioactive species located in ranges of 0.4 and 0.5 may be considered compounds including a P–P bond.

The relative mobility of target material itself was smaller at electromigration using usual background buffer solution (0.45 in the case of monoester and 0.60 in diester). Although retention product which is $^{32}$P-labeled target material was clearly found in autoradiography, the activities corresponding to that was less than a few percents, which was in agreement with the values theoretically calculated.2) Irradiating monoester, we always obtained a compound which behaved electrophoretically in a manner similar to F-1,6-P. This seems to be an organic diphosphorus compound which resulted from an attack of recoil phosphorous atom on the target materials. From the facts that isomerization of hexose by $\gamma$-rays or neutrons was not found and that the dihalogenide was obtained by neutron irradiation of monohalogenide,15,16) we can safely suppose the formation of diphosphorus compound in which the positions of phosphorus-32 will be probably statistically distributed. Branching of *U*-pH curves of irradiated materials shows the formation of phosphorous esters of hexoses. The characteristic results obtained from irradiated F-1,6-P(Ba) was the

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12) S. Ohashi, "Topics in Phosphorus Chemistry," Vol. 1, John Wiley, New York (1964), p. 113.

13) T. R. Sato and H. H. Strain, *J. Inorg. Nucl. Chem.*, **18**, 24 (1964).

14) J. Fenger, *Risö Report* (Denmark), No. 95 (1964).

15) J. C. W. Chien and J. E. Willard, *J. Am. Chem. Soc.*, **79**, 4872 (1957).

16) T. E. Gilroy, G. Miller and D. F. P. Shaw, *ibid.*, **86**, 5033 (1964).

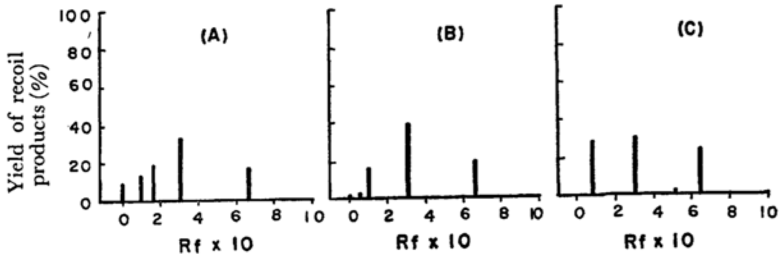

Fig. 5. Effect of water on recoil products.
(A) : glucose-6-phosphate, (B) : glucose-6-phosphate(20 mg) + $H_2O$(0.5m*l*)
(C) : glucose-6-phosphate sodium salt(17mg)+$H_2O$(0.1m*l*).

absence of $^{32}$P-labeled monophosphoric ester of hexose. Generally speaking, the $^{32}$P-labeled organic compounds containing the same number of phosphorus as target material or more than one are formed in the recoil event of phosphorus, and the oxidation state and the position of phosphorus in a molecule depend on chemical form of target materials.

The results of irradiated phosphate ester which is free from metallic element was shown in Fig. 5A.

In this case, lower oxidation products of phosphorus were obtained in abundance. An addition of a small amount of water to a target material also showed similar tendency as shown in Figs. 5B and C. Although the addition of water into the sample to be irradiated favored formation of hypophosphorous acid, the addition of excess of water, that is, dilution with water, lowerd.

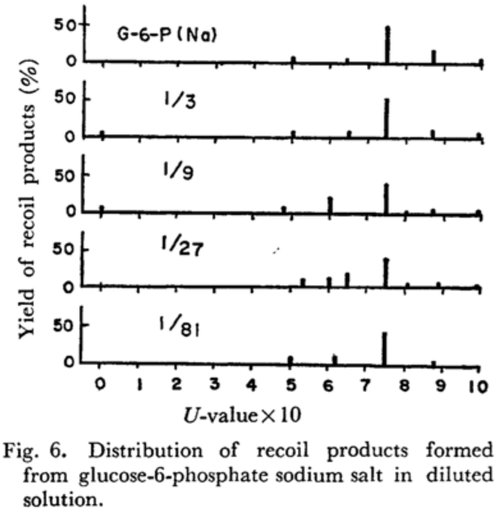

Fig. 6. Distribution of recoil products formed from glucose-6-phosphate sodium salt in diluted solution.

The effects of dilution with water on recoil products are shown in Fig. 6.

As the irradiation position in the reactor and the phase of target material were different from those of the irradiation without water, we can not strictly compare the results shown in Fig. 6 with that shown in Fig. 5, but we can say that the working of water molecule at the recoil event are not simple. Although it was said in early days that the presence of crystal water or of ammonium group in the irradiation sample favors formation of compounds in lower oxidation state, this experimental rule can be applied only within a limited range.

In the case of very diluted solution, the main product was phosphoric acid and the yields of phosphorous and hypophosphorous acids were very small. In addition to the characteristics mentioned above, an abnormal tailing in paper chromatogarm increased with increasing dilution. Such phenomenon was also observed in the irradiation of aqueous solution of phosphorus oxyacids.*4 The abnormal tailing may be due to the formation of an unstable complex by the recoil phosphorus atom with water molecules. A more detailed study on the dilution effect is in progress and will be reported in a near furure.

The authers are grateful to Professor S. Ohashi of Kyushu University for the generous gift of hypophosphoric acid.

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*4 The results of phosphorus oxyacids were presented at the 11th Symposium on Radiochemistry, Tokyo, October, 1967.