# 39. *Chemical Action of Ionising Radiations in Solution. Part XVIII.** *Action of X-Rays (200 kv) on α- and β-Glycerophosphate in Aqueous Solution.*

By G. Scholes, W. Taylor, and J. Weiss.

The chemical effects of *X*-rays (200 kv) on α- and β-glycerophosphate in aqueous solution have been investigated. Inorganic phosphate is liberated and, in the presence of molecular oxygen, formaldehyde is produced. α-Glycerophosphate gives some dihydroxyacetone phosphate, and β-glycerophosphate a phosphate ester which is labile to acid at elevated temperatures.

In quantitative studies the effects of pH and other factors have been examined. The mechanism of the radiolysis of glycerophosphate is discussed.

Although fairly extensive studies have been made of the chemical action of ionising radiations on aqueous solutions of a number of more complex organic phosphate compounds, *e.g.*, ribonucleic and deoxyribonucleic acids [1-3] and monoribonucleotides,[2,4] comparatively little has been reported on the subject for relatively simple organic phosphates. Acetyl phosphate and acetaldehyde are formed on irradiation of solutions of ethyl dihydrogen phosphate acid with *X*-rays,[5] the former product only when dissolved molecular oxygen is present. Using 1 Mev electrons and $^{60}$Co γ-rays, Wilkinson and Williams [6] showed that for a number of alkyl dihydrogen phosphates the yield of acyl compound decreases with increase in chain length, obviously owing to oxidation at sites other than the α-position. In a preliminary publication,[7] we reported the presence of labile phosphate esters in solutions of α- and β-glycerophosphate irradiated with *X*-rays. These two substances have now been investigated in more detail.

Although the chemical effects of ionising radiations in dilute aqueous solutions are due, primarily, to the reaction with H and OH radicals ($H_2O \rightsquigarrow H + OH$),[8] a small part of the radiation energy absorbed by the water results in the direct formation of molecular hydrogen and hydrogen peroxide,[9] $2H_2O \longrightarrow H_2 + H_2O_2$, and this " molecular yield " must be taken into account in the interpretation of the experimental results. The yields are, as usual, given in *G* values (molecules/100 ev). For 200 kv *X*-rays $G(Fe^{3+}) = 15{\cdot}5$ has been taken,[10] which leads to a maximum radical yield, $G(R)$, $= 3{\cdot}6$ and to a molecular yield, $G(M)$, $= 0{\cdot}5$.

## Results.

*α-Glycerophosphate.*—Irradiation with *X*-rays in aqueous solution leads to the formation of inorganic phosphate and, in addition, to a product (labile ester) giving rise to a post-irradiation release of inorganic phosphate which continues for about 30 hr. at room temperature at pH ~7. The labile ester was found to be strongly alkali-labile, being completely hydrolysed in N-sodium hydroxide in about 20 min. at room temperature. Hydrolysis of this intermediate was also affected by acid; in N-sulphuric acid at 100° the time of 50% change was approx. 33 min. Such behaviour to acid and base is exhibited by dihydroxyacetone phosphate [11,12] and by

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* Part XVII, preceding paper.

[1] Scholes, Stein, and Weiss, *Nature*, 1949, **164**, 709; Scholes and Weiss, *ibid.*, 1950, **166**, 640.

[2] Scholes and Weiss, *Exp. Cell. Res.*, 1952, **2**, Suppl. 2, 219; *Biochem. J.*, 1953, **53**, 567; 1954, **56**, 65.

[3] Butler and Simpson, Radiobiology Symposium, Liège, 1954.

[4] Daniels, Scholes, and Weiss, *Experientia*, 1955, **11**, 219.

[5] Scholes and Weiss, *Nature*, 1954, **173**, 267.

[6] Wilkinson and Williams, *J. Chim. phys.*, 1955, **52**, 600.

[7] Scholes and Weiss, *Nature*, 1953, **171**, 920.

[8] Weiss, *Nature*, 1944, **153**, 748; *Brit. J. Radiol.*, 1947, Suppl. 1, 56.

[9] Allen, *Ann. Rev. Phys. Chem.*, 1952, **3**, 57.

[10] Farmer, Rigg, and Weiss, *J.*, 1955, 582.

[11] Meyerhof and Lohmann, *Biochem. Z.*, 1934, **271**, 89.

[12] Kiessling, *Ber* , 1934, **67**, 869.

glyceraldehyde 3-phosphate,[13] both of which could conceivably be products of irradiation (by oxidation at $C_{(2)}$ and $C_{(3)}$ respectively). The following reaction distinguishes between these two substances.[11] On treatment with iodine in mildly alkaline solution, glyceraldehyde 3-phosphate is oxidised to β-phosphoglyceric acid, which is alkali-stable: dihydroxyacetone phosphate is not attacked under these conditions. When the irradiated α-glycerophosphate solutions were treated with iodine in this manner,.there was no loss of alkali-labile phosphate. Thus, we conclude that dihydroxyacetone phosphate is the labile product formed on irradiation.

Figs. 1 and 2 show some typical yield–dose plots for the production of inorganic phosphate and of total phosphate (*i.e.*, inorganic + labile phosphate) on irradiation of α-glycerophosphate solutions. The air-saturated solutions were irradiated in a thermostatically controlled vessel (at 24·5°), to ensure constant oxygen concentrations in the individual experiments. The oxygen-saturated solutions were irradiated at room temperature; it was shown that the radiation-induced reaction exhibited no significant temperature coefficient over the range 15—30°. It can be seen from Figs. 1 and 2, that the yields in oxygenated and in aerated solutions are *initially* identical; in air, the changes which occur as the total dose is increased can be attributed to the effects of the gradually diminishing oxygen concentration.

FIG. 1.

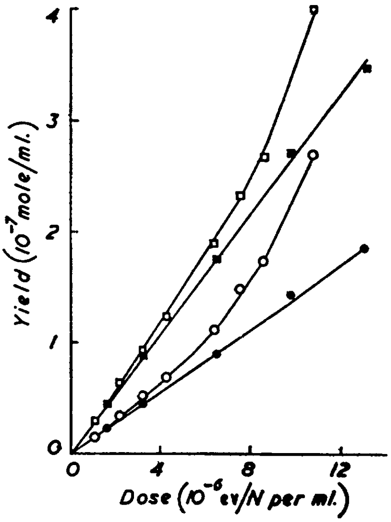
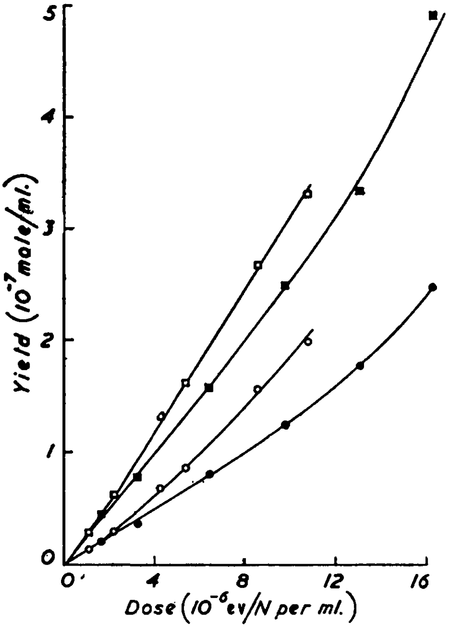


FIG. 2.



FIGS. 1 *and* 2. *Irradiation of α-glycerophosphate* ($10^{-2}$M-*sodium salt*) *with* X-*rays* (200 kv).
pH = 1·2 (Fig. 1) *and* 6·8 (Fig. 2).
*Inorganic phosphate*: ● *in the presence of* $O_2$ (1 *atm.*); ○ *in air* (*at* 24·5°).
Total phosphate (after alkali-treatment): ■ *in the presence of* $O_2$ (1 *atm.*); □ *in air* (*at* 24·5°).

From such yield–dose plots, the marked dependence on pH of the initial yields of inorganic and dihydroxyacetone phosphate in oxygen-saturated solutions has been obtained (Fig. 3); the largest amounts of dihydroxyacetone phosphate ($G \sim 1{\cdot}9$) are formed at pH 4—5; formation of total phosphate, however, is little dependent on pH, except in the more strongly acid solutions.

The yields of hydrogen peroxide in the irradiated α-glycerophosphate solutions, for oxygenated and aerated solutions at pH 1·2 and pH 6·8, are shown in Fig. 4. Under acid conditions, the peroxide yields are a linear function of dose. In the air-equilibrated solutions, however, the rate of formation changes after a total dose of about $6 \times 10^{-6}$ ev/*N* per ml. (" break point "),

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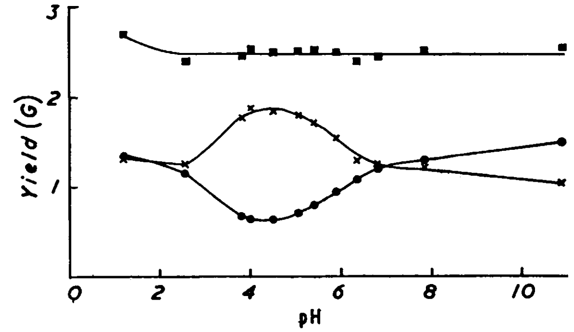
[13] Fischer and Baer, *Ber.*, 1932, **65**, 337, 1040; Ballou and Fischer, *J. Amer. Chem. Soc.*, 1955, **77**, 3329.

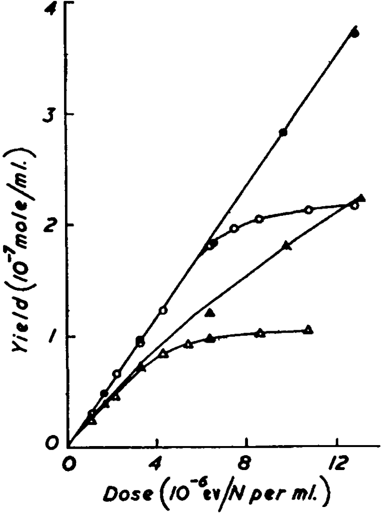
owing to the exhaustion of the dissolved oxygen. It is of interest to ascertain from these results whether all the available oxygen is eventually reduced to hydrogen peroxide, or whether some of it is incorporated into other radiation products. For this purpose, it is necessary to establish the position of the " break point " in the peroxide yield–dose plot; this, although not very well defined, may be taken as occurring after a dose of $7{\cdot}5 \times 10^{-6}$ ev/*N* per ml. (Fig. 4). If no decomposition had occurred before the " break point," the maximum yield of peroxide expected here would have been $2{\cdot}11 \times 10^{-7}$ mole per ml., *i.e.*, the same as that in solutions saturated with oxygen (1 atm.). Included in this value is the " molecular yield," which, up to this point, amounts to $0{\cdot}36 \times 10^{-7}$ mole per ml. Thus the peroxide formed by radical processes is $1{\cdot}75 \times 10^{-7}$ mole per ml. Under these experimental conditions (24·5°) the total available oxygen present in the solutions saturated with air was $2{\cdot}6 \times 10^{-7}$ mole per ml. Therefore, $0{\cdot}85 \times 10^{-7}$ mole per ml. (corresponding to $G \sim 1$) have entered into reactions involving the solute molecules and are eventually incorporated in the final products.

FIG. 3. pH-*Dependence of the* initial *yields of inorganic, of total, and of labile phosphate* (*dihydroxyacetone phosphate*) *from* α-*glycerophosphate* ($10^{-2}$M-*sodium salt*) *irradiated with* X-*rays* (200 kv) *in the presence of oxygen* (1 *atm.*).



● *Inorganic phosphate.* ■ *Total phosphate.*
× *Labile phosphate* (*dihydroxyacetone phosphate*).

FIG. 4. *Formation of hydrogen peroxide by irradiation of aqueous solutions of* α-*glycerophosphate* ($10^{-2}$M-*sodium salt*) *with* X-*rays* (200 kv).



*At* pH 1·2 :
● *in the presence of* $O_2$ (1 *atm.*)
○ *in air* (*at* 24·5°)
*At* pH 6·8 :
▲ *in the presence of* $O_2$ (1 *atm.*).
△ *in air* (*at* 24·5°).

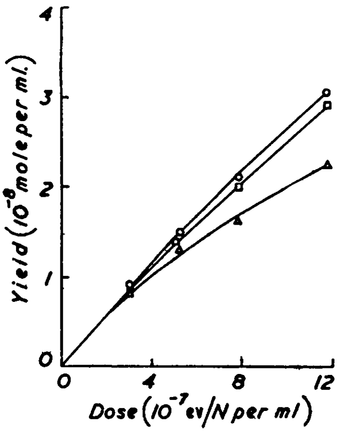
At pH 6·8 (Fig. 4), peroxide formation is no longer linearly dependent upon dose; the initial yields, nevertheless, seem to be the same in air- and oxygen-saturated solutions. In solutions of pH > 2, it was necessary to go to much lower doses in order to determine the *initial* peroxide yields. Fig. 5 shows a series of yield–dose plots for oxygen-saturated solutions, irradiated at various pH values, with doses varying from about 3 to $12 \times 10^{-7}$ ev/*N* per ml. It can be seen that, over the pH range investigated, the initial yield of hydrogen peroxide is independent of pH and corresponds to *G* 2·9.

Identical results were obtained, here, by either the titanium sulphate or the iodide method for determination of peroxide, indicating the absence of organic hydroperoxides in the irradiated solutions.

The dependence of the yields of inorganic phosphate, of total phosphate, and of hydrogen peroxide on the concentration of α-glycerophosphate, in solutions irradiated in the presence of oxygen, at pH 1·2, is shown in Fig. 6. A low dose ($1{\cdot}6 \times 10^{-6}$ ev/*N* per ml.) was used in order to avoid the possibility of secondary processes and, in fact, the yield–dose plots for solute concentrations as low as $2 \times 10^{-5}$M were linear up to a dose of $4{\cdot}5 \times 10^{-6}$ ev/*N* per ml. The amounts of hydrogen peroxide formed are constant over a wide range of solute concentration. The yields of total and inorganic phosphate, however, are a function of the concentration, the increase in total phosphate above about $10^{-3}$M being predominantly due to increased formation of the labile ester.

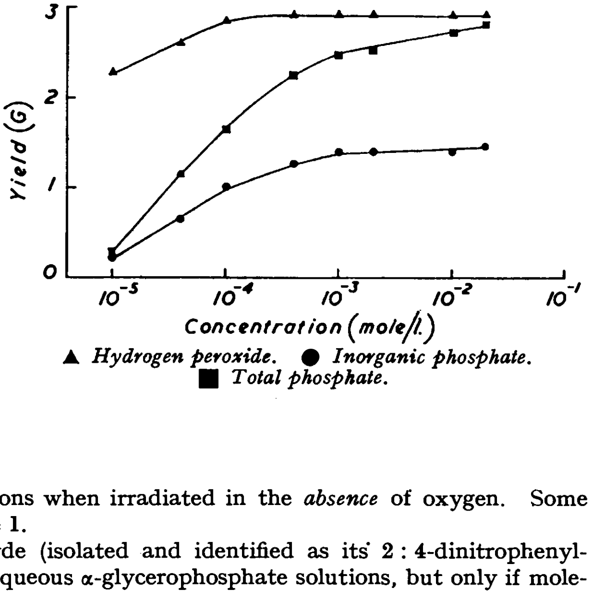
Irradiations have also been carried out with α-glycerophosphate solutions *in vacuo*. Yields of inorganic phosphate, total phosphate, and molecular hydrogen (Fig. 7) are linearly dependent upon dose. Greater amounts of hydrogen are produced in acid solution: at pH 1·2, $G(H_2)$ = 3·1; at pH 6·8, $G(H_2)$ = 1·3. The yield of hydrogen peroxide at pH 1·2 corresponds roughly to the molecular yield, while in neutral evacuated solutions the peroxide yields are very low, owing, presumably, to secondary decomposition. The absence of oxygen from these solutions leads to an increase in the yield of inorganic phosphate and to a decrease in that of labile phosphate; the total inorganic phosphate yields, however, are greater *in vacuo* than in presence of oxygen, particularly under acid conditions. The labile phosphate is decreased to a much greater extent in neutral solutions when irradiated in the *absence* of oxygen. Some comparative *G* values are given in Table 1.

FIG. 5. *Effect of* pH *on the* initial *yields of hydrogen peroxide produced by irradiation of aqueous solutions of* α-*glycerophosphate* ($10^{-2}$M-*sodium salt*) *with* X-*rays* (200 kv) *in the presence of oxygen* (1 *atm.*).



○ pH = 3·4. □ pH = 5·6.
△ pH = 8·5.

FIG. 6. *Dependence of* initial *yields on concentration of* α-*glycerophosphate* (*sodium salt*) *in aqueous solutions irradiated with* X-*rays* (200 kv) *in the presence of oxygen* (1 *atm.*) *at* pH 1·2. *Total dose* = 1·6 × $10^{-6}$ ev/*N*/*per ml*



▲ *Hydrogen peroxide.* ● *Inorganic phosphate.*
■ *Total phosphate.*

It has been found that formaldehyde (isolated and identified as its 2 : 4-dinitrophenylhydrazone) is formed on irradiation of aqueous α-glycerophosphate solutions, but only if molecular oxygen is present. The yields, determined by a paper-chromatographic technique described on p. 246, varied only slightly over the pH range investigated, falling from $G(CH_2O)$ ~ 0·8 at pH ~ 1 to ~ 0·6 at pH ~ 7; in these experiments doses of the order of 1 × $10^{-5}$ ev/*N* per ml. were used.

TABLE 1. *Comparison of the* initial *yields on irradiation with* X-*rays* (200 kv) *of* α-*glycerophosphate* ($10^{-2}$M-*sodium salt*) *in the presence of oxygen* (1 *atm.*) *and* in vacuo.

| | Initial yield (*G*) | | | |
|---|---|---|---|---|
| | In the presence of $O_2$ (1 atm.) | | *In vacuo* | |
| Product | pH 1·2 | pH 6·8 | pH 1·2 | pH 6·8 |
| Inorganic phosphate | 1·36 | 1·21 | 3·85 | 2·70 |
| Labile phosphate (dihydroxyacetone phosphate) | 1·34 | 1·25 | 1·05 | 0·40 |
| Total phosphate | 2·70 | 2·46 | 4·90 | 3·10 |

Tests, by Grant's method,[14] for the presence of formic acid in the irradiated solutions

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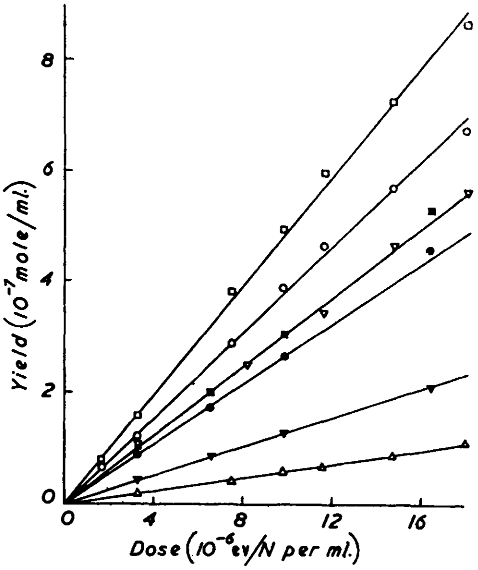
[14] Grant, *Ind. Eng. Chem. Analyt.*, 1946, **18**, 729; 1948, **20**, 267.

disclosed almost negligibly small amounts ($G < 0{\cdot}1$), and it is possible that these arise from some secondary oxidation of formaldehyde.

It is known [5,6] that certain phosphate esters containing the grouping $-CH_2{\cdot}O{\cdot}PO_3H_2$ can be oxidised to the corresponding acyl phosphate ($-CO{\cdot}O{\cdot}PO_3H_2$) on irradiation in aqueous solutions containing oxygen. Since this particular grouping occurs in α-glycerophosphate, solutions of this substance, irradiated in the presence of oxygen, were tested for acyl phosphate. A suitable method involves using, separately, the methods of phosphate determination described by Lowry and Lopez [15] and by Berenblum and Chain; [16] the former determines inorganic phosphate in the presence of acyl phosphate (*e.g.*, acetyl phosphate [15]), but under the conditions of the latter acyl phosphate is rapidly hydrolysed.[17] These two methods, applied to the irradiated α-glycerophosphate solutions, gave results which agreed within the limits of experimental error (±2%). Hence it was concluded that no *stable* acyl compound was produced in these solutions.

FIG. 7. *Irradiation of α-glycerophosphate* ($10^{-2}$M-*sodium salt*) *with* X-*rays* (200 kv) in vacuo.

| | pH 1·2 | pH 6·8 |
|---|---|---|
| *Total phosphate* ..................... | □ | ■ |
| *Inorganic phosphate* ............ | ○ | ● |
| *Hydrogen* ........................ | ▽ | ▼ |
| *Hydrogen peroxide* .............. | △ | • |



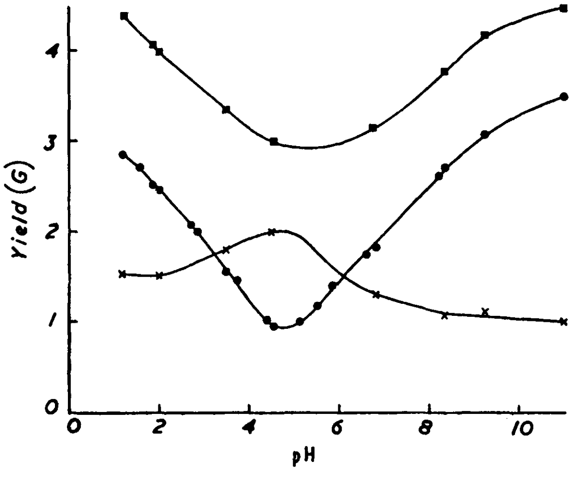


FIG. 8. pH-*Dependence of the* initial *yields of inorganic, of total, and of labile phosphate from β-glycerophosphate* ($10^{-2}$M-*sodium salt*) *irradiated with* X-*rays* (200 kv) *in the presence of oxygen* (1 *atm.*)

■ *Total inorganic phosphate* (*after acid treatment*).
● *Inorganic phosphate.*
× *Labile phosphate.*

β-*Glycerophosphate*.—A qualitative survey of the products formed on irradiation of β-glycerophosphate solutions, in the presence of oxygen, showed that inorganic phosphate was liberated,

[15] Lowry and Lopez, *J. Biol. Chem.*, 1946, **162**, 421.
[16] Berenblum and Chain, *Biochem. J.*, 1938, **32**, 295.
[17] Weil Malherbe and Green, *ibid.*, 1951, **49**, 286.

but that there was no significant post-irradiation release of phosphate, or any alkali-lability of the type found in irradiated solutions of the α-isomer. However, on hydrolysis at 100° in N-sulphuric acid, further amounts of inorganic phosphate were liberated; under these conditions, approx. 2 hr. were required to release the phosphate, the time of 50% change being ~36 min. This additional phosphate was not due to reactions between hydrogen peroxide and β-glycerophosphate since, in these experiments, an amount of hydrogen peroxide equivalent to that formed during the irradiation, was added to a blank solution, which was then hydrolysed in the same manner: the hydrogen peroxide had an observable effect,[18] but this was quite small. (In the quantitative experiments reported below correction was made for this by the addition of hydrogen peroxide to the blank.)

The presence of formaldehyde in solutions of β-glycerophosphate irradiated with *X*-rays in the presence of oxygen was established by isolation and identification as its 2 : 4-dinitrophenylhydrazone.

On irradiation *in vacuo*, the yields of inorganic phosphate were much greater than in the presence of oxygen as with α-glycerophosphate; on the other hand, the yields of acid-hydrolysable phosphate were almost negligible and no formaldehyde could be detected under these conditions.

Yield–dose plots for the formation of inorganic phosphate and of hydrogen peroxide in oxygenated and in aerated solutions, at various pH values, exhibited characteristics very similar to those obtained with the α-isomer. The "oxygen loss," at pH 1·2, in air-saturated solutions, as determined from the position of the "break point" in the peroxide yield–dose plots was a little higher, corresponding to $G \sim 1{\cdot}7$. The initial yields of hydrogen peroxide were obtained from dose-curves with low radiation doses; again, the peroxide yields were independent of pH and, here, corresponded to a value of $G(H_2O_2) = 2{\cdot}95$.

FIG. 9.

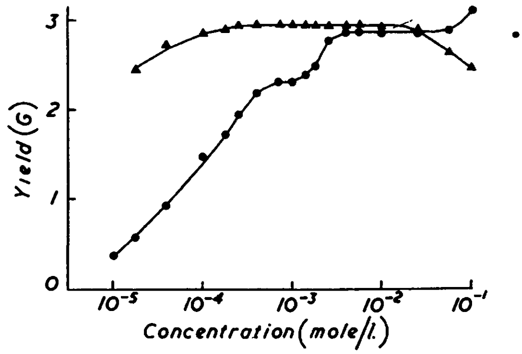
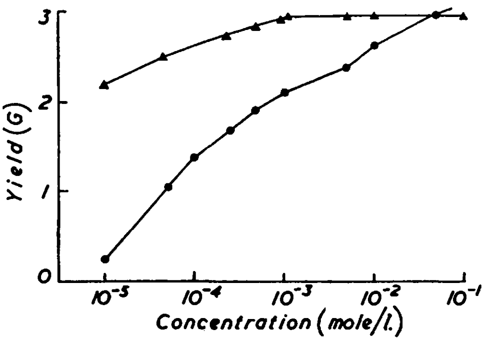


FIG. 10.



FIGS. 9 *and* 10. *Dependence of* initial *yields on concentration of β-glycerophosphate (sodium salt) in aqueous solutions irradiated with* X-*rays* (200 kv) *in the presence of oxygen* (1 *atm.*). FIG. 9: pH 1·2. FIG. 10: pH 8·2.

● *Inorganic phosphate.* ▼ *Hydrogen peroxide.*

Fig. 8 shows the influence of pH on the yields of inorganic, of total and of acid-labile phosphate. In contrast to α-glycerophosphate, the yield of total phosphate depends upon pH and exhibits a minimum around pH 5; in addition, rather high total yields ($G \sim 4{\cdot}5$) were obtained in the more acid and more alkaline pH regions. It is of interest, however, that the amounts of labile ester formed from both α- and β-glycerophosphate are very similar at all pH values.

Concentration–yield curves for β-glycerophosphate solutions, irradiated in the presence of oxygen, are given in Fig. 9 (pH 1·2) and Fig. 10 (pH 8·2). In acid solutions there is, around solute concentrations of $10^{-3}$M (cf. Fig. 9), a rather unusual break in the continuity of the phosphate yield curve and, at concentrations higher than $\sim 3 \times 10^{-2}$M, the hydrogen peroxide yields fall off noticeably. At pH 8·2, the initial yield of inorganic phosphate varies continuously with solute concentration, up to the highest concentrations (0·1M) employed.

The amounts of formaldehyde produced in solutions, irradiated in the presence of oxygen,

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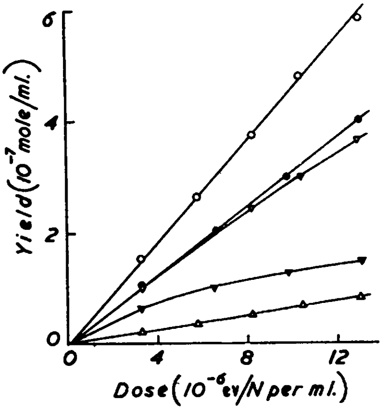
[18] Cf. Neumann, *Rec. Trav. chim.*, 1948, **67**, 101.

varied little over the pH range 1—8; the yield on the average was $G(CH_2O) \sim 0{\cdot}8$. The yields of formic acid were again very small ($G < 0{\cdot}1$).

Figs. 11—13 show some results obtained on the irradiation of β-glycerophosphate solutions *in vacuo*. In this case, the yield of hydrogen is not linearly dependent upon dose, particularly in neutral solution (Fig. 11); again, however, the *initial* yield is greater in acid solutions, at pH 1·2, $G(H_2) = 3{\cdot}1$, falling at pH 6·8 to $G(H_2) < 2$. Removal of oxygen, before irradiation, increases the yield of inorganic phosphate, but, under these conditions, the amount of acid-hydrolysable phosphate is so much reduced as to be almost insignificant (cf. Table 2 and Fig. 12). Fig. 12 shows that *in vacuo* there is a continuous decrease in inorganic phosphate with increasing pH, and, at all pH's investigated, the amounts of acid-labile phosphate were very small.

FIG. 11. *Irradiation of β-glycerophosphate* ($10^{-2}$M-*sodium salt*) *with* X-*rays* (200 kv) in vacuo.

| | pH 1·2 | pH 6·8 |
|---|---|---|
| *Inorganic phosphate* .................. | ○ | ● |
| *Hydrogen* .............................. | ▽ | ▼ |
| *Hydrogen peroxide* ..................... | △ | |



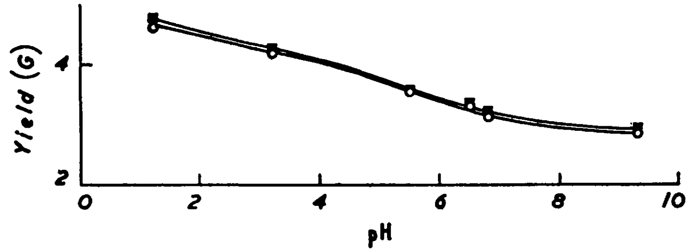


FIG. 12. pH-*Dependence of the yields of inorganic and of total phosphate from β-glycerophosphate* ($10^{-2}$M-*sodium salt*) *irradiated with* X-*rays* (200 kv) in vacuo. *Dose* = $1{\cdot}6 \times 10^{-6}$ ev/*N per ml.*

■ *Total phosphate* (*after acid treatment*).

○ *Inorganic phosphate.*

FIG. 13. *Dependence of* initial *yields on concentration of β-glycerophosphate* (*sodium salt*) *in aqueous solutions irradiated with* X-*rays* (200 kv) in vacuo *at* pH 1·2. *Dose* = $3 \times 10^{-6}$ ev/*N* per ml.

● *Inorganic phosphate.* ▼ *Hydrogen.*

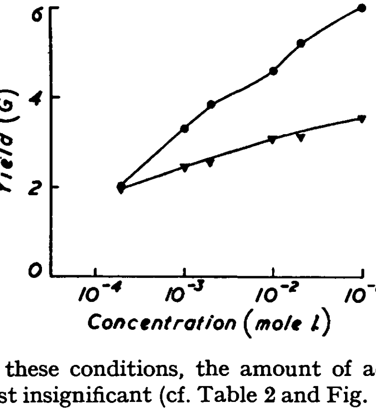


TABLE 2. *Comparison of the* initial *yields from the irradiation with* X-*rays* (200 kv) *of β-glycerophosphate* ($10^{-2}$M-*sodium salt*) *in the presence of oxygen* (1 *atm.*) *and* in vacuo.

| | Initial yield (*G*) | | | |
|---|---|---|---|---|
| | In the presence of $O_2$ (1 atm.) | | *In vacuo* | |
| Product | pH 1·2 | pH 6·8 | pH 1·2 | pH 6·8 |
| Inorganic phosphate ........................ | 2·85 | 1·85 | 4·60 | 3·12 |
| Labile phosphate (acid-hydrolysable)... | 1·55 | 1·30 | 0·15 | 0·10 |
| Total phosphate ............ | 4·40 | 3·15 | 4·75 | 3·22 |

At low pH (1·2) the yields of hydrogen and of inorganic phosphate, *in vacuo* (Fig. 13), depend on the concentration of β-glycerophosphate, up to the highest concentrations (0·1M) employed.

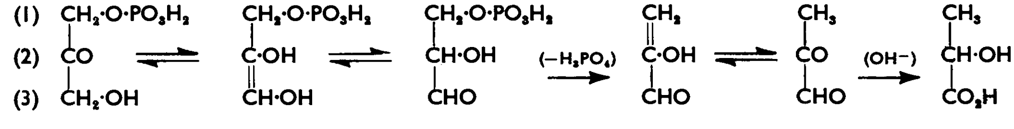
## DISCUSSION

*Formation of Labile Phosphate Ester.*—It has been shown that irradiation of α- and β-glycerophosphate in aqueous solution leads to marked chemical changes. Inorganic phosphate is liberated and various other products are formed.

From α-glycerophosphate, some dihydroxyacetone phosphate is produced, *viz.*:

$$HO{\cdot}CH_2{\cdot}CH(OH){\cdot}CH_2{\cdot}O{\cdot}PO_3H_2 \longrightarrow HO{\cdot}CH_2{\cdot}CO{\cdot}CH_2{\cdot}O{\cdot}PO_3H_2$$

The lability of dihydroxyacetone phosphate, particularly towards basic reagents, may be associated with an elimination reaction rather than with an ordinary hydrolytic process. Linstead, Owen, and Webb[19] have shown that compounds of the general type $YO{\cdot}CRR'{\cdot}CHR''X$, where X is an electron-attracting group and Y is, for example, $PO_3H_2$, Ac, or $NO_2$, can, in the presence of alkali, eliminate YOH with the formation of an unsaturated product $CRR'{:}CR''X$. It is known that dihydroxyacetone phosphate decomposes to lactic acid in alkaline solution[11] and this can be interpreted in terms of a β-elimination, subsequent to enolisation, as follows:



The product formed on irradiation of β-glycerophosphate is not alkali-labile, but is hydrolysed in acid solution at elevated temperatures. Oxidation at $C_{(1)}$ should (by analogy with α-glycerophosphate) lead to glyceraldehyde 2-phosphate, $HO{\cdot}CH_2{\cdot}CH(O{\cdot}PO_3H_2){\cdot}CHO$. This phosphate is not known, but it may be expected that its hydrolytic behaviour lies between those of glycollaldehyde phosphate[20] and glucose 2-phosphate,[21] which do not readily lose their phosphate groups in alkaline solution at ***room temperature*** (it is apparent from their structures that here no β-elimination can take place) but can do so, for example, in acid solution at elevated temperatures. We have attempted to separate the labile ester from the irradiated β-glycerophosphate solution by means of paper chromatography, using a method described by Cohen and McNair-Scott,[22] but, so far, without success. More experimental work is required to establish that glyceraldehyde 2-phosphate is a product of irradiation.

It has been shown that although oxygen is not essential for the production of dihydroxyacetone phosphate from α-glycerophosphate, greater yields were obtained in its presence. Chemically, this can be readily understood, since the formation of a keto-group from a secondary alcohol group requires two oxidation steps:

$$>CH{\cdot}OH + OH \longrightarrow >\dot{C}{\cdot}OH + H_2O \quad . \; . \; . \; . \; . \; . \; . \; . \quad (1a)$$

$$>\dot{C}{\cdot}OH + X{\cdot} \longrightarrow >CO + XH \quad . \; . \; . \; . \; . \; . \; . \; . \; . \quad (1b)$$

where X· represents an *oxidising* radical available under vacuum conditions. Under the present conditions, it is unlikely that X· is an OH radical since the reaction with the solute (reaction 1*a*) is probably the only important process into which the OH radicals enter.

In the presence of oxygen, hydrogen atoms, produced from the water, react with

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[19] Linstead, Owen, and Webb, *J.*, 1953, 1211.

[20] Fleury, Courtois, and Desjobert, *Bull. Soc. chim. France*, 1948, **15**, 694.

[21] Farrar, *J.*, 1949, 3131.

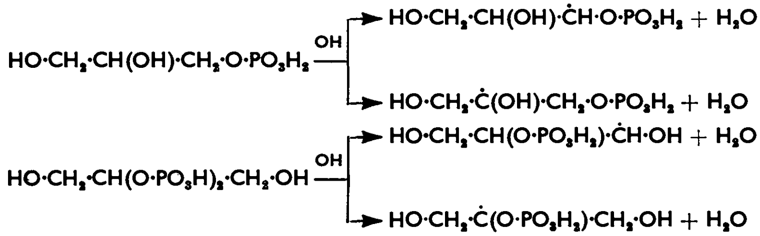
[22] Cohen and McNair-Scott, *Science*, 1950, **111**, 543.

oxygen ($H + O_2 \longrightarrow HO_2$), and thus the effective number of OH radicals may be increased by inhibiting back-reactions involving H atoms (*e.g.*, $H + OH \longrightarrow H_2O$). Possibly, and more importantly, the yield may be increased by a reaction of the initially formed organic radicals with molecular oxygen :

$$>\dot{C}\cdot OH \xrightarrow{O_2} >\overset{O\cdot O}{\dot{C}}\cdot OH \xrightarrow{X\cdot} >CO + XH + O_2 \quad . \; . \; . \; . \; . \; . \; . \quad (2)$$

that is, *via* an organic peroxy-radical, which can eventually lead to the carbonyl compound according to reaction (2). The rôle of oxygen in this case would be mainly inhibition of the reduction of the primarily formed organic radical. This reasoning, of course, also applies to the β-glycerophosphate; in this case, oxygen has a much more profound effect, the yields of labile ester in the absence of this substance being extremely low. This question is discussed below.

*Reaction Mechanism.*—We may, in the first instance, expect attack to take place at the carbon atom carrying the phosphate group. However, one cannot say *a priori*, that the neighbouring carbon atom will not also be attacked, particularly when it carries a primary or secondary alcohol group. Indeed, the experimental observation that dihydroxyacetone phosphate is produced on irradiation of α-glycerophosphate indicates the vulnerability of this atom. At this stage, therefore, we must represent the initial processes as follows :



$$HO\cdot CH_2\cdot CH(OH)\cdot CH_2\cdot O\cdot PO_3H_2 \xrightarrow{OH} \begin{cases} HO\cdot CH_2\cdot CH(OH)\cdot \dot{C}H\cdot O\cdot PO_3H_2 + H_2O \\ HO\cdot CH_2\cdot \dot{C}(OH)\cdot CH_2\cdot O\cdot PO_3H_2 + H_2O \end{cases}$$

$$HO\cdot CH_2\cdot CH(O\cdot PO_3H)_2\cdot CH_2\cdot OH \xrightarrow{OH} \begin{cases} HO\cdot CH_2\cdot CH(O\cdot PO_3H_2)\cdot \dot{C}H\cdot OH + H_2O \\ HO\cdot CH_2\cdot \dot{C}(O\cdot PO_3H_2)\cdot CH_2\cdot OH + H_2O \end{cases}$$

Attack at the 3-position in α-glycerophosphate has been excluded on the grounds that no glyceraldehyde 3-phosphate could be detected in the irradiated solutions.

(a) *In the absence of oxygen.* A striking feature here is the high yields of total phosphate, particularly at low pH; these can be so high that they cannot be accounted for solely by the number of OH radicals produced. It seems therefore that, under these conditions, hydrogen atoms as well as OH radicals can react with the substrate. In this respect it is important to note that, because of the equilibrium[23] $H + H^+ \rightleftharpoons H_2^+$, the hydrogen atom can, in acid solution, become an effective oxidising species. If, thus, we assume that the radical-ion $H_2^+$ can also oxidise the glycerophosphate molecule, one can account, at the same time, for the dependence of the total phosphate yield on pH (cf. Fig. 12).

In this respect, mention may be made of the pH-dependence of the formation of dihydroxyacetone phosphate *in vacuo*, which falls from a yield of $G \sim 1$ in acid solutions to a value of $G \sim 0{\cdot}3$ in neutral solutions (cf. Table 1). One may well expect such a change if the production of the labile ester is likewise associated with an $H_2^+$ reaction, *viz.* :

$$HO\cdot CH_2\cdot \dot{C}(OH)\cdot CH_2\cdot O\cdot PO_3H_2 + H_2^+ \longrightarrow HO\cdot CH_2\cdot CO\cdot CH_2\cdot O\cdot PO_3H + H^+ + H_2$$

The absence of significant amounts of labile phosphate in solutions of β-glycerophosphate, irradiated in the absence of oxygen, is somewhat puzzling. If the labile product in oxygenated solution is actually glyceraldehyde 2-phosphate, one would expect formation of this compound *in vacuo*, in yields at least equivalent to those of dihydroxyacetone phosphate. In these circumstances, therefore, one can only conclude that (*a*) oxidation of the intermediate β-glycerophosphate radical by the $H_2^+$ ion does not lead to glyceraldehyde 2-phosphate, or (*b*) the β-glycerophosphate radicals themselves are unstable and in

[23] Weiss, *Nature*, 1950, **165**, 728.

the absence of molecular oxygen (which may introduce some element of stability owing to the formation of a peroxy-radical) undergo hydrolysis with the liberation of inorganic phosphate.

On the basis of the above discussion we may represent the overall reaction processes *in vacuo* as follows (HRO ph = α- or β-glycerophosphate; DHAP = dihydroxyacetone phosphate; R′ = unspecified end products):

$$\mathrm{OH + HRO\,ph \longrightarrow \cdot RO\,ph + H_2O} \quad \ldots\ldots\ldots \quad (3)$$

$$\mathrm{H_2^+ + HRO\,ph \longrightarrow \cdot RO\,ph + H^+ + H_2} \quad \ldots\ldots\ldots \quad (4)$$

$$\mathrm{H_2^+ + \cdot RO\,ph \longrightarrow DHAP + H^+ + H_2} \quad \ldots\ldots\ldots \quad (5)$$

$$\mathrm{H_2^+ + \cdot RO\,ph \longrightarrow R' + H^+ + H_2 + H_3PO_4} \quad \ldots\ldots \quad (6a)$$

or

$$\mathrm{H_2^+ + \cdot ROH \longrightarrow R' + H^+ + H_2} \quad \ldots\ldots\ldots \quad (6b)$$

$$\mathrm{H + \cdot RO\,ph \longrightarrow R' + H_3PO_4} \quad \ldots\ldots\ldots \quad (7a)$$

or

$$\mathrm{H + \cdot ROH \longrightarrow R'} \quad \ldots\ldots\ldots \quad (7b)$$

$$\mathrm{\cdot RO\,ph \xrightarrow{H_2O} \cdot ROH + H_3PO_4} \quad \ldots\ldots\ldots \quad (8)$$

$$\mathrm{2\dot{R}OH \longrightarrow (ROH)_2} \quad \ldots\ldots\ldots \quad (9)$$

$$\mathrm{2H \longrightarrow H_2} \quad \ldots\ldots\ldots \quad (10)$$

**(Reaction 5 applies specifically to the α-compound only.)**

Reactions of the type (7*a* or *b*) must certainly be invoked, particularly in neutral solutions, where the yields of hydrogen gas are much less than could be obtained from recombination of the available hydrogen atoms [*viz.*, $G(\mathrm{H_2})_{\mathrm{max.}} = G(\mathrm{R})/2 + G(\mathrm{M}) = 2{\cdot}3$].

The decrease in the phosphate yields and also, to some extent, the decrease in the hydrogen yields with increasing pH, must be ascribed to a fall-off in the $H_2^+$ reactions. At higher pH's, in addition to an increase in the extent of the reductive processes (7*a* or *b*), recombination of hydrogen atoms, reaction (10), may also become more important.

In the suggested mechanism, for vacuum conditions, it has been assumed that the phosphate groups are removed before any possible interactions of organic radicals. This could account for the absence of the type of pH-dependence which was observed in solutions irradiated in the presence of oxygen (see below). The interaction of dephosphorylated substrate radicals may possibly lead to a dimer, reaction (9); some support for this seems to arise from the observation that some butane-2 : 3-diol is formed on irradiation of aqueous-ethanol solutions *in vacuo*.[24] As yet, no attempts have been made to look for the presence of polyhydric $C_6$ alcohols in the irradiated glycerophosphate solutions; some work in this direction may be rewarding.

(b) *In the presence of oxygen.* Outstanding among the results obtained under these conditions is the effect of pH on the initial yields (Figs. 3 and 8). In general, pH-dependence of the initial radiation yields would be observed if the pH of the solution influences any of the following processes: (i) primary radical formation from water; (ii) primary radical–substrate interaction; (iii) subsequent reactions involving the substrate radicals. There is, at present, no evidence that (i) is a significant factor. On the other hand, in the case of the glycerophosphates, the pH of the solution governs the dissociation of the phosphate group and it is, therefore, not unlikely that either (ii) and/or (iii) may be of some importance. However, it is very difficult to unravel, on any theoretical basis, the *effects* of such a dissociation equilibrium.

Until this aspect is better understood, little value is gained by setting down a scheme which accounts for the stoicheiometry and the mechanism at a single pH only. At this stage, it may also be pointed out that additional complications arise in the interpretation of the effects of the solute concentration (Figs. 6, 9, 10), a situation which also exists in

[24] Jayson, Ph.D. Thesis, Durham, 1955.

the experiments *in vacuo* (Fig. 13). We have already pointed out [25] that such a concentration–yield dependency cannot be fully interpreted on the earlier simple views of the mechanism of radiation reactions in aqueous systems.

In the presence of molecular oxygen, hydrogen atoms are converted into $HO_2$ radicals. If all of these react with the substrate according to $RH + HO_2 \longrightarrow R\cdot + H_2O_2$, the total yield of hydrogen peroxide should be $G(H_2O_2) = G(R) + G(M) = 4{\cdot}1$. The observed yield of peroxide [which is independent of pH (Fig. 5) and of glycerophosphate concentration over a fairly wide range (cf. Figs. 6, 9, and 10)] is less than this [$G(H_2O_2) \approx 2{\cdot}9$] and is not very different from that which would be expected if all the $HO_2$ radicals recombine according to reaction (13) [$G(H_2O_2) = G(HO_2)/2 + G(M) = 2{\cdot}3$]. One may conclude, therefore, that the primary attack on the solute is essentially concerned with OH radicals. Rather than dehydrogenate the substrate, $HO_2$ radicals could react in any of the following possible ways :

$$RO_2\cdot + HO_2 \longrightarrow RO_2^+ \ldots\ldots HO_2^- \tag{11a}$$

$$RO_2^+ \ldots\ldots HO_2^- + H_2O \longrightarrow ROH + H_2O_2 + O_2 \tag{11b}$$

$$RO_2\cdot + O_2^- \longrightarrow RO_2^- + O_2 \tag{12a}$$

$$RO_2^- + H^+ \rightleftharpoons RO_2H \tag{12b}$$

$$2HO_2 \longrightarrow H_2O_2 + O_2 \tag{13}$$

where $RO_2\cdot$ is a peroxy-radical derived from the substrate.

Again, when oxygen is present, primary dehydrogenation by OH radicals will be followed by the addition of molecular oxygen to form peroxy-radicals ($RO_2\cdot$). These may then interact as such, or react after dismutation, *e.g.*, according to $2RO_2\cdot \longrightarrow 2RO\cdot + O_2$.

Formaldehyde is presumed to arise as a result of oxidation at position 2 in both α- and β-glycerophosphate, the presence of oxygen being necessary. The reaction, in many ways, resembles the similar cleavage of the benzene ring [26] and of the indole ring of tryptophan.[27] It has been suggested that the latter processes also involve the intermediate formation of hydroxy-peroxy(or hydroxy-alkoxy)-radicals. Any hydroperoxides, if formed, must be very unstable, since none could be found in the irradiated glycerophosphate solutions. Some of the observed " oxygen loss " could be associated with the production of formaldehyde in this way.

The possibility that β-phosphoglyceric acid is the labile material from β-glycerophosphate is ruled out in view of the known resistance of this substance to acid hydrolysis.[28]

## EXPERIMENTAL

*Irradiations.*—The solutions were irradiated with *X*-rays generated from a Victor Maximar therapy tube operating at 200 kv and 15 mA, the irradiation arrangements being similar to those described in Part I.[29] The dose rate was $3{\cdot}8 \times 10^{-7}$ ev/*N* per ml. per min. as determined by the ferrous dosimeter, $G(Fe^{3+})$ being taken [10] as 15·5. To determine the initial yields of hydrogen peroxide, the dose rate was cut down to $1{\cdot}3 \times 10^{-7}$ ev/*N* per ml. per min. by placing the irradiation vessel on a plastic stand at a distance from the displacement cone. For the experiments at controlled temperatures, a special double-walled vessel [24] was used, water from a large thermostatically controlled reservoir being pumped through the outer jacket throughout the period of irradiation; the dose rate in this case was $2{\cdot}2 \times 10^{-7}$ ev/*N* per ml. per min.

Solutions were prepared in triply-distilled water, ordinary distilled water being redistilled in an all-glass still from alkaline permanganate and then from phosphoric acid. Where it was necessary to adjust the pH of the solutions, sulphuric acid (" AnalaR ") and sodium hydroxide (" AnalaR ") were used.

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[25] Johnson, Scholes, and Weiss, *Nature*, 1956, **177**, 883.

[26] Stein and Weiss, *J.*, 1949, 3254; Daniels, Scholes, and Weiss, *J.*, 1956, 832.

[27] Jayson, Scholes, and Weiss, *Biochem. J.*, 1954, **57**, 386.

[28] Meyerhof and Kiessling, *Biochem. Z.*, 1933, **264**, 40; Warweg and Stearns, *J. Biol. Chem.*, 1936, **115**, 567.

[29] Farmer, Stein, and Weiss, *J.*, 1949, 3241.

Irradiations were carried out in the presence of air or of oxygen (the solutions being saturated by passage of oxygen for about 15 min.) and also after evacuation as follows: The solution was pumped out by means of a two-stage "Hyvac" oil pump, shaken for a few minutes, and next morning evacuated with a mercury diffusion pump.

*Materials.*—Sodium β-glycerophosphate (supplied by British Drug Houses Ltd.) was recrystallised from water before use. The purity of the recrystallised material, determined by Burmaster's method,[30] was >99%.

Sodium α-glycerophosphate (supplied by Messrs. Boots Ltd.) was found to be 98% pure by the Burmaster technique and was used without further purification.

*Identification and Determination of Formaldehyde.*—The irradiated solution (50 ml.) was treated with 10 ml. of a 0·25% (w/v) solution of 2 : 4-dinitrophenylhydrazine in 30% (v/v) perchloric acid and then kept for 45 min. at room temperature, an orange precipitate (mainly osazone) being slowly formed. The solution was extracted with carbon tetrachloride (2 × 20 ml.) and the combined tetrachloride extracts filtered to remove any osazone. The extract was then washed with 10% aqueous sodium carbonate (10 ml.), 30% perchloric acid (10 ml.), and finally water (twice). After being dried ($Na_2SO_4$), the organic solvent was removed *in vacuo* and the residue taken up in ~1 ml. of carbon tetrachloride. This final solution was used for paper chromatography on Whatman No. 1 paper with light petroleum–methanol–water (10 : 9·5 : 0·5) as solvent.[31]

The hydrazone from solutions of α- and β-glycerophosphate, irradiated in the presence of oxygen, gave spots at $R_F$ values 0·46 and 0·63, comparing well with the $R_F$ 0·45 and 0·64 for synthetic formaldehyde 2 : 4-dinitrophenylhydrazone run on the same paper. Further confirmation that the derivative from the irradiated solutions was formaldehyde 2 : 4-dinitrophenylhydrazone was obtained by comparison of the spectrum of an ethanol eluate of the main spot ($R_F$ 0·46) with that from the corresponding spot from the synthetic sample. In the solutions irradiated *in vacuo* no formaldehyde could be detected; the 2 : 4-dinitrophenylhydrazine derivatives ran further on the paper than the synthetic sample.

Formaldehyde was measured quantitatively by this method as follows: An accurately known volume of the solution of the hydrazone in carbon tetrachloride was applied to the paper, by using an "Agla" micrometer syringe which was calibrated to deliver volumes down to 0·01 ml. After development of the chromatogram, the main spot was eluted with ethanol (10 ml.), and the absorption measured at 348 mμ. Calibration was made by chromatography of known amounts of pure formaldehyde 2 : 4-dinitrophenylhydrazone and elution in the same manner.

*Quantitative Methods.*—(a) *Inorganic phosphate.* Berenblum and Chain's spectrophotometric method [16] was used, with the precautions indicated earlier.[32] Lowry and Lopez's method [15] was used without further modification. In each case calibration was made with "AnalaR" disodium hydrogen phosphate.

(b) *Hydrogen peroxide.* This was estimated with titanium sulphate by Eisenberg's method,[33] with a "Spekker" colorimeter and blue-violet filter (Kodak 601), and by iodide according to Hochenadel's procedure,[34] with a Unicam S.P.500 spectrophotometer and measurement of optical density at 353 mμ. Calibration was made with pure (unstabilised) hydrogen peroxide, standardised with potassium permanganate.

(c) *Hydrogen.* The gas-analysis apparatus used was similar to that described by Stein and Weiss.[35] After irradiation *in vacuo*, the gases were pumped from the vessel, through a liquid-air trap, by means of a semiautomatic Töpler pump, and the hydrogen was determined manometrically after combustion in the presence of excess of oxygen.

Our thanks are offered to the North of England Council of the British Empire Cancer Campaign and to the Rockefeller Foundation, for financial support. We are very grateful to Messrs Boots Ltd. (Nottingham) for a gift of sodium α-glycerophosphate.

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[*Received, June 14th,* 1956.]

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[30] Burmaster, *J. Biol. Chem.*, 1946, **164**, 233.
[31] Cf. Meigh, *Nature*, 1952, **170**, 579.
[32] Daniels, Scholes, and Weiss, *J.*, 1956, 3771.
[33] Eisenberg, *Ind. Eng. Chem. Analyt.*, 1943, **15**, 327.
[34] Hochenadel, *J. Phys. Chem.*, 1952, **56**, 587.
[35] Stein and Weiss, *J.*, 1949, 3245.