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STUDIES IN PEROXIDASE ACTION—XII*

TRANSIODINATION AND RELATED PROCESSES

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Abstract—The investigations reported here suggest that the enzyme peroxidase (which is known to exist in the mammalian thyroid gland[1]) may be concerned in a fundamental way with the transport of iodine. Further studies with systems containing both the enzyme and iodine suggest possible connections between peroxidase action, iodine metabolism and natural phosphorylation processes and the formation of disulphide links.

ACCORDING to a recent suggestion by Ljunggren,[2] a peroxidase may be involved in the formation of thyroxine from di-iodotyrosine. Daniels and Saunders[3] showed that the peroxidase system at pH 4·5 converted *p*-chloroaniline into 2-amino-5-*p*-chloroanilinobenzoquinone di-*p*-chloroanil and tetra-*p*-chloroazophenine. This took place with the facile removal of the relatively stable chlorine atom from *p*-chloroaniline as chloride ion. It was furthermore shown that the chloride ion was produced quantitatively. *p*-Iodoaniline behaved somewhat similarly in giving the corresponding crystalline organo-iodine compounds; here, however, the iodine, which was first eliminated as I^-, was then oxidised completely to I_2 (an oxidation known to be accelerated by peroxidase). This immediately iodinated some unchanged iodoaniline to give 2:4-di-iodoaniline. Thus we had an example of de-iodination followed by re-iodination. The reaction has now been extended so that a compound other than the original substrate becomes the "iodine acceptor." Thus the peroxidase system is able to effect a process that we may call *transiodination*.

We have accordingly investigated a number of reactions involving this intermolecular transfer of iodine under the influence of the peroxidase system; particular attention has been paid to reactions with compounds similar to those present in the thyroid gland. Our results are shown in Table 1.

The reactions were carried out at room temperature by the intermittent addition of peroxidase and hydrogen peroxide to mixtures of iodine donor and acceptor in weakly acid buffer solutions. Under these conditions the use of phenolic ethers as iodine acceptors gave products containing only traces of iodine. These results would seem to be in agreement with those of Gemmill,[4] who observed that iodination of phenolic amino acids in aqueous solution led to substitution at positions *ortho* to phenolic hydroxyl groups but not at positions *ortho* to ether linkages. Higher yields of

* Paper XI, G. M. K. Hughes and B. C. Saunders *J. Chem. Soc.* 3814 (1956).

[1] E. De Robertis and R. Grasso, *Endocrinology* **38**, 137 (1946).

[2] J.-G. Ljunggren, *Acta Chem. Scand.* **11**, 1072 (1957).

[3] D. G. H. Daniels and B. C. Saunders, *J. Chem. Soc.* 822 (1953); B. C. Saunders, *Peroxidase Action and Use in Organic Synthesis.* Royal Institute of Chemistry Lectures, Monographs and Reports No. 1, London (1957).

[4] C. L. Gemmill, *Arch. Biochem. Biophys.* **63**, 177 (1956).

iodinated acceptors could be obtained by reaction in multi-phase systems, when, for instance, phenol gave a good yield of tri-iodophenol.

TABLE 1. RESULTS OF TRANSIODINATION REACTIONS

Iodine donor	Iodine acceptor	Transiodinated product
p-Iodoaniline	Pyrrole	Tetraiodopyrrole
4-Iodo-2:6-dimethylphenol	Indole	3-Iodoindole
4-Iodo-2:6-dimethylaniline	Phenol	Tri-iodophenol
4-Iodo-2:6-dimethylaniline	*p*-Hydroxyphenylacetic acid	3:5-Di-iodo-4-hydroxy-phenylacetic acid
4-Iodo-2:6-dimethylphenol	Tyrosine	Mono- and di-iodotyrosines
Di-iodotyrosine	Thyronine	3′-Iodothyronine
Di-iodotyrosine	3:5-Di-iodothyronine	Thyroxine
3-Iodotyrosine	3-Iodotyrosine	3:5-Di-iodotyrosine

We next decided to investigate other reactions in which peroxidase systems in the presence of iodine might be utilised. McCombie *et al.*[5] obtained quantitative yields of phosphoroiodidates by the action of iodine on trialkyl phosphites:

$$(RO)_3P + I_2 = (RO)_2POI + RI$$

The same workers observed, however, that, when dialkyl hydrogen phosphites were used in place of the trialkyl phosphites, negligible reaction occurred. They suggested that the hydrogen iodide first formed interfered with the formation of phosphoroiodidate, perhaps by virtue of a reversible reaction:

$$(RO)_2POH + I_2 \rightleftharpoons (RO)_2POI + HI$$

Gerrard and Jeacocke[6] suggested that dealkylation of the hydrogen phosphite by the hydrogen iodide formed as above prevented the formation of phosphoroiodidate. It seemed then that, if the reaction between iodine (or an iodine donor) and a dialkyl phosphite could be carried out in the presence of peroxidase, then a high yield of the phosphoroiodidate should result. This has indeed been found to be the case.

Since phosphoroiodidates react with amines to give dialkyl phosphoramidates and iodide,[5] it was thought worth while to investigate the formation of phosphorus–nitrogen bonds in the presence of the enzyme. Such a system was found to give good yields of phosphoramidate.

It is known that the controlled hydrolysis of phosphorochloridates yields tetraalkyl pyrophosphates and halide.[7] Here again it seemed that the enzyme system might be used in the formation of P–O–P links. Preliminary experiments showed that careful hydrolysis of diethyl phosphoroiodidate led to the formation of tetraethyl pyrophosphate (TEPP). Separate experiments showed that addition of iodine to aqueous solutions of tri*cyclo*hexyl phosphite gave di*cyclo*hexyl hydrogen phosphate, formed by hydrolysis of the phosphoroiodidate. On the other hand, a small yield of TEPP could be obtained by careful addition of peroxidase and peroxide to solutions containing

[5] H. McCombie, B. C. Saunders and G. J. Stacey, *J. Chem. Soc.* 921 (1945).
[6] W. Gerrard and G. J. Jeacocke, *J. Chem. Soc.* 3647 (1954).
[7] A. D. F. Toy, *J. Amer. Chem. Soc.* **70**, 3882 (1948).

triethyl phosphite and iodide in the presence of a limited amount of water. In this latter reaction, iodide was continually oxidised by the enzyme to iodine, which could re-enter the cycle:

$$(RO)_3P + I_2 = (RO)_2POI + RI$$
$$(RO)_2POI + H_2O = (RO)_2P(O)OH + HI$$
$$(RO)_2POI + (RO)_2P(O)OH = (RO)_2P(O)OP(O)(OR)_2 + HI$$
$$2HI + H_2O_2 \xrightarrow{\text{peroxidase}} I_2$$

Phosphorylation reactions involving dialkyl phosphites have also been considered. As explained above, the removal of hydrogen iodide is essential for the complete formation of phosphoroiodidate. In our work, hydrogen iodide has been removed either by oxidation with the peroxidase system, or by precipitation as a tertiary base hydriodide. In the reaction scheme given below, a cyclic formation and oxidation of iodide continually occurs; thus the peroxidase system is able to bring about the formation of phosphates, pyrophosphates and phosphoramidates in the presence of only catalytic amounts of iodine:

$$(RO)_2POH + I_2 = (RO)_2POI + HI$$
$$(RO)_2POI + H_2O = (RO)_2P(O)OH + HI$$
$$(RO)_2POI + (RO)_2P(O)OH = (RO)_2P(O)OP(O)(OR)_2 + HI$$
$$(RO)_2POI + R'NH_2 = (RO)_2P(O)NHR' + HI$$
$$4HI + 2H_2O_2 \xrightarrow{\text{peroxidase}} 2I_2$$

Some typical results may be summarised thus. Reaction of peroxidase, peroxide and iodine with di*cyclo*hexyl phosphite in the presence of much water gave di*cyclo*-hexyl hydrogen phosphate; diethyl phosphite in the presence of a limited amount of water gave TEPP in small yield. Higher yields of TEPP could be obtained by precipitation of hydrogen iodide with pyridine, in the presence of a limited amount of water; moreover, addition of aniline to the phosphoroiodidate thus formed gave diethyl N-phenylphosphoramidate, while gradual addition of iodine (again from peroxidase oxidation) to mixtures of benzylamine and di*tert.*-butyl phosphite gave di*tert.*-butyl N-benzylphosphoramidate.

Brief study has also been made of the formation of disulphide linkages from sulphydryl groups by the action of peroxidase and iodine. Here again, hydrogen iodide is formed, oxidised and returned to an earlier part of the cycle as iodine:

$$2RSH + I_2 = R\text{-}S\text{-}S\text{-}R + 2HI$$
$$2HI + H_2O_2 \xrightarrow{\text{peroxidase}} I_2$$

Thus cysteine was readily converted to cystine by the action of peroxidase and hydrogen peroxide, in the presence of di-iodotyrosine as iodine donor. This type of oxidation may be of interest in connexion with the recent description by Mills[8] of a "glutathione peroxidase" and his suggestion that this enzyme may have the effect of protecting haemoglobin from oxidative breakdown.

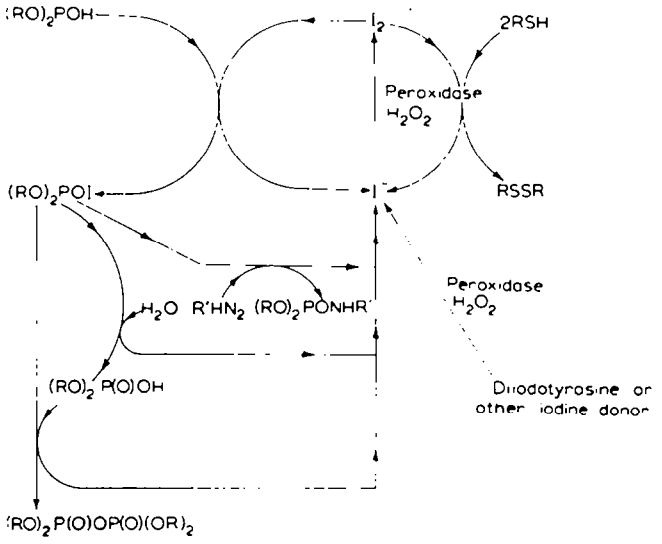
It is now convenient to summarise the action of iodine in phosphorylation and oxidation processes. It is known that some 8 μg of iodine are present in 100 ml of

[8] G. C. Mills, *J. Biol. Chem.* **229**, 189 (1957).

systemic blood, and the present work may serve to indicate possible functions of this circulating iodine. Furthermore, we have emphasised the triple function of peroxidase in this connexion: (*a*) the release of iodine from an iodine donor, (*b*) the actual transfer of iodine—"transiodination," (*c*) the oxidation of iodide to iodine, hence the circulation of iodine is ensured.

All these processes may conveniently be represented by the scheme shown in Table 2.

TABLE 2. PEROXIDASE OXIDATION-PHOSPHORYLATION CYCLES



In general, in the transiodinations described above the yields were low, but it must be remembered that these *in vitro* reactions have been carried out entirely in aqueous media, whereas the physical many phase structure of the animal cell may well permit a more efficient iodine transport process.

It may be argued that some of the substances used in the phosphorylation reactions are somewhat artificial, but nevertheless they serve to represent certain new general principles, which may be capable of extension to materials having a more biological significance.

EXPERIMENTAL

Peroxidase solution. Two enzyme preparations were used; one was a concentrated solution (P.N. ca. 500) of horse-radish peroxidase, kindly supplied by Professor Keilin, and the other was a less active enzyme preparation (P.N. ca. 10) obtained from turnips.[9]

Transiodinations

(a) *From p-iodoaniline to pyrrole.* Turnip peroxidase and hydrogen peroxide (20 volume) were added at intervals to *p*-iodoaniline[10] (21·9 g, 0·1 mole) and pyrrole (3·35 g, 0·05 mole) in acetate buffer solution (2900 ml, 0·1 M, pH 4·7), a total of 150 ml

[9] F. G. Mann and B. C. Saunders, *Practical Organic Chemistry* (3rd. Ed.) p. 420. Longmans, Green, London (1952).

[10] R. Q. Brewster, *Organic Syntheses* Vol. XI, p. 62. Wiley, New York (1931).

of enzyme solution and 70 ml of peroxide being added. The mixture was filtered and the precipitate was suspended in dilute hydrochloric acid (500 ml, 3 N) and extracted with ether (4 × 250 ml). The ether-soluble material was dried and washed with chloroform (50 ml at 0°) and extracted with benzene (150 ml). The benzene-soluble material was extracted with ethanol (150 ml) and the dissolved material was chromatographed on alumina, when crude tetraiodopyrrole was obtained. Repeated recrystallisation from aqueous ethanol gave pure 2:3:4:5-tetraiodopyrrole (85 mg, 3 per cent), m.p. 140–150° (dec.) (Found: C, 8·9; H, 0·4; N, 2·3. Calc. for C_4HNI_4: C, 8·4; H, 0·2; N, 2·5 per cent).

(b) *From* 4-*iodo*-2:6-*dimethylphenol to indole.* This reaction was carried out in a manner similar to that described above; turnip peroxidase (31 ml) and hydrogen peroxide (23 ml, 20 volume) were added to indole (1·17 g, 0·01 mole) and 4-iodo-2:6-dimethylphenol[11] (4·96 g, 0·02 mole) in acetate buffer solution (2 l., 0·1 M, pH 4·7). Extraction with ethanol, acetone and ether, followed by chromatography, gave 3-iodoindole (170 mg, 7 per cent), m.p. 71–72° (dec.).

(c) *From* 4-*iodo*-2:6-*dimethylaniline to phenol.* Turnip peroxidase (16 ml) and hydrogen peroxide (10 ml, 20 volume) were added during 12 hr to 4-iodo-2:6-dimethylaniline[12] (14·82 g, 0·06 mole) and phenol (1·82 g, 0·02 mole) in phosphate–citrate buffer solution[13] (2 l., pH 5·4). The mixture was then allowed to stand for 14 hr, acidified and extracted exhaustively with ether (250 ml). The ethereal solution was washed with 10% sodium hydroxide solution (5 × 100 ml), and the combined washings were acidified and extracted with ether (6 × 100 ml). The combined ethereal solutions were dried and evaporated; the resulting solid when recrystallised thrice from aqueous ethanol gave 2:4:6-triiodophenol (346 mg, 7·3 per cent), m.p. and mixed m.p. 156°.

(d) *From* 4-*iodo*-2:6-*dimethylaniline to* p-*hydroxyphenylacetic acid.* Turnip peroxidase (72 ml) and hydrogen peroxide (28·5 ml, 20 volume) were added during 31 hr to 4-iodo-2:6-dimethylaniline (12·35 g, 0·05 mole) and *p*-hydroxyphenylacetic acid[14] (0·76 g, 0·005 mole) in phosphate–citrate buffer solution (1 l., pH 5·9). The mixture was made alkaline and filtered. The filtrate was extracted with ether (10 × 50 ml), and the aqueous portion was acidified and extracted with ether (10 × 50 ml). Evaporation of the second ether extract gave a solid (0·916 g), which was dissolved in alkali and reprecipitated with acid. Repeated recrystallisation from aqueous ethanol gave 4-hydroxy-3:5-di-iodophenylacetic acid (545 mg, 27 per cent), m.p. 253–256° (Found: C, 23·7; H, 1·9. Calc. for $C_8H_6O_3I_2$: C, 23·8; H, 1·5 per cent).

(e) *From* 4-*iodo*-2:6-*dimethylphenol to tyrosine.* (i) Turnip peroxidase (42 ml) and hydrogen peroxide (27 ml, 20 volume) were added in portions to 4-iodo-2:6-dimethylphenol (4·96 g, 0·02) mole and tyrosine (1·81 g, 0·01 mole) in phosphate–citrate buffer solution (2 l., pH 5·0). Alkali was then added until the solution had pH 7·0, and the mixture was extracted with ether (4 × 250 ml); the aqueous portion was examined by paper chromatography—ascending chromatograms were run overnight on Whatman No. 1 filter-paper, and the dried chromatograms were sprayed with diazotised sulphanilic acid.[15] Two phenolic amino acids were detected and were found to be identical

[11] K. Heicken, *Angew. Chem.* **52**, 263 (1939).
[12] B. M. Roberts, Ph. D. Dissertation Cambridge University (1956).
[13] T. C. McIlvaine, *J. Biol. Chem.* **49**, 183 (1921).
[14] J. H. Wilkinson, *Biochem. J.* **63**, 601 (1956).
[15] E. C. Albright, F. C. Larson and W. P. Deiss, *Proc. Soc. Exp. Biol., N. Y.* **84**, 240 (1953).

with tyrosine (R_F 0·31 ± 0·03 in butan-1-ol–2 N formic acid[4]) and 3-iodotyrosine (R_F 0·55 ± 0·05).

(ii) Peroxidase (41 ml) and hydrogen peroxide (19 ml) were gradually added to tyrosine (0·905 g, 0·005 mole) and 4-iodo-2:6-dimethylphenol (4·96 g, 0·02 mole) in phosphate–citrate buffer solution (2500 ml, pH 5·0). Alkali was now added until the solution had pH 7·4, and the mixture was extracted with ether (4 × 300 ml). Paper chromatography of the aqueous phase revealed the presence of 3-iodotyrosine and 3:5-di-iodotyrosine (R_F 0·65 ± 0·05).

(f) *From* 3:5-*di-iodotyrosine to thyronine.* Turnip peroxidase and hydrogen peroxide (20 volume) (2·4 ml of each) were added in portions to 3:5-di-iodotyrosine (21·1 mg, 0·05 mmole) and thyronine (10·4 mg, 0·038 mmole) in phosphate–citrate buffer solution (200 ml, pH 5·4). Paper chromatography under the same conditions as before, but with butan-1-ol–6 N ammonia solution[4] as solvent, revealed the presence of 3′-iodothyronine (R_F 0·50 ± 0·03) and thyronine (R_F 0·59 ± 0·03).

(g) *From* 3:5-*di-iodotyrosine to* 3:5-*di-iodothyronine.* Turnip peroxidase (1·5 ml) and hydrogen peroxide (1·0 ml, 20 volume) were added to 3:5-di-iodotyrosine (55 mg, 0·13 mmole) and 3:5-di-iodothyronine (4·6 mg, 0·009 mmole) in phosphate–citrate buffer solution (1 l., pH 5·4). The pH of the solution was adjusted to 8·0, and the mixture was examined by paper chromatography as described under (f). Thyroxine (R_F 0·70 ± 0·03) was found to be present in addition to another material (R_F 0·85 ± 0·05), which was probably either 3:5:3′-tri-iodothyronine or 3:5-di-iodothyronine.

Peroxidase-catalysed oxidation of 3-*iodotyrosine*

Turnip peroxidase (8 ml) and hydrogen peroxide (3·5 ml, 20 volume) were added during 3 hr to 3-iodotyrosine[16] (92 mg, 0·3 mmole) in phosphate–citrate buffer solution (200 ml, pH 5·9); sodium fluoride (2·0 g) was added and the mixture was allowed to stand for 3 hr and was then chromatographed on Whatman No. 1 filter-paper in butan-1-ol–2 N formic acid as in (e) above. Diazotised sulphanilic acid revealed the presence of 3:5-di-iodotyrosine (R_F 0·65 ± 0·03) and of 3-iodotyrosine (R_F 0·55 ± 0·03).

Tetraethyl pyrophosphate from diethyl phosphoroiodidate

Iodine (25·4 g, 0·1 mole) in ether (250 ml) was added at 0° to triethyl phosphite[17] (16·6 g, 0·1 mole) in ether (50 ml). The resulting solution of diethyl phosphoroiodidate was treated at 0° with a mixture of pyridine (7·9 g, 0·1 mole) and water (0·9 g, 0·05 mole). The mixture was warmed to 35° during 20 min, then cooled and filtered and the filtrate was distilled (after removal of solvent under reduced pressure) to give tetraethyl pyrophosphate (6·6 g, 45 per cent), b.p. 135–140°/1 mm. The infra-red absorption spectrum of this material was identical with that of an authentic sample of the pyrophosphate prepared by the method of Toy.[7]

Tricyclohexyl phosphite

A solution of phosphorus trichloride (20·6 g, 0·15 mole) in ether (40 ml) was added during 45 min to a stirred solution of *cyclo*hexanol (45 g, 0·45 mole) and dimethylaniline (55 g, 0·45 mole) in ether (100 ml) at 0°. The mixture was allowed to stand for

[16] R. Pitt-Rivers, *Chem. & Ind.* 21 (1956).
[17] H. McCombie, B. C. Saunders and G. J. Stacey, *J. Chem. Soc.* 380 (1945).

2 hr and it was then filtered; the filtrate was dried (sodium sulphate) and filtered, and then warmed under reduced pressure to remove low-boiling liquids. Distillation of the residual material gave tri*cyclo*hexyl phosphite (35 g, 72 per cent), b.p. 164–165°/0·6 mm as a translucent very deliquescent solid. (Arbuzov and Valitova[18] reported b.p. 175–176°/1 mm for the ester prepared by a different method.)

Iodine (2·54 g, 0·01 mole) in ether (25 ml) was added gradually to a solution of tri*cyclo*hexyl phosphite (3·28 g, 0·01 mole) in ether (25 ml) at 0°; a trace of free iodine was present at the end of the addition. A solution of aniline (1·86 g, 0·02 mole) in ether (10 ml) was now added; a white precipitate of anilinium iodide separated at once. The mixture was filtered and the filtrate was warmed under reduced pressure; the residual material was *di*cyclo*hexyl N-phenylphosphoramidate*, which, after recrystallisation from aqueous ethanol, had m.p. 121·5° (Found: C, 64·0; H, 8·6; N, 4·1. $C_{18}H_{28}O_3NP$ requires C, 64·0; H, 8·3; N, 4·2 per cent).

*Conversion of tri*cyclo*hexyl phosphite to di*cyclo*hexyl phosphate*

Tri*cyclo*hexyl phosphite (6·29 g, 0·019 mole) in light petroleum (40 ml, boiling range 60–80°) was added during 40 min to a mixture of *cyclo*hexylamine (3·8 g, 0·038 mole), acetic acid (3·8 ml), iodine (4·90 g, 0·019 mole) and potassium iodide (20 g) in water (200 ml) and light petroleum (200 ml). After a further 45 min the layers were separated and the organic layer was washed with hydrochloric acid and then with water. Removal of solvent gave a nitrogen-free oil, which crystallised slowly on standing. Recrystallisation from aqueous ethanol gave di*cyclo*hexyl hydrogen phosphate (1·55 g, 31 per cent), m.p. 80–81° (Found: C, 55·5; H, 8·9. Calc. for $C_{12}H_{23}O_4P$: C, 55·0; H, 8·8 per cent).

Conversion of triethyl phosphite to tetraethyl pyrophosphate by the peroxidase system

(i) Horse-radish peroxidase was added in portions to a stirred, cooled mixture of potassium iodide (25·72 g, 0·155 mole), barium peroxide (13·13 g, 0·0775 mole), acetate buffer (4·20 ml, 1 M, pH 4·7), triethyl phosphite (12·86 g, 0·0775 mole) and pyridine (6·27 g, 0·08 mole) in light petroleum (100 ml, boiling range 60–80°). After 2 hr the mixture was filtered and the filtrate was warmed under reduced pressure to remove low-boiling liquids. Distillation of the residual liquid gave tetraethyl pyrophosphate (1·24 g, 11 per cent), b.p. 125–130°/0·5 mm.

(ii) A second experiment was carried out, essentially as in (i); double quantities of reactants were used, but with omission of pyridine. A yield of 1·58 g, 7 per cent of tetraethyl pyrophosphate was obtained.

*Conversion of di*cyclo*hexyl phosphite to di*cyclo*hexyl phosphate*

Turnip peroxidase (10 ml) and hydrogen peroxide (2 ml, 20 volume) were added gradually to di*cyclo*hexyl phosphite[19] (0·78 g, 0·0032 mole) and iodine (0·40 g, 0·0016 mole) in ether (20 ml) and acetate buffer solution (30 ml, 0·33 M, pH 4·7). After 100 min the layers were separated, and aniline (0·56 g, 0·0032 mole) in ether (10 ml) was added to the organic layer; needles of *anilinium di*cyclo*hexyl phosphate*

[18] A. E. Arbuzov and F. G. Valitova, *Izv. Akad. Nauk SSSR Otdel. Khim. Nauk* 801 (1952).
[19] H. G. Cook, J. D. Ilett, B. C. Saunders, G. J. Stacey, H. G. Watson, I. G. E. Wilding and S. J. Woodcock, *J. Chem. Soc.* 2921 (1949).

(0·70 g, 61 per cent) separated (Found: C, 60·7; H, 8·7; N, 3·8. $C_{18}H_{33}O_4NP$ requires C, 60·9; H, 8·4; N, 3·9 per cent).

Conversion of dibenzyl phosphite to dibenzyl phosphate

Turnip peroxidase (18 ml) and hydrogen peroxide (6 ml, 20 volume) were added during 3 hr to dibenzyl phosphite (2·62 g, 0·01 mole) and iodine (0·254 g, 0·001 mole) in ether (100 ml) and acetate buffer solution (100 ml, 0·1 M, pH 4·7). The layers were separated and the aqueous portion was washed with ether (4 × 25 ml). The combined organic layers were evaporated to dryness and the residual material recrystallised from a mixture of chloroform and pentane to give dibenzyl hydrogen phosphate (0·93 g, 33 per cent), m.p. 78–80° (Found: C, 60·2; H, 5·4. Calc. for $C_{14}H_{15}O_4P$: C, 60·4; H, 5·6 per cent).

Peroxidase-induced formation of diethyl N*-phenylphosphoramidate from triethyl phosphite*

Horse-radish peroxidase (21·1 ml, P.N. 5) and hydrogen peroxide (6 ml, 20 volume) were added during 9 hr to 3:5-di-iodotyrosine (3·009 g, 0·007 mole) in acetate buffer (4 l., 0·1 M, pH 4·7). The mixture was allowed to stand for 14 hr and it was then extracted with ether (5 × 200 ml). The combined ether layers were added to a cooled solution of triethyl phosphite (0·208 g, 1·25 mmoles) in ether (50 ml); aniline (0·233 g, 2·5 mmole) in ether (10 ml) was added to the resulting mixture, whereupon a precipitate of anilinium iodide rapidly separated. The mixture was filtered and the filtrate was evaporated to dryness to give diethyl N-phenylphosphoramidate (150 mg), m.p. 96°.

Conversion of diethyl phosphite to tetraethyl pyrophosphate in the presence of peroxidase.

Horse-radish peroxidase (1·5 ml, P.N. 500) and hydrogen peroxide (12·0 ml, 100 volume) were added during 12 hr to a stirred cooled solution of diethyl phosphite (13·80 g, 0·1 mole) and iodine (2·54 g, 0·01 mole) in dioxan (100 ml). The mixture was left at 0° for a further 12 hr and it was then filtered through sodium sulphate, and the filtrate was warmed under reduced pressure to remove volatile liquids. The residual material was distilled to give TEPP (1·54 g, 5 per cent), b.p. 125–130°/0·5 mm.

Conversion of diethyl phosphite to TEPP in the presence of pyridine

A solution of iodine (30·6 g, 0·12 mole) in ether (300 ml) was added with stirring during 2 hr to a solution of diethyl phosphite (15·66 g, 0·12 mole), pyridine (18·96 g, 0·24 mole) and water (1·08 g, 0·06 mole) in ether (70 ml) at 0°. The mixture was then warmed to 35° during 30 min and then cooled to 0° and filtered. The filtrate was warmed under reduced pressure and the residual liquid was distilled to give tetraethyl pyrophosphate (7·87 g, 46 per cent), b.p. 129–311°/0·35 mm.

Preparation of phosphoramidates from diethyl phosphite

A solution of iodine (5·1 g, 0·02 mole) in ether (50 ml) was added during 15 min to a stirred cooled solution of diethyl phosphite (2·76 g, 0·02 mole) and pyridine (1·58 g, 0·02 mole) in ether (30 ml). The mixture was kept at 0° for a further 15 min and then filtered. The filtrate was treated with a solution of aniline (3·72 g, 0·04 mole) in ether

(10 ml) and the mixture was allowed to stand for 3 hr and then filtered. The filtrate was washed with water, sodium hydroxide solution, water, hydrochloric acid and water again, and finally evaporated. The white solid thus obtained was recrystallised from aqueous ethanol to give diethyl N-phenylphosphoramidate (2·98 g, 65 per cent), m.p. 96° (McCombie *et al.*[17] reported m.p. 95·5–96·5°).

A solution of iodine (5·1 g, 0·02 mole) in ether (50 ml) was added during 45 min to a solution of benzylamine (6·42 g, 0·06 mole) and di*tert.*-butyl phosphite[20] (3·88 g, 0·02 mole) in ether (30 ml). After 1 hr the mixture was filtered and the filtrate was washed, evaporated and recrystallised as described above to give di*tert.*-butyl N-benzylphosphoramidate (2·45 g, 41 per cent), m.p. 96–98° (Goldwhite and Saunders[20] reported m.p. 97–98°).

Peroxidase-catalysed oxidation of cysteine

Cysteine hydrochloride (158 mg, 0·001 mole) and 3:5-di-iodotyrosine (217 mg, 0·0005 mole) were dissolved in acetate buffer solution (500 ml, 0·1 M, pH 4·7); turnip peroxidase (8 ml) and hydrogen peroxide (3 ml, 2 volume) were added during 2 hr. A precipitate of almost pure cystine (102 mg, 85 per cent) separated, which was chromatographically identical with an authentic sample. A trace of free iodine was present at the end of the experiment. Omission of iodine donor gave a much slower oxidation, while omission of enzyme or of both enzyme and di-iodotyrosine led to very slow reaction.

Acknowledgements—One of us (B. P. S.) is indebted to the Department of Scientific and Industrial Research for a maintenance allowance.

[20] H. Goldwhite and B. C. Saunders, *J. Chem. Soc.* 2409 (1957).