734. *Chemical Action of Ionising Radiations in Solutions. Part XVI.* Formation of Labile Phosphate Esters from Purine and Pyrimidine Ribonucleotides by Irradiation with X-Rays in Aqueous Solution.*

By M. Daniels, G. Scholes, and J. Weiss.

Irradiation of purine and pyrimidine ribonucleotides in aqueous solution with *X*-rays (200 kv) results in the formation of intermediate labile phosphate esters which lead to a post-irradiation release of inorganic phosphate. This release of phosphate, which occurs after irradiation in the presence or absence of oxygen, follows a first-order rate law and is strongly base-catalysed. It is suggested that the radiation-induced lability of the nucleotides is due to the formation of activating carbonyl groups in the sugar components.

Previous studies [1] of the action of *X*-rays (200 kv) on purine and pyrimidine ribonucleotides in aqueous solutions have shown that a number of chemical reactions take place. Both the base and the pentose constituents are attacked, ammonia and inorganic phosphate being formed. Some free base is also liberated but no nucleoside or free pentose is produced. Such chemical changes induced by ionising radiations in dilute aqueous systems are due, in the first instance, to the production of free radicals from the water according to the net process : [2]

$$H_2O \longrightarrow\!\!\!\!\!\sim\!\!\!\!\!\rightarrow H + OH \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

The radicals thus formed may then react with the solute. Because of the possible rôle of labile phosphate esters in the radiation-induced degradation of nucleic acids in aqueous systems,[3] we have studied the effects of *X*-rays on a number of phosphate esters. The ribonucleotides are of obvious interest from this point of view and in the following we report our observations with adenylic and cytidylic acid, which are typical of purine and pyrimidine ribonucleotides respectively.

Results

Irradiations were carried out on 0·1% (w/v) nucleotide solutions (~0·003M) adjusted to pH 6·6—7·0. In all cases, in addition to the formation of inorganic phosphate during irradiation, there was a post-irradiation release of inorganic phosphate, which at 25° and pH ~7 continued for about 30 hours. Hydrolysis of the labile ester was markedly base-catalysed, treatment with N-sodium hydroxide for 20—30 min. at room temperature being sufficient to liberate the phosphate completely; this alkali-lability provided a quick and convenient method for the estimation of this compound.

Figs. 1—3 show yield–dose plots for the production of inorganic phosphate and of the labile phosphate from aqueous solutions of the (commercial) nucleotides irradiated both in the presence and in the absence of oxygen. The labile material is a primary product of irradiation, as shown by the fact that the yield is initially proportional to the dose; the yields (G = molecules/100 ev) are summarised in Table 1. For the purine-3′ and the pyrimidine-3′ nucleotide the yields of

* Part XV, *J.*, 1956, 832.

[1] Scholes and Weiss, *Exp. Cell. Res.*, 1952, *Suppl.* **2**, 219; *Biochem. J.*, 1953, **53**, 567.
[2] Weiss, *Nature*, 1944, **153**, 748; *Brit. J. Radiol.*, 1947, *Suppl.* **1**, 56.
[3] Scholes and Weiss, *Nature*, 1953, **171**, 920.

labile phosphate are decreased on removal of oxygen from the solution before irradiation; with adenosine-5′ phosphate about equal amounts are formed *in vacuo* and in the presence of oxygen. 3′- and 5′-Nucleotides differ in their dependence of yield of inorganic phosphate on radiation dose: with the (commercial) 3′-nucleotides there is an induction-like period, whereas the production of inorganic phosphate from the 5′-compound is initially a linear function of dose.

In solutions irradiated in the presence of oxygen, hydrogen peroxide could be readily

FIG. 1.

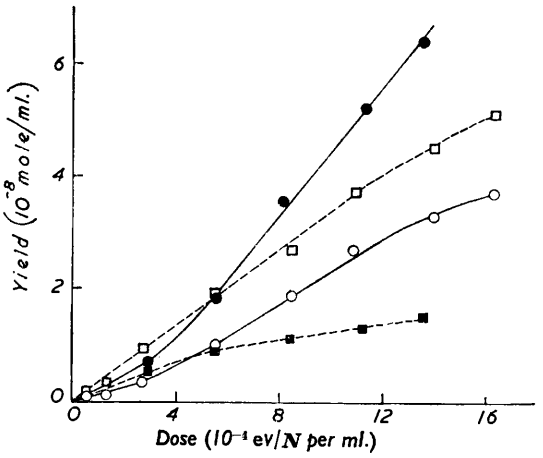


FIG. 2.

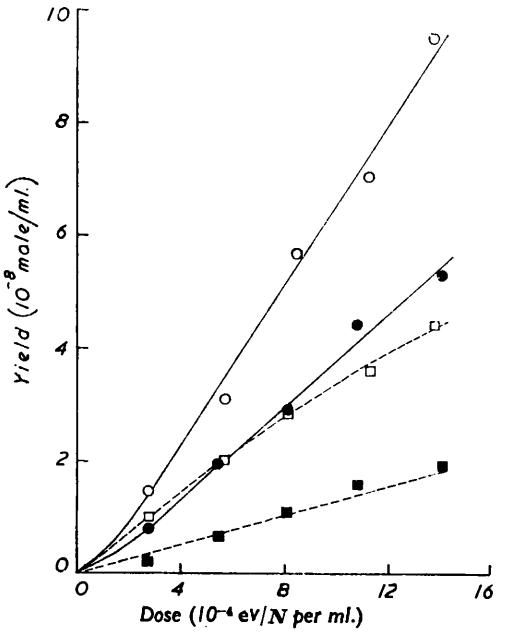
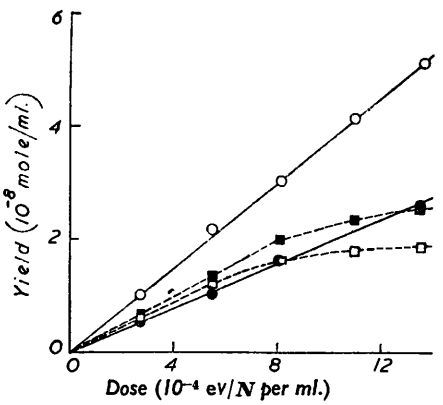


FIG. 3.



FIGS. 1—3. *Formation of inorganic phosphate and labile phosphate by X-irradiation* (200 kv) *of aqueous solutions* (0·1% w/v) *of (commercial)* (*Fig.* 1) *adenosine-3′ phosphate at* pH 6·7, (*Fig.* 2) *cytidine-3′ phosphate at* pH 6·9, *and* (*Fig.* 3) *adenosine-5′ phosphate at* pH 6·6.

Inorganic phosphate { ○ *in presence of oxygen* (1 *atm.*). ● in vacuo.

Labile phosphate ... { □ *in presence of oxygen* (1 *atm.*). ■ in vacuo.

detected: up to total doses of about 8×10^{-6} ev/N per ml. there was a linear dependence and the initial yield $G(H_2O_2)$ was of the order of 1·5. *In vacuo*, on the other hand, much smaller quantities of peroxide were formed, in yields [$G(H_2O_2) \simeq 0{\cdot}5$] comparable with the so-called "molecular yield."[4] Finally, the yields of hydrogen gas, in solutions irradiated *in vacuo*,

TABLE 1. *Initial yield of labile phosphate produced on irradiation of some ribonucleotides* (*commercial*) *in aqueous solution* (0·1% *w/v*) *with* X-*rays* (200 kv) *at* pH = 6·6—7·0.

	Yield (G = molecules/100 ev)	
Nucleotide	In presence of O_2 (1 atm.)	*In vacuo*
Adenosine-3′ phosphate	0·34	0·18
Cytidine-3′ phosphate	0·36	0·13
Adenosine-5′ phosphate	0·23	0·25

were found to be initially linear with a smooth tail-off at higher doses; in all cases the initial yield $G(H_2) \simeq 1{\cdot}0$.

[4] Johnson and Allen, *J. Amer. Chem. Soc.*, 1952, **74**, 4147.

It has been found that the rate of post-irradiation release of inorganic phosphate follows a first-order law. Table 2 shows the values of the rate constants for this process at 25° and pH ~7; the rate is the same whether the solutions are irradiated in the presence of oxygen or *in vacuo*, indicating that the same labile product is produced under both conditions. One may conclude, on this evidence alone, that the production of an organic hydroperoxide (RO_2H) is not a necessary prerequisite for the formation of the labile material since, in general, hydroperoxides should only be formed if molecular oxygen is present during irradiation. It has been found, in the case of the purine nucleotides, that the same peroxide values are obtained by both the titanium and the iodide method of estimation. Since hydroperoxides, in general, can oxidise iodide but do not necessarily produce a colour with the titanium sulphate reagent, this suggests that no stable hydroperoxide is produced in these solutions. With cytidylic acid, on the other hand, irradiation in the presence of oxygen leads to the formation of a hydroperoxide ($G \simeq 0{\cdot}3$) which could be detected by this procedure; this indicates a specific effect due to the base and it is not unreasonable to suppose that the pyrimidine, but not the purine, can form a hydroperoxide.

None of these findings is compatible with the assumption that this post-irradiation release of phosphate is the result of a (slow) attack by the hydrogen peroxide in the solution on some relatively stable product of irradiation. If this was the case, phosphate liberation should take place according to second-order kinetics. The hydrogen peroxide does, in fact, disappear during the post-irradiation period but in accordance with a first-order law. In order to show conclusively that hydrogen peroxide is not responsible for the observed effect, experiments were carried out in which the hydrogen peroxide in the irradiated solution was removed by freeze-drying. Some typical results are recorded in Table 3. A single freeze-drying reduced

TABLE 2. *Rate constants (k) at 25° of post-irradiation release of inorganic phosphate from 0·1% (w/v) solutions of ribonucleotides (commercial) irradiated with X-rays (200 kv) at pH 6·8.*

Nucleotide	10^3k (min.$^{-1}$) In presence of O_2 (1 atm.)	10^3k (min.$^{-1}$) *In vacuo*
Adenosine-3′ phosphate	1·87	1·75
Cytidine-3′ phosphate	2·05	2·06
Adenosine-5′ phosphate	1·30	1·27

TABLE 3. *Freeze-drying experiments with solutions of adenosine-3′ phosphate (0·1% w/v) (commercial sample) irradiated with X-rays (200 kv) in the presence of oxygen (1 atm.). Total dose = $8{\cdot}4 \times 10^{-6}$ ev/N per ml. Hydrolyses carried out at pH 6·8 and 25°.*

Irradiated solution	H_2O_2 (10^{-6} mole per 100 ml.)	Inorganic phosphate before hydrolysis (10^{-6} mole/100 ml.)	Inorganic phosphate after hydrolysis (10^{-6} mole/100 ml.)	Rate of post-irradiation phosphate release ($10^{-3}k$, min.$^{-1}$)
Untreated	12·5	1·9	4·1	1·87
After 1st freeze-drying	2·0	2·0	4·15	1·80
	1·75	1·8	4·10	—
After 2nd freeze-drying ...	0·5	1·9	4·18	1·92

the hydrogen peroxide concentration to about one-sixth of its original value and a small quantity of " residual " peroxide remained after a second treatment. This was found to be the case when the same freeze-drying procedure was carried out on an unirradiated solution of adenylic acid containing comparable amounts of hydrogen peroxide. It can be seen (cf. Table 3) that the rate, as well as the amount, of phosphate release is unaffected by freeze-drying and both are thus independent of the amount of hydrogen peroxide present. One must conclude, therefore, that the post-irradiation phosphate is produced directly from a labile compound formed during irradiation.

It has been shown [5,6] that certain phosphate esters containing the grouping $-CH_2{\cdot}O{\cdot}PO(OH)_2$ can be oxidised to the corresponding acyl phosphate [$-CO{\cdot}O{\cdot}PO(OH)_2$] on irradiation in aqueous solutions containing oxygen. Since this particular grouping occurs in adenosine-5′ phosphate, solutions of this substance which had been irradiated in the presence of oxygen were tested for

[5] Scholes and Weiss, *Nature*, 1954, **173**, 267.
[6] Wilkinson and Williams, *J. Chim. phys.*, 1955, **52**, 600.

the presence of acyl phosphate. A weak Lipmann reaction [7] was found, but this was also observed in irradiated solutions of the 3′-nucleotides. Thus, because of the apparently non-specific nature of this reaction when applied to these compounds, no definite conclusions can be drawn.

Another method for the detection of acyl phosphates is based on the release of inorganic phosphate under suitable conditions. It is known that acetyl phosphate is catalytically decomposed by molybdate,[8] particularly in acid solutions; *e.g.*, its phosphate group is completely split off under the conditions prevailing in the method of phosphate estimation described by Berenblum and Chain.[9] This catalysis, however, is not quite so marked at pH ~4, and, if

FIG. 4.

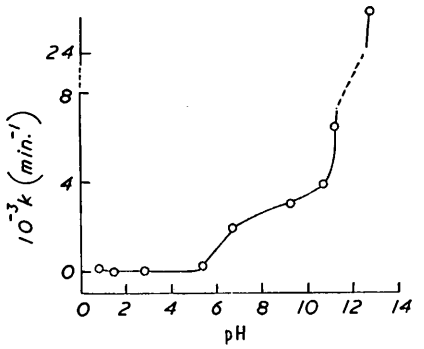
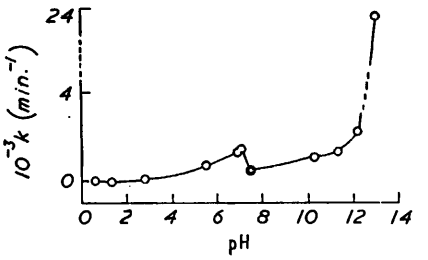


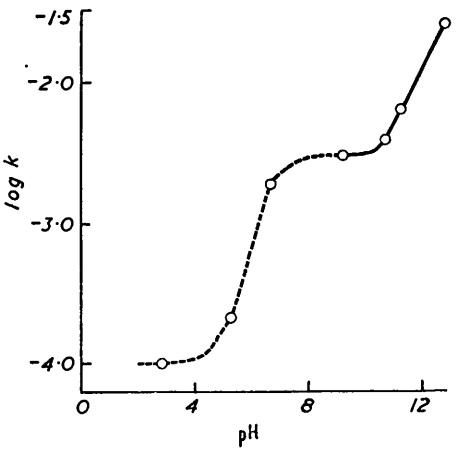
FIG. 5.



FIGS. 4 *and* 5. *Irradiation of aqueous solutions* (0·1% w/v) *of* (*Fig.* 4) *adenosine-3′ phosphate* (*commercial*) *and* (*Fig.* 5) *adenosine-5′ phosphate* (*commercial*) *with* X-*rays* (200 kv) *in the presence of oxygen* (1 *atm.*), *at* pH 6·8—7·0. *Dose* = $8{\cdot}6 \times 10^{-6}$ ev/*N per ml. First-order rate constant* (*k*) *of the post-irradiation phosphate release at* 25° *as a function of* pH.

FIG. 6. *Plot of* log k *against* pH *for post-irradiation phosphate release from adenosine-3′ phosphate* (*commercial*).

○ *Experimental points.*
– – – – *Theoretical curve.*



care is taken, inorganic phosphate can be determined in the presence of acetyl phosphate.[10] If, as seems likely, other acyl phosphates exhibit similar behaviour, this could provide a sensitive test for these compounds. However, in the case of the 5′-nucleotide, it was found that the yields of inorganic phosphate were identical when either Berenblum and Chain's or Lowry and Lopez's method [10] was used; this confirms the absence of any *stable* acyl compound in irradiated adenosine-5′ phosphate solutions. Indeed, it was found that decomposition of the labile 5′-ester was not molybdate-catalysed, either in N-sulphuric acid or at pH 4·1, as was also found to be the case with the labile products from the 3′-nucleotides.

In order to gain some further insight into the nature of the labile compounds the pH-dependence of the rate of hydrolysis was studied. The total amount of inorganic phosphate released

[7] Lipmann and Tuttle, *J. Biol. Chem.*, 1943, **159**, 21.
[8] Weil-Malherbe and Green, *Biochem. J.*, 1951, **49**, 286.
[9] Berenblum and Chain, *ibid.*, 1938, **32**, 295.
[10] Lowry and Lopez, *J. Biol. Chem.*, 1946, **162**, 421.

was constant and followed a first-order law over the whole range of pH in which it was possible to measure it. Figs. 4 and 5 show the variation of the rate of phosphate release with pH in the cases of the irradiated (commercial) adenosine-3′ and -5′ phosphate respectively. In both instances the reaction is strongly base-catalysed and extremely slow in acid solutions. For the 3′-isomer, the behaviour in the region pH 5—9 is rather suggestive of the dissociation of a weak acid and this appears more striking when the logarithm of the rate constant is plotted against pH (Fig. 6). With the 5′-isomer there is a rather striking inflexion in the region pH 7—8 where the rate constant decreases from $1\cdot3 \times 10^{-3}$ min.$^{-1}$ at pH 7·0 to $0\cdot50 \times 10^{-3}$ min.$^{-1}$ at pH 7·5, thereafter slowly increasing with pH until base-catalysis predominates in the region of pH 12.

At this point it was necessary to consider the fact that the commercial nucleotide-3′ phosphates are actually mixtures of the closely related 2′- and 3′-isomers.[11] *A priori*, there was no reason to expect that only one of the isomers would give rise to labile phosphate on irradiation; to investigate this, some experiments were carried out on pure samples of adenosine-3′ phosphate (isomer *b*) and adenosine-2′ phosphate (isomer *a*). The results obtained (Table 4) show the somewhat surprising fact that, under otherwise identical experimental

TABLE 4. *Irradiation of aqueous solutions* (0·1% *w/v*) *of ribonucleotides with* X-*rays* (200 kv) *in the presence of oxygen* (1 *atm.*) *at* pH 6·8—7·0. *Dose* $= 8\cdot67 \times 10^{-6}$ ev/*N per ml.*

Nucleotide	Inorganic phosphate (10^{-8} mole/ml.)	"Labile phosphate" (*i.e.*, post-irradiation release of inorganic phosphate) (10^{-8} mole/ml.)	$10^{-3}k$ (min.$^{-1}$) at pH 6·75 and at 25°
Adenosine-2′ phosphate (isomer *a*) ...	1·9	0·65	~10
Adenosine-3′ phosphate (isomer *b*) ...	2·60	5·2	1·7

conditions, eight times as much labile phosphate is obtained from the 3′- as from the 2′-isomer. The release of phosphate from the 3′-phosphate fits a first-order plot and that from the 2′-ester does so approximately, although in the latter case it was difficult to make very accurate measurements because of the rather small amounts of phosphate released. At 25° and pH 7, the rate constant (k) of phosphate release from the 3′-isomer ($1\cdot7 \times 10^{-3}$ min.$^{-1}$) is almost identical with that observed for the mixture at the same pH ($1\cdot85 \times 10^{-3}$ min.$^{-1}$) whilst that for the 2′-isomer is about six times greater ($\sim 1\cdot0 \times 10^{-2}$ min.$^{-1}$). If it is assumed [11] that the commercial specimen is a 3 : 2 (w/w) mixture of 3′- and 2′-nucleotide and that the radiation-induced attack is divided between these two compounds solely according to their proportions, the yields obtained from the mixture can be accounted for. Further, in view of the small amounts (both relative and absolute) of labile phosphate formed from the 2′-isomer and also because of the greater rate of hydrolysis of this material, it seems legitimate to assume that in the irradiation of the commercial 3′-nucleotide the measured rate of hydrolysis is that of the labile ester from the 3′-isomer.

The rate of release of phosphate from irradiated solutions of adenosine-3′ phosphate (commercial) has also been studied as a function of temperature. After irradiation, the solutions were hydrolysed at 15°, 25° and 35° and separate runs were carried out at pH 8·5 and 11·2. In all cases, inorganic phosphate was released according to a first-order law and, as expected, log k was a linear function of $1/T$. This gives a value of 26·3 kcal./mole for the overall activation energy of hydrolysis at pH 8·5 and of 26·6 kcal./mole at pH 11·2.

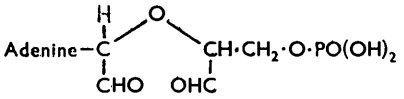
DISCUSSION

In a consideration of the chemical nature of the labile phosphate esters produced on irradiation, the observed lability towards alkali appears to be of special significance. Fleury *et al.*[12] have investigated the pH-dependence of the hydrolysis of a wide variety of phosphate esters and pointed out that alkali-lability (but only slight acid-lability) is characteristic of esters containing a free carbonyl group. In addition, it is well known that phosphate esters of β-keto- and β-aldehydo-alcohols, *e.g.*, 3-glyceraldehyde 1-phosphate, readily lose their phosphate on treatment with alkali; [13] lability in the latter case

[11] Carter and Cohn, *Fed. Proc.*, 1948, **8**, 190; Khym and Cohn, *J. Amer. Chem. Soc.*, 1954, **76**, 1818.
[12] Fleury, Courteois, and Desjobert, *Bull. Soc. chim. France*, 1948, **15**, 694.
[13] Meyerhof and Lohmann, *Biochem. Z.*, 1934, **271**, 89.

is probably associated with a β-elimination [14] rather than with a normal hydrolytic process, *viz.*:

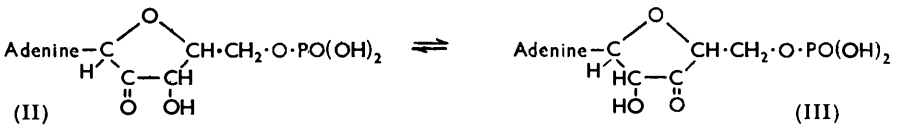
This suggests that the radiation-induced lability of the nucleotides is due to the formation of activating carbonyl groups in the sugar components. In this respect, some experiments



(I)

by Brown, Fried, and Todd [15] on the periodate oxidation of adenosine-5′ phosphate are of interest. This reagent oxidises the nucleotide to a dialdehyde of the structure (I) in which the phosphate group is in β-position to the activating carbonyl (formyl) group; these authors found that the phosphate could be removed in mildly alkaline solution at room temperature, a lability which is comparable to that of the irradiation products studied here.

It must be pointed out, however, that the alkali-lability of the 3′- and the 5′-nucleotide irradiation products does not necessarily imply that the carbonyl group is always formed in a β-position to the $>CH\cdot O\cdot PO(OH)_2$ or $-CH_2\cdot O\cdot P\cdot O(OH)_2$ group respectively, since, under the alkaline conditions where release of phosphate occurs, the possibility of enolisation cannot be ignored. This may be illustrated as follows: if the initial attack on, for example, adenosine-5′ phosphate leads to a carbonyl group at the 2′-position, the product (II) thus formed may not be expected to be particularly labile. However, if enolisation takes place, this could possibly result in the formation of a 3′-keto-ester (III) which can



(II) (III)

undergo β-elimination as discussed above. Such additional complexities, therefore, make it difficult, on the basis of these experiments alone, to determine the exact position of attack in the sugar moiety.

The variation of the rate constant of phosphate release with pH (Figs. 4 and 6) can be accounted for on the assumption that the rates of hydrolysis of the various ionised forms of the labile phosphate esters are rather different in magnitude.[16] In particular, the variation of the rate in the pH region 5—8 can be attributed to the ionisation of the mononegative to the dinegative form. On this basis and in the case of adenosine-3′ phosphate (commercial), a theoretical curve can be calculated which fits the experimental results. It can be readily shown that, if the two different ions are hydrolysed at different rates (rate constants k_1 and k_2):

$$R\cdot PO_4H^- + H_2O \xrightarrow{k_1} R\cdot OH + H_2PO_4^- \quad . . . . . (2)$$

$$R\cdot PO_4^{2-} + H_2O \xrightarrow{k_2} R\cdot OH + HPO_4^{2-} \quad . . . . . (3)$$

then

$$k_{exp.} = k_1/\{1 + (K_2/[H^+])\} + k_2/\{1 + ([H^+]/K_2)\} \quad . . . . . (4)$$

where $k_{exp.}$ is the experimentally determined rate constant and K_2 is the second dissociation constant of the labile phosphate ester. Depending on the relative values of k_1, k_2, and K_2,

[14] Linstead, Owen, and Webb, *J.*, 1953, 1211.
[15] Brown, Fried, and Todd, *J.*, 1955, 2206.
[16] Cf. Bacher and Kauzmann, *J. Amer. Chem. Soc.*, 1952, **74**, 3779.

the experimental rate constant will, at a certain acid pH, be closely approximated by k_1 and towards the alkaline region by k_2. From Fig. 6 it can be seen that $k_1 \ll 1 \times 10^{-4}$ min.$^{-1}$ and $k_2 = 3{\cdot}02 \times 10^{-3}$ min.$^{-1}$ at 25°. For a given value of K_2 both terms on the right-hand side of the equation (4) can be evaluated as a function of pH and $k_{exp.}$ thus calculated. By successive approximation it was found that $pK_2 = 6{\cdot}6 \pm 0{\cdot}05$. In Fig. 6 the experimental points and the theoretical curve are plotted together and these show satisfactory agreement. The pK_2 value, which arises from this treatment, is somewhat different from that of adenosine-3′ phosphate (6·0—6·2). It is of interest that the direction of change in pK_2 in going from adenylic acid to the labile product is similar to that found in going from glycerol 1-phosphate (pK_2 6·44) to glyceraldehyde 3-phosphate (pK_2 6·75), *i.e.*, for a process involving the conversion of $-CH_2{\cdot}OH$ into $-CHO$. These observations may support the view that the labile ester contains an activating carbonyl group. In the case of adenosine-5′ phosphate (Fig. 5) a behaviour similar to that of the 3′-isomer is observed in the pH range 5—7; however, above this pH (where ionisation to the dinegative form is complete) additional complications set in which tend to obscure the above simple statement. The fall in the rate constant above pH ~7 may perhaps be connected with ring fission in the sugar moiety leading to a product which is hydrolysed more slowly.

Following the primary radiation process [reaction (1)] we may represent the initial oxidation of the nucleotide (RH) by OH radicals as :

$$RH + OH \longrightarrow R\cdot + H_2O \quad \ldots\ldots\ldots\ldots \quad (5)$$

This leads to the organic radical R·.

Dehydrogenation of the nucleotide by hydrogen atoms, *viz.*, $RH + H \longrightarrow R\cdot + H_2$, appears to be inconsistent with the results since this, in general, should lead to rather high yields of hydrogen. *In vacuo*, therefore, the hydrogen atoms must either recombine or, if they do react with the nucleotide, it must be in such a way as not to yield molecular hydrogen. After reaction (5), the organic radicals may undergo oxidation or reduction, *viz.* :

$$R\cdot + OH \longrightarrow ROH \quad \ldots\ldots\ldots\ldots \quad (6)$$

$$R\cdot + H \longrightarrow RH \quad \ldots\ldots\ldots\ldots \quad (7)$$

$$2R\cdot \longrightarrow (R^+ + R^-) \longrightarrow RH + ROH \quad \ldots\ldots\ldots \quad (8)$$

In the presence of dissolved molecular oxygen, hydrogen atoms are converted into HO_2 radicals,

$$H + O_2 \longrightarrow HO_2 \quad \ldots\ldots\ldots\ldots \quad (9)$$

and primary dehydrogenation of the nucleotide [reaction (5)] can be followed by the addition of oxygen to form organic peroxy-radicals :

$$R\cdot + O_2 \longrightarrow RO_2\cdot \quad \ldots\ldots\ldots\ldots \quad (10)$$

Thus, under these latter conditions, we have the following possible (overall) reactions :

$$RO_2 + OH \longrightarrow ROH + O_2 \quad \ldots\ldots\ldots \quad (11)$$

$$RO_2 + RO_2 \longrightarrow RO_2^+ \ldots RO_2^- \quad \ldots\ldots\ldots \quad (12)$$

$$RO_2 + HO_2 \longrightarrow RO_2^+ \ldots HO_2^- \quad \ldots\ldots\ldots \quad (13)$$

$$RO_2^+ \ldots RO_2^- + H_2O \longrightarrow ROH + RO_2H + O_2 \quad \ldots\ldots \quad (14)$$

$$RO_2^+ \ldots HO_2^- + H_2O \longrightarrow ROH + H_2O_2 + O_2 \quad \ldots\ldots \quad (15)$$

$$HO_2 \rightleftharpoons H^+ + O_2^- \quad \ldots\ldots\ldots \quad (16)$$

$$RO_2 + O_2^- \longrightarrow RO_2^- + O_2 \quad \ldots\ldots\ldots \quad (17)$$

$$RO_2^- + H^+ \rightleftharpoons RO_2H \quad \ldots\ldots\ldots \quad (18)$$

From this scheme, it can be seen that, whatever the detailed mechanism may be, the only effective change in the absence of oxygen is of the type $RH \longrightarrow ROH$, whereas in the presence of oxygen two products may possibly arise, namely, ROH and RO_2H.

As regards specific attack on the ribose residue at position 5′ on the one hand, and at position 2′, 3′, and 4′ on the other, the possible products are summarised as in Table 5.

Its interesting feature is that ROH, which is formed in both the presence and the absence of oxygen, leads in all cases to a carbonyl compound and this is in agreement with the experimental finding that the formation of a labile ester does not depend on the presence of oxygen. Since there was no evidence for the presence of hydroperoxides in the irradiated solutions of the purine nucleotides and since that observed in the cytidylic acid solutions is

TABLE 5. *Possible products from the oxidation of the pentose component.*

Position of attack	Products: *via* ROH	Products: *via* RO_2H
5′-CH_2·OH	$-CH(OH)_2 \longrightarrow -CHO$	$-CH\langle^{OH}_{O_2H} \longrightarrow \{-CHO \text{ or } -CO_2H$
2′-, 3′-, or 4′- >CH·OH	$>C(OH)_2 \longrightarrow >CO$	$>C\langle^{OH}_{O_2H} \longrightarrow >CO$

probably connected with the base, it seems that any hydroxyhydroperoxides, if formed at all in the pentose components, decompose rather rapidly. Possible modes of decomposition of these compounds are indicated in Table 5.

It seems reasonable to suppose that inorganic phosphate is formed during the irradiation (cf. Figs. 1—3) by direct attack of the radicals on the carbon atom to which the phosphate group is attached, *i.e.*, at position 3′ in the 3′-nucleotides and at 5′ in the 5′-nucleotide. The overall picture here is, however, not quite straightforward. It may be noted, for instance, that the yield of inorganic phosphate *increases* in the absence of oxygen in the case of the purine-3′ nucleotide, but *decreases* in that of the pyrimidine-3′ nucleotide; this seems to indicate a specific effect due to the base. Further, the yield–dose plots of inorganic phosphate from the 3′-nucleotides are non-linear, the G(inorganic phosphate) increasing with increasing dose; this suggests that primary radical attack leads to a product which, as its concentration builds up, is then preferentially attacked so that the rate of formation of inorganic phosphate increases.

EXPERIMENTAL

Irradiations.—The source of X-rays was a Victor Maximar therapy tube, without filters and operating at 200 kv and 15 ma. The dose-rate was $2{\cdot}7 \times 10^{-7}$ ev/N per ml. per min., as determined by the ferrous sulphate dosimeter ($G_{Fe^{3+}}$ being taken as 15·5).[18] Irradiations were carried out in Pyrex glass vessels as previously described.[19] For experiments in the presence of oxygen the solutions were saturated with the gas. Evacuation was effected by pumping out with a two-stage oil-pump backed by a mercury diffusion pump; by this means it was shown that the pressure of oxygen in equilibrium with the solution did not exceed 10^{-5} mm.

Triply distilled water, used in all experiments, was prepared by distilling ordinary distilled water from alkaline permanganate and then from phosphoric acid in a " Baraglass " still of the type described by Smith.[20] The nucleotide solutions were brought to pH 6·5—7·0 by the addition of " AnalaR " sodium hydroxide.

Freeze-drying.—The apparatus used was based on a model described by Holtzman.[21] The solution (100 ml.) was frozen in thin layers in four 250 ml. flasks which were then attached to a central flask immersed in solid carbon dioxide–methanol. The whole apparatus was continuously evacuated by an oil-pump until sublimation into the central vessel was complete. A water reservoir was included in the apparatus, so that at the end of the freeze-drying a little water vapour could be condensed on the powder remaining; this prevented losses which were likely to occur on opening of the apparatus to the air.

Inorganic Phosphate.—For the estimation of inorganic phosphate in the presence of labile phosphate a slight modification of the method of Berenblum and Chain [9] was employed. To an aliquot part (10 ml.) were added 10N-sulphuric acid (1 ml.), water (4 ml.), 5% (w/v) ammonium molybdate (5 ml.), and butan-2-ol (20 ml.). The whole was shaken for 1 min., the acid aqueous layer run off, and the butanol extract washed with N-sulphuric acid (2 × 10 ml.). The inorganic

[17] Mackinnon and Waters, *J.*, 1953, 323.
[18] Farmer, Rigg, and Weiss, *J.*, 1955, 582.
[19] Farmer, Stein, and Weiss, *J.*, 1949, 3241.
[20] Smith, *Chem. and Ind.*, 1938, **57**, 936.
[21] Holtzman, *Science*, 1950, **3**, 550.

layer was transferred to a second flask (reserved for this stage) where reduction of the phosphomolybdate was accomplished by shaking with a 0·4% (w/v) solution (30 ml.) of stannous chloride in N-sulphuric acid for 30 sec. The blue butanol layer was then made up to 25 ml. with ethanol and the extinction measured in a Spekker colorimeter with a red (Kodak 608) filter. Calibration was with "AnalaR" disodium hydrogen phosphate.

To prevent "false" colours it was necessary to purify the butan-2-ol and ethanol; these were refluxed over calcium hydroxide and sodium hydroxide and then fractionally distilled. To avoid interference by silicomolybdate the molybdate reagent was stored in a polythene bottle. Polythene vessels were used for the hydrolyses, this being particularly essential for alkaline solutions.

Lowry and Lopez's method was used as described by these authors.[10]

Hydrogen Peroxide.—This was estimated with titanium sulphate according to Eisenberg's method,[22] with a Spekker colorimeter and blue-violet filter (Kodak 601) and by iodide according to the procedure described by Hochenadel [23] with a "Unicam" spectrophotometer (S.P. 500) (optical density measured at 353 mμ).

Materials.—Wherever possible "AnalaR" reagents were used. Commercial preparations of adenosine-3′ phosphate, cytidine-3′ phosphate, and adenosine-5′ phosphate were supplied by Roche Biochemical Products. The samples of the adenylic acid *a* and *b* were obtained from Schwarz Laboratories (New York).

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[22] Eisenberg, *Ind. Eng. Chem., Analyt.*, 1943, **15**, 327.
[23] Hochenadel, *J. Phys. Chem.*, 1952, **56**, 587.