[CONTRIBUTION FROM THE CHEMISTRY DIVISION, OAK RIDGE NATIONAL LABORATORY]

Ester Interchange for the Preparation of P^{32}-Labeled Butyl Phosphates[1,2]

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Phosphorus-32 labeled butyl phosphate esters result from the ester interchange between tributyl phosphate and orthophosphoric acid-P^{32} at elevated temperatures. The amount of each ester at equilibrium is primarily dependent upon the reactant ratio, while the rate at which equilibrium is attained is mainly a function of the temperature and the acidity of the reaction mixture. Anion exchange methods were effective for the separation and identification of the acidic components. The stability of the esters to heat is reported.

The successful use of P^{32}-labeled phosphate esters assumes the stability of these esters. Relatively little is known of the ester interchange reactions between tertiary phosphates and phosphoric acid (I). The study of the reaction between tributyl phosphate (IV) and labeled phosphoric acid-P^{32} (I-P^{32}) was undertaken (1) to determine the limits within which tracer IV can be used reliably without fear of errors caused by interchange and (2) to investigate the feasibility of ester interchange as a method for producing the three butyl esters labeled with P^{32}.

Although no ester interchange was reported[3] for the reactions between several tertiary phosphate esters and phosphate ions in solution, Cherbuliez and Leber[4] presented evidence to show ester interchange when an intimate mixture of triethyl phosphate and phosphoric acid was heated. The ester interchange between tributyl phosphate (IV) and phosphoric acid-P^{32} (I-P^{32}) has been exploited to prepare the esters monobutyl dihydrogen phosphate (II), dibutyl hydrogen phosphate (III), and tributyl phosphate (IV) labeled with P^{32}. The over-all reaction can be summarized:

$$\underset{\text{I-}P^{32}}{H_3P^{32}O_4} + \underset{\text{IV}}{(C_4H_9O)_3PO} \longrightarrow$$

$$\underset{\text{I-}P^{32}}{H_3P^{32}O_4} + \underset{\text{II-}P^{32}}{C_4H_9OP^{32}(O)(OH)_2} + \underset{\text{III-}P^{32}}{(C_4H_9O)_2P^{32}(O)OH} + \underset{\text{IV-}P^{32}}{(C_4H_9O)_3P^{32}O}$$

Variables influencing the extent of the interchange are temperature, heating time, ratio of the reactants, and the history of the phosphoric acid (heating to form polyphosphoric acids). The conditions of the reaction can be varied sufficiently to direct a large portion of the P^{32} activity into any of the esters desired. When equilibrium (constant specific activity for all products) is attained the primary factor influencing the yield of each product is the starting ratio of reactants, while factors which determine when equilibrium will be reached are temperature and the acidity of the starting mixture (reactant ratio).

The method for separating linear polyphosphates by selective elution from an anion exchange resin[5] was successfully employed for the separation of the acidic ester products of the exchange reaction. The acid esters II and III, separated from the reaction mixture by solvent extraction methods, were identified by equivalent weight and the preparation of amine salts.

Essentially no interchange occurred at 100° under the conditions used (Test 1, Table I). This result, coupled with the findings of Lukovnikov, *et al.*,[3] indicates that ester interchange will not introduce serious errors in most experiments where tributyl phosphate-P^{32} is employed as tracer.

Extensive interchange did occur at higher temperatures. A mixture having a reactant ratio of 4 (4 mmoles of IV to 1 mmole of I-P^{32}) after being heated one hour at 206° yielded more than 4 times as much IV-P^{32} and more than 1.5 times as much III-P^{32} as at 176° (Tests 3 and 2, Table I). From a plot of the results of Tests 2, 4, and 5, Table I (Figure 1) and a comparison of the specific activities (calculated in these runs using the carrier phosphorus in IV, III, and the sum of I and II as determined by titration of the reaction products) it was found that the results of 1 hour at 206° (Test 3, Table I) were comparable to the equilibrium values obtained at 176°. Increasing the temperature from 176° to 206° greatly reduced the reaction time necessary for the interchange.

The above results obtained with a reactant ratio of 4 (IV/I) indicated that a larger ratio would improve the yield of labeled IV. Increasing the ratio from 4 to 40 (Tests 4 and 6, Table I) increased the yield of P^{32} in IV from 38 to 63%. The logical extension of the ratio to the point where the high specific activity phosphoric acid-P^{32} (without additional carrier) was heated with IV (ratio $\sim 2 \times 10^3$) yielded only 33% of the P^{32} in fraction IV (Test 7, Table I). The unexpectedly low result was thought to be due either to the lower acidity, or the

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[2] Presented at the 128th Meeting of the American Chemical Society, Minneapolis, Minn., Sept. 14, 1955.

[3] Lukovnikov, Medvedev, Neĭman, Nesmeyanov, and Shaverdina, *Doklady Akad. Nauk, S.S.S.R.*, **70**, 43 (1950); *Chem. Abstr.*, **44**, 4315 (1950).

[4] Cherbuliez and Leber, *Helv. Chim. Acta*, **35**, 644 (1952)

[5] Higgins and Baldwin, *Anal. Chem.*, **27**, 1780 (1955).

TABLE I

REACTION BETWEEN TRIBUTYL PHOSPHATE AND PHOSPHORIC ACID-P^{32}

EXPERIMENTAL CONDITIONS[a]			DISTRIBUTION OF P^{32}, (%)				
Test No.	I-P^{32} mmoles	Time, hours	I	II	Poly-phosphates	III	IV
1[b]	1	3	98.7	0.4	0.7	<0.1	0.0
2	1	1	20.4	38.5	3.7	29.0	8.9
3[b]	1	1	0.5	8.9	0.4	48.3	41.3
4	1	3	0.7	10.6	0.4	47.8	38.3
5	1	8	0.6	10.7	0.6	49.6	35.5
6	0.1	3	0.7	5.2	0.1	28.9	63.0
7	$\sim 2 \times 10^{-3}$	3	2.7	12.2	0.8	42.8	32.7
8	1[c]	1	9.8	34.4	3.5	39.7	12.9
9	0.01	2	8.7	33.8	0.9	43.6	12.1
10	0.01	3	3.2	13.3	0.8	42.9	36.3
11	0.01	8	1.3	2.9	0.2	12.9	84.0
12	2	3	1.8	20.0	1.6	58.0	18.6
13	8	3	75.8	0.0	24.4	0.0	0.0
14	4	3	20.3	37.7	13.0	25.6	2.6

[a] In each experiment, 4 mmoles of IV were used. [b] All tests were made at 176° except Test 1 (100°) and Test 3 (206°). [c] Tracer and carrier H_3PO_4 heated together for 25 hours at 176°. The P^{32} was distributed as 49% ortho-, 40% pyro-, 10% tri-, 2% tetra-, and 0.2% penta-phosphate.[5]

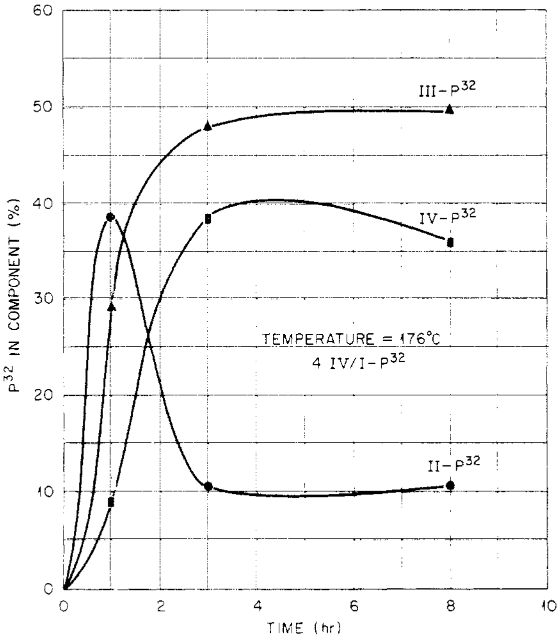

FIG. 1.—THE DISTRIBUTION OF P^{32} IN THE ESTERS AFTER HEATING MIXTURES OF TRIBUTYL PHOSPHATE AND ORTHOPHOSPHORIC ACID-P^{32}.

lesser probability of forming inorganic polyphosphates (as possible intermediates for further reaction), or both.

A mixture of polyphosphoric acids accelerated the interchange (compare Tests 8 and 2, Table I) but whether the formation of polyphosphoric acids in the reaction mixture was prerequisite for the interchange remained unknown.

An experiment was devised to shed further light on this problem. In this case, 40 mmoles of IV and 10 mmoles of I were heated under reflux for 4 hours at 176°. After cooling the reaction mixture, the apparatus was washed with methanol. Water in the mixture was determined by Karl Fisher reagent. The yield of 1 mg. of water, out of a possible yield of 90 mg., showed that the formation of inorganic polyphosphates was not essential to the interchange.

The effect of reaction time at a high reagent ratio (IV/I-P^{32} = 400) revealed that the reaction proceeded at a lower rate (Tests 9, 10, and 11, Table I). After 8 hours the yield of IV-P^{32} had reached 84%. The premise that an increase in the reactant ratio (in favor of tributyl phosphate) would result in greater yields of IV-P^{32} thus was substantiated; however, a longer heating time was required by the slower rate of interchange in the mixtures of lower acidity. The time required to attain equilibrium can be shortened by increasing the temperature.

The best yields of labeled tributyl phosphate resulted from the heating of ester with acid in a ratio of 40 to 1 at 206° for 1 hour. This procedure has now supplanted the method described previously[6] since the over-all yields are higher (85 + % *vice* 60%), the operations are much simpler, and the time required is shorter (time is important since P^{32} has a 14.3 day half-life and this frequently determines the experimental usefulness of a P^{32}-labeled compound).

Labeled dibutyl hydrogen phosphate in 50% yield resulted at the reactant ratio of 4 when the mixture was heated at 176° for 3 or 8 hours, or 206° for 1 hour. The theoretical reactant ratio to form III is 2, since the mixture would contain 2 butyl groups and 1 hydrogen for each phosphate group. After this mixture was heated 3 hours at 176° a 58% yield of III-P^{32} was obtained (Test 12, Table I). The remainder of the P^{32} was distributed largely between II and IV.

Reactant ratios were varied further in an attempt to improve the yield of monobutyl dihydrogen

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[6] Baldwin and Higgins, *J. Am. Chem. Soc.*, **74**, 2431 (1952).

phosphate-P^{32} (II-P^{32}). When the reactant ratio was reduced to 0.5 (theoretical for II) the reaction mixture foamed badly during the first half hour at 176° and in three hours complete dealkylation had taken place (Test 13, Table I), leaving only ortho- and poly-phosphoric acids. At a reactant ratio of 1 (3 hours at 176°, Test 14, Table I) 38% of the P^{32} appeared in II.

There was ample evidence to indicate that the esters themselves would undergo extensive changes when heated alone. A mixture of II-P^{32} and III-P^{32} was prepared by ester interchange and separated by the use of solvent extraction (to be independent of the ion exchange method). These were heated at 176° (Table II).

TABLE II

DISTRIBUTION OF P^{32} AFTER PYROLYSIS OF ESTERS

	ESTER			
	IV-P^{32}	III-P^{32}		II-P^{32a}
Time, hours	1	0.5	3	0.5
Temperature, ° C.	206	176	176	176
$H_3P^{32}O_4$, %	0.05	0.6	1.9	32.3
II-P^{32}, %	0.3	15.8	21.5	33.2
III-P^{32}, %	2.8	68.0	57.7	14.2
IV-P^{32}, %	95.7	16.1	18.9	0.8
Polyphosphates, %	<0.1	0.0	0.0	20.4

[a] 95% pure, contaminated with small amounts of $H_3P^{32}O_4$ and dibutyl hydrogen phosphate-P^{32}.

II-P^{32} at 176° in one-half hour underwent extensive dealkylation to form much $H_3P^{32}O_4$ and polyphosphates. Some interchange resulted in the formation of 14% III-P^{32} and a trace of IV-P^{32}; however, dealkylation was more extensive.

III-P^{32} was more stable to heating. One-half hour at 176° produced equivalent amounts of II-P^{32} and IV-P^{32} (16%) and only a trace of $H_3P^{32}O_4$. Continued heating for 3 hours produced slightly more interchange and a small amount of dealkylation.

IV-P^{32} was the most stable to heating, as expected. After 1 hour at 206° 95.7% of the IV-P^{32} was recovered. The III-P^{32} fraction accounted for 2.8%, II-P^{32} 0.3%, and $H_3P^{32}O_4$ 0.05%.

EXPERIMENTAL

Exchange. Anhydrous phosphoric acid (usually 1 mmole containing ~1 mc. of P^{32} as high specific activity tracer), prepared as previously described,[5] was dissolved in tributyl phosphate (4 or 40 mmoles) and the solution was heated in a refluxing liquid bath (boiling water, *p*-cymene, or tetralin for observed temperatures of 100°, 176°, or 206°, respectively). A platinum crucible served as the reactor for most of the tests. Later it was found that Pyrex vessels were satisfactory for most conditions.

Labeled esters (IV-P^{32}, III-P^{32}, and II-P^{32}) were also heated separately (see Table II). After the reaction the products were washed into a 250-ml. beaker with 30 ml. of CCl_4 and 50 ml. of water in several portions. Electrometric titration with standard sodium hydroxide revealed the amounts of III and the sum of II and I that were present in the mixture.

Tributyl phosphate-P^{32} (IV-P^{32}). After the above neutralization the CCl_4 layer was separated and the aqueous phase was extracted with an equal volume of CCl_4 to remove the small amounts of dissolved tributyl phosphate. The combined CCl_4 layers were washed with water, adjusted to 100 ml., and an aliquot was removed for determination of the *beta* activity. The aliquot, dissolved in dioxane-ethanol solution of such composition as to give a specific gravity of 1.00 for the sample to be counted, was assayed as described.[5] The labeled tributyl phosphate was easily isolated, after removal of the CCl_4, by distillation at 87–88° under less than 1 mm. pressure.

Found, $n_D^{25.8}$ 1.4220; Reported, n_D^{25} 1.4224.[7]

Estimation of the acids. The aqueous solution containing the sodium salts was diluted to 200 ml. (*p*H 9) and an aliquot containing about 7 μc. of P^{32} was passed through an anion exchange resin column (in the chloride form). The phosphates were retained at the top of the resin. Orthophosphoric acid (I) and II were eluted in the order named with 0.05 *M* HCl and III was eluted with 0.5 *M* KCl. A typical elution curve is shown in Figure 2. The methods used for the estimation of the yields and the identification by co-elution of the components with authentic specimens have already been described.[5]

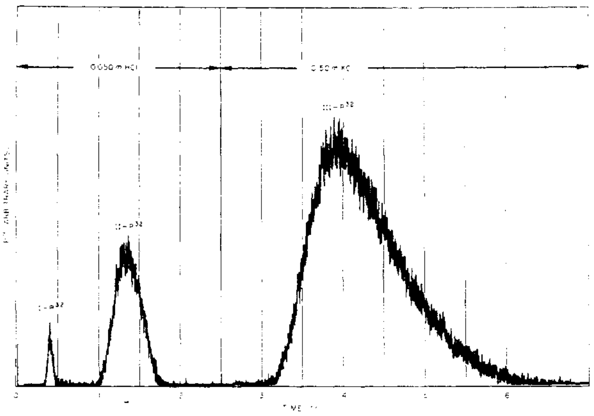

FIG. 2.—A TYPICAL ELUTION CURVE OF THE SEPARATION OF ACIDS: resin (Dowex 1, 100–200 mesh, 8x, Cl^- form) loaded with an aliquot at *p*H 9 containing 0.054 mg.-atom of P, 7.2 μc. of P^{32}; bed—0.85 sq. cm. × 15.5 cm.; rate—1.0 ml./min.

Frequently, a small portion (usually less than 4%) of the total activity was eluted by the 0.5 *M* KCl before III. A fraction of this portion was found to be pyrophosphate; the remainder was not identified.

Dibutyl hydrogen phosphate-P^{32} (III-P^{32}). Tributyl phosphate (8.00 g., 30 mmoles) and phosphoric acid-P^{32} (1.48 g., 15 mmoles containing P^{32}, 20.6 μc./mg.-atom of total P) were mixed well in a Pyrex flask fitted with a condenser. The flask and its contents were heated at 176° for 3 hours. The reaction products were treated as described above. The aqueous solution of the sodium salts (after extraction of the neutral ester) was acidified with a slight excess of sulfuric acid, then extracted with ether. (Stopcock grease was avoided in all operations since any grease dissolving in the ether would be retained during concentration of the product). The ether layer was washed five times with one-fifth volume of distilled water each time. Aliquots of the water washes were titrated electrometrically. The washing operation was completed when the titration curve of the

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(7) Evans, Davies, and Jones, *J. Chem. Soc.*, 1310 (1930).

fifth water wash showed only one inflection. The ether layer was filtered into a small flask, the ether was removed at the water pump, and III-P^{32} finally was dried at room temperature and 1 mm.

The yield of III-P^{32} (4.16 g., 19.8 mmoles, 20.2 μc. P^{32}/mmole) accounted for 44% of the total phosphorus in the experiment. Elution of an aliquot from anion exchange resin as described previously revealed 99.9% III-P^{32} and 0.1% II-P^{32}.

Anal. Calc'd for $C_8H_{19}O_4P$: MR, 51.0; Neut. equiv., 210. Found: n_D^{25} 1.4266; d_4^{25} 1.054; MR, 51.1; Neut. equiv., 211.

The *aniline salt* (20.4 μc. of P^{32}/mmole) melted at 71° when alone and when mixed with an authentic sample.

Dibutyl hydrogen phosphate (III). An authentic sample of III was prepared by hydrolysis of dibutyl chlorophosphate (0.74 mole) with 1 *N* sodium hydroxide (1.7 l.) at room temperature. The III was isolated as described above in 90% yield.

Anal. Calc'd for $C_8H_{19}O_4P$: MR, 51.0; Neut. equiv., 210. Found: n_D^{25} 1.4268; d_4^{25} 1.054; MR, 51.1; Neut. equiv., 211.

The *aniline salt* when crystallized from ligroin melted at 71°.

Anal. Calc'd for $C_{14}H_{26}NO_4P$: Equiv. wt.,[8] 303. Found: Equiv. wt., 303.

Monobutyl dihydrogen phosphate-P^{32} (II-P^{32}). The acidic aqueous solution, from which III-P^{32} had been removed, was extracted with ether continuously for two days. The ether extract was dried as before. The II-P^{32} (1.20 g., 7.8 mmoles, 23.1 μc. of P^{32}/mmole) accounted for 17% of the total phosphorus. Elution of an aliquot from an anion exchange resin as described above revealed 95.6% II-P^{32}, 0.3% I-P^{32}, and 4.6% III-P^{32}.

Anal. Calc'd for $C_4H_{11}O_4P$: MR, 32.4; Neut. equiv., 154. Found: n_D^{25} 1.4309; d_4^{25} 1.212; MR, 32.9; Neut. equiv., 159.

The *dibenzylamine salt* (23.8 μc. of P^{32}/mmole) melted at 120°, when mixed with an authentic specimen melting was observed at 120–121°.

Monobutyl dihydrogen phosphate (II). An authentic specimen of II was prepared by the hydrolysis of butyl dichlorophosphate (0.26 mole) in water at room temperature. Barium hydroxide then was added until the solution was pink to phenolphthalein. Due to the limited solubility of the barium salt of II (BaII) most of the product precipitated in this step. The solids were filtered off, and the BaII in solution in the filtrate was precipitated by the addition of alcohol. This crop (20 g., 26% yield) was recrystallized by dissolving in water, filtering, and precipitating by the addition of alcohol. No change in equivalent weight was noted after the first recrystallization.

Anal. Calc'd for $C_4H_9BaO_4P$: Equiv. wt.,[8] 145. Found: Equiv. wt., 145.

The barium salt of II was used as the form in which to store the acid. The acid was readily liberated by stirring 3 g. of BaII in 50 ml. of water with the H^+ form of Nalcite HCR at room temperature for 30 minutes when solution was complete. The solution was washed through a column of the same resin. The effluent was extracted with ether continuously for 2 days yielding 1.36 g. (86%) II.

Anal. Calc'd for $C_4H_{11}O_4P$: MR, 32.4; Neut. equiv., 154. Found: n_D^{25} 1.4306; d_4^{25} 1.224; MR, 32.5; Neut. equiv., 154.

The *dibenzylamine salt* when crystallized from ethyl acetate melted at 121°.

Anal. Calc'd for $C_{18}H_{26}NO_4P$: Equiv. wt., 176. Found: Equiv. wt., 179.

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(8) The equivalent weights of the salts were determined by the use of a column of Nalcite HCR in the hydrogen form. The salt, dissolved in a small amount of water (when necessary 70% ethanol may be used to dissolve it) was washed quantitatively through the resin. The effluent containing the liberated acid was titrated potentiometrically. See O. Samuelson, *Ion Exchangers in Analytical Chemistry,* John Wiley and Sons, Inc., New York, N. Y., 1953, pp. 117–135.