[CONTRIBUTION FROM THE BIOCHEMISTRY BRANCH, CHEMICAL CORPS MEDICAL LABORATORIES]

The Reaction of Phosphorus-containing Enzyme Inactivators with Phenols and Polyphenols

BY BERNARD J. JANDORF, THEODOR WAGNER-JAUREGG, JOHN J. O'NEILL AND MARVIN A. STOLBERG

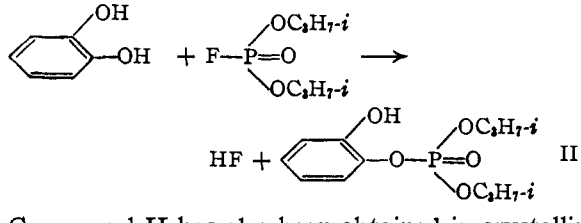
Phenol reacts slowly, and catechol more rapidly, with diisopropyl fluophosphate (DFP) and diisopropyl chlorophosphate (DClP), at room temperature and in neutral or slightly alkaline aqueous solution, to give monophosphorylated derivatives. Evidence is presented that pyrogallol reacts even more rapidly than catechol, to form a diphosphorylated compound. The presence of at least two unsubstituted hydroxyl groups in ortho-position in the polyphenols is required for high reactivity.

In a previous paper from this Laboratory,[1] it was shown that the powerful enzyme (esterase) inactivators diisopropyl fluophosphate[2] (DFP) and tetraethyl pyrophosphate[3] (TEPP) react under the proper conditions with certain amino acid derivatives and amines to yield N-phosphorylated products. The significance of these findings was discussed in relation to a mechanism for the reaction between these inactivators and the enzymes cholinesterase and chymotrypsin.[4] It was further noted in preliminary experiments that phenolic hydroxyl groups might also be of importance in the reaction between proteins and DFP and TEPP. The results of more complete studies in this latter connection are presented here.

Phenol was found to be phosphorylated by DFP in aqueous potassium carbonate solution to a small extent. However, it was not possible to isolate pure phenyl diisopropylphosphate under these conditions because of rather extensive side reactions. Better results were obtained when the chloro analog of DFP, diisopropyl chlorophosphate (DClP) was allowed to react with phenol in benzene as solvent, and in the presence of triethylamine[5]

$$C_6H_5OH + ClP(O)(OC_3H_7\text{-}i)_2 + (C_2H_5)_3N \longrightarrow (C_2H_5)_3N\cdot HCl + C_6H_5OP(O)(OC_3H_7\text{-}i)_2 \quad (I)$$

The reactivity of the phenolic OH group with DFP is increased very markedly by the introduction of a second OH group in the ortho-position. Thus catechol reacts readily with DFP in aqueous solution at *p*H 9 according to the equation



Compound II has also been obtained in crystalline form by the reaction between catechol and DClP in chloroform in the presence of triethylamine. There has been no evidence for the formation of the diphosphorylated product, even in the presence of two equivalents of DClP.

The reaction between catechol and DFP was followed manometrically in $NaHCO_3$–CO_2 buffer, *p*H 7.4. With equimolar quantities of DFP and catechol, a rate of acid production somewhat higher than that due to spontaneous hydrolysis of DFP was found. With increasingly larger excesses of catechol over DFP the rate of reaction increased progressively; however, even using ratios of DFP to catechol as high as 20:1 no evidence of a reaction of more than one mole of DFP per mole of catechol could be found.

The high reactivity of catechol toward DFP depends on the presence of the two free hydroxyl groups in the ortho-relationship on the benzene ring. Thus, resorcinol and hydroquinone show no reactivity toward DFP under the conditions of the manometric experiments, and the same is true for phenol. Likewise, a series of phenol derivatives, with the NO_2, NH_2, COOH, CH_3, CH_2OH and OCH_3 group in the ortho-position to the free phenolic OH group, showed no reactivity under comparable conditions. On the other hand, 3,4-dihydroxyphenylalanine (DOPA) gave a reaction rate identical with that of catechol.

Phenols with three vicinal OH groups, such as pyrogallol or gallic acid, show a reactivity toward DFP which is approximately 5 times that of catechol under comparable conditions. Figure 1 shows a marked increase in the rate of acid production over that due to the spontaneous hydrolysis of DFP in the mixture containing equimolar amounts

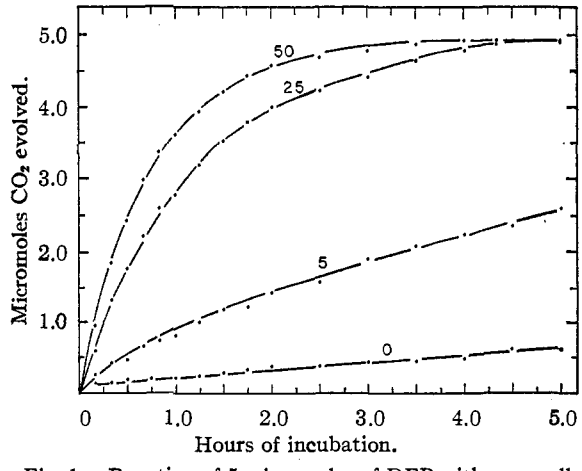


Fig. 1.—Reaction of 5 micromoles of DFP with pyrogallol at *p*H 7.4 and 25°. Figures on curves = micromoles of pyrogallol added to reaction mixture at zero time.

(1) T. Wagner-Jauregg, J. J. O'Neill and W. H. Summerson, THIS JOURNAL, **73**, 5202 (1951).

(2) H. McCombie and B. C. Saunders, *Nature*, **157**, 287 (1946).

(3) G. Schrader, BIOS Final Report No. 714, U. S. Dept. of Commerce, OTS, PB-87923 R (1945); K. P. DuBois and G. H. Mangun, *Proc. Soc. Expll. Biol. Med.*, **64**, 137 (1948).

(4) E. F. Jansen, M.-D. F. Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 201 (1949); B. J. Jandorf, W. H. Summerson, J. H. Fleisher and D. Johnson, Abstracts of the 116th Meeting, American Chemical Society, Atlantic City, New Jersey, September, 1949, p. 42c; H. O. Michel and S. Krop, *J. Biol. Chem.*, **190**, 119 (1951).

(5) The preparation of monoalkyl diaryl phosphates in high yields has recently been reported by H. R. Gamrath (to Monsanto Chemical Co.), U. S. Patents 2,504,120 (1950); *C. A.*, **44**, 6196 (1950), and 2,504,121 (1950); *ibid.*, **44**, 6435 (1950).

of reactants, and completeness of the reaction when 1 mole of acid per mole of added DFP has been produced.

When the reaction between equimolar amounts of pyrogallol and DFP is allowed to go to completion, the resulting mixture gives a red color in Arnow's[6] test for *o*-dihydroxyphenols which shows the same spectral characteristics as that produced by free catechol; free pyrogallol under the same conditions yields only a weak brown color with no absorption maxima. This may indicate that monophosphorylation of pyrogallol involves the hydroxyl group in position 1 (or 3) rather than 2. With an excess of DFP, a reaction corresponding to approximately 2 moles of DFP per mole of pyrogallol may be demonstrated, the resulting mixture then failing to give a color in Arnow's test while still yielding a violet color with $FeCl_3$. Since in catechol the second OH group is not phosphorylated by DFP, it seems likely that the hydroxyl groups in positions 1 and 3 are involved in the diphosphorylation of pyrogallol.

As far as the *mechanism* of phosphorylation is concerned, it may be assumed that the phenols react in their ionized form (as phenolate, catecholate, etc.) since the rate of reaction with any given phenol increases sharply with increasing *p*H. The higher reactivity of *o*-polyhydroxybenzenes as compared with phenol may be explained at least in part by hydrogen bonding in the catechol molecule, and the involvement of the chelated structure in the initial step of the reaction. Another possibility would be complex formation, by hydrogen or by covalency bonding, between catechol and DFP, with formation of an unstable intermediate. In either case, substitution of one hydroxyl group prevents a reaction of the remaining one with excess of the phosphorylating agent. An analogous reaction mechanism may be formulated for pyrogallol, leading, on exhaustive reaction, to the introduction of two dialkylphosphate moieties into the pyrogallol molecule, probably in positions 1 and 3.

It may be noted here that the described reaction of catechol and some of its derivatives is, to the best of our knowledge, the first example of a phosphorylation with DFP which proceeds at considerable speed and good yield even in aqueous solution at neutral or slightly alkaline reaction. There exists the possibility that enzymes which are inactivated by DFP might contain groups which are chemically related to the aromatic ortho-dihydroxy structure. However, we know of no biochemical evidence at the present time which would support such a possibility.

Experimental

Reaction of Phenol with DClP.—To a mixture of 2 g. of phenol (0.021 mole) and 2.9 ml. of triethylamine in 5 ml. of absolute benzene, 3.6 ml. of DClP (0.021 mole) was added. Separation of triethylamine hydrochloride occurred immediately. After 24 hours standing at room temperature the crystals were collected on a buchner funnel and washed with benzene. The yield was 2.6 g., with an additional 0.1 g. after another 24 hours (total, 95%). The crude product decomposed at 240–245° (uncor.); recorded m.p. of triethylamine hydrochloride, 253–256°.

(6) L. E. Arnow, *J. Biol. Chem.*, **164**, 321 (1946).

Ether was added to the filtrate, the mixture was washed with water and the ethereal layer dried over anhydrous sodium sulfate. After evaporation of the solvent, 3.5 ml. of a liquid distilled at 110–120° (1–2 mm.). On redistillation the following fractions were obtained: (A) 0.5 ml.; b.p. 103–109° (1–2 mm.). (B) 1.4 ml.; b.p. 109–116° (1–2 mm.); $n^{26}D$ 1.4708; *Anal.* Calcd. for $C_{12}H_{19}O_4P$: C, 55.8; H, 7.4; P, 12.0. Found: C, 56.5; H, 7.6; P, 11.1. (C) 1.5 ml.; b.p. 116–122° (1–2 mm.).

Fraction B was not further investigated.[7] Fraction C was soluble in ether, petroleum ether and alcohol, insoluble in hot or cold water or *N* NaOH. It reduces ferric chloride in aqueous or dilute alcoholic solution with the formation of a white precipitate. Even when a small amount of phenol was added to a sample of fraction C there was no color reaction with $FeCl_3$. The latter reagent probably is used up for the dehydrogenation of two molecules of the phosphorylated phenol, with the formation of a diphenyl derivative, in a fashion comparable to the synthesis of dithymol.

For purification, C was separated in two fractions as follows: 1.1 ml. of C was diluted with alcohol to 2 ml. and with distilled water to 3 ml. Separation into 1.2 ml. of upper layer and 1.8 ml. of lower layer occurred. On evaporation of the solvent in a high vacuum, the upper layer yielded 0.1 ml. of an oil, containing some solid particles, and the lower layer yielded 1 ml. of an oil. The latter was mixed with 4 ml. of alcohol and 5 ml. of distilled water. The centrifuged upper layer, after evaporation of the solvent, yielded 0.5 ml. of an oil with a refractive index $n^{25}D$ 1.4640. The lower layer, after removal of the solvent, gave 0.5 ml. of an oil with an $n^{25}D$ 1.4684.

Anal. Calcd. for $C_{12}H_{19}O_4P$: P, 12.0. Found: P, 12.0.

Reaction of Phenol with DFP.—To 2.4 g. of phenol, dissolved in 20 ml. of H_2O and 3.6 ml. of saturated K_2CO_3 solution, 4.8 ml. of DFP was added. The mixture was stirred for 3.5 hours. After this time 2 ml. of acetone was added and stirring was continued for 3 more hours; the *p*H now was about 8. The oily upper layer was taken up with ether, and dried over Na_2SO_4.

The reaction product was fractionated twice in a high vacuum, giving the following two fractions: ~0.4 ml.; b.p. 102–108° (1–2 mm.); ~0.5 ml.; b.p. 108–112° (1–2 mm.); $n^{25}D$ 1.4708. *Anal.* Found: P, 10.85; F, 0.05.

Diisopropyl *o*-Hydroxyphenylphosphate (II). a.—To 4 g. of catechol, dissolved in 10 ml. of 6.7% aqueous Na_2CO_3, 1 ml. of DFP was added with stirring. Further additions of 10 ml. of Na_2CO_3 and of 1 ml. of DFP were made alternately, over a total period of 10 minutes, until the total amount added consisted of 60 ml. of Na_2CO_3 and 6 ml. of DFP. The alkalinity at the beginning was above *p*H 8 and dropped to about *p*H 7–7.5 after 1 hour of stirring. After this time the heavy oil on the bottom of the reaction mixture was taken up in $CHCl_3$, the solution washed successively with water, dilute acetic acid, and water again, then dried over Na_2SO_4. After evaporation of the solvent in a vacuum, 5.7 g. of an oil remained which crystallized at room temperature. For purification the product was repeatedly precipitated with petroleum ether at −20° from its solution in a small amount of methylene chloride. The pure product melts at 68–70°, is soluble in ether, 2% Na_2CO_3, dilute NH_3, 2 *N* NaOH and 5 *N* acetic acid, insoluble in 2 *N* HCl, very sparingly soluble in water. With dilute aqueous $FeCl_3$ a light violet color is formed. It shows no color reaction in the Arnow test.[6]

Anal. Calcd. for $C_{12}H_{19}O_5P$: C, 52.5; H, 7.0; P, 11.3; *i*-OC_3H_7, 43.1. Found: C, 52.3; H, 7.0; P, 11.2; *i*-OC_3H_7, 42.1.

b.—4.4 g. (0.04 mole) of catechol was dissolved in 25 ml. of chloroform plus 5.6 ml. (0.04 mole) of triethylamine and 7.6 ml. (0.04 mole) of DClP was added to the solution. After refluxing 2 hours the reaction mixture was washed with water and dried over sodium sulfate. After evaporation of the chloroform the remaining viscous liquid crystallized upon pouring into another vessel. After recrystallization from methylene chloride plus petroleum ether the melting point was 68–69°.

(7) From the analytical figures of fraction B, we presume that the crude reaction mixture of phenol with DClP might contain, besides diisopropyl phenyl phosphate, some triisopropyl phosphate and isopropyl diphenyl phosphate, formed by disproportionation of the original reaction product.

Anal. Calcd. for $C_{12}H_{19}O_5P$: P, 11.3. Found: P, 11.0.

Manometric Determination of Reactivity.—These experiments were carried out in conventional Warburg respirometers, carrying conical vessels of 17–20-ml. capacity with one or two vented side-arms. The phenolic compound under investigation was dissolved in water, neutralized to phenol red if necessary, and pipetted into the main part of the vessel. Sufficient water and $NaHCO_3$ solution were added to achieve a final volume of 2.2 ml. and a concentration of 0.025 *M* $NaHCO_3$. The vessels were gassed on the manometers for 10 minutes with a stream of 5% CO_2 in nitrogen. At the end of this time, solutions of the required strength of DFP in 0.025 *M* $NaHCO_3$ were prepared and added to the side arms which were then stoppered. The manometers were closed off and shaken in a constant temperature-bath at 25° for 10 minutes. After mixing the contents of main vessel and side arm, the rate of CO_2 evolution was followed at suitable intervals in the usual manner.

ARMY CHEMICAL CENTER, MARYLAND

RECEIVED AUGUST 23, 1951

[CONTRIBUTION FROM THE KEDZIE CHEMICAL LABORATORY, MICHIGAN STATE COLLEGE]

The Origin of the Methyl Carbon of Nicotine Formed by *Nicotiana Rustica L.*[1]

BY STEWART A. BROWN[2] AND RICHARD U. BYERRUM

Tracer experiments with C^{14} have established that the methyl carbon of methionine can act as a precursor of the nicotine methyl carbon in intact *Nicotiana rustica* plants. A lesser incorporation of formate carbon into the methyl group of nicotine was observed. It is considered probable that formate is employed by the plant in the synthesis of labile methyl groups, which then undergo transmethylation to nicotine.

Transmethylation has been well established in the animal,[3] but although its existence has been postulated[4] the reaction has not been established by direct experimentation in the higher plant. Barrenscheen and von Vályi-Nagy[5] have obtained evidence, *in vitro*, for its occurrence in wheat germ, and Dawson[6] formerly considered that the origin of nornicotine in the tobacco leaf could best be explained by a transmethylation reaction, but recently he has shown that this reaction is a relatively non-specific N-dealkylation of nicotine. Kirkwood and Marion,[7] after feeding experiments with C^{14}-methyl choline, concluded that the N-methyl groups of the alkaloid hordenine do not arise from the choline–methionine system in barley.

We have administered C^{14}-methyl-labeled methionine, a methyl donor in the animal,[8] to intact plants of *Nicotiana rustica*, and have succeeded in establishing the transfer of the methyl carbon to the methyl group of nicotine. In addition, we have obtained some evidence, through the use of C^{14}-formate, that this transfer does not take place with an intermediate oxidation and reduction.

Experimental

Synthesis of C^{14}-Labeled Compounds.—DL-C^{14}-Methyl methionine was synthesized from C^{14}-methyl iodide (purchased from Tracerlab, Inc., Boston) essentially according to the method of Melville, Rachele and Keller.[9] The DL-S-benzyl homocysteine used in this synthesis was prepared from commercially available DL-homocystine as described by du Vigneaud and Patterson.[10] The C^{14}-formate was purchased as the sodium salt from the Oak Ridge laboratories of the United States Atomic Energy Commission.

Preparation of the Tobacco Plants.—*Nicotiana rustica* var. humilis, a high nicotine strain, was used in these studies. The seeds were planted in flats in the greenhouse, and the seedlings were transplanted after about three weeks into small pots, where they were grown until they had reached a height of at least six inches. During this period, from two to three months, they were occasionally supplemented with commercial plant food mixture as required.

To prepare the plants for hydroponic administration of the radioactive materials, as much as possible of the adhering soil was removed from the roots, first by shaking and then by washing under a stream of tap-water. The roots were then immersed in a 0.01% solution of Wyandotte detergent germicide No. 1528[11] for at least one hour, with occasional agitation. Tests carried out by the Department of Horticulture at this institution have shown that this compound has no deleterious effect on plant growth,[12] and its use was considered advantageous to reduce the bacterial population. Following a brief rinse under tap water, the roots of each plant were placed in 50 ml. of an inorganic nutrient medium in a 125-ml. erlenmeyer flask. This medium was prepared by diluting, with two parts water, one part of a stock solution the composition of which was as follows: water, 1 l.; calcium nitrate, 1 g.; potassium chloride, 250 mg.; magnesium sulfate, 250 mg.; ammonium sulfate, 250 mg.; potassium dihydrogen phosphate, 250 mg.; ferric chloride, 2 mg. The weights are of the anhydrous salt.

During the administration of the isotope it was considered advisable to grow the plants in a hood, because of a possible health hazard from $C^{14}O_2$ liberated through respiration. Two 36-inch, 30-watt fluorescent tubes and a 100-watt incandescent bulb were placed about 14 inches above the tops of the plants, and the light intensity at the level of the upper leaves was found to be 200 to 250 foot-candles. The light was left on approximately 12 hours out of 24 while the plants were growing. Distilled water was added as required.

Although Steinberg[13] has shown that methionine can exert a toxic influence on tobacco plants under certain conditions, this appears not to be the case with the much lower concentrations and shorter exposure times employed here.

(1) This paper is based on a thesis presented by Stewart A. Brown in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in the Graduate School of Michigan State College. The work was supported in part by an All College Research Grant and the U. S. Atomic Energy Commission.

(2) Eastman Kodak Fellow, 1950–1951.

(3) (a) V. du Vigneaud, J. P. Chandler, M. Cohn and G. B. Brown, *J. Biol. Chem.*, **134**, 787 (1940); (b) V. du Vigneaud, M. Cohn, J. P. Chandler, J. R. Schenck and S. Simmonds, *ibid.*, **140**, 625 (1941); (c) J. R. Schenck, S. Simmonds, M. Cohn, C. M. Stevens and V. du Vigneaud, *ibid.*, **149**, 355 (1943).

(4) (a) R. Robinson, *J. Roy. Soc. Arts*, **96**, 795 (1948); (b) H. B. Vickery, *Biological Symposia*, **5**, 3 (1941).

(5) H. K. Barrenscheen and T. von Vályi-Nagy, *Z. physiol. Chem.*, **277**, 97 (1942).

(6) (a) R. F. Dawson, *Am. J. Botany*, **32**, 416 (1945); (b) R. F. Dawson, THIS JOURNAL, **73**, 4218 (1951).

(7) S. Kirkwood and L. Marion, *Can. J. Chem.*, **29**, 30 (1951).

(8) E. B. Keller, J. R. Rachele and V. du Vigneaud, *J. Biol. Chem.*, **177**, 733 (1949).

(9) D. B. Melville, J. R. Rachele and E. B. Keller, *ibid.*, **169**, 419 (1947).

(10) V. du Vigneaud and W. I. Patterson, *ibid.*, **109**, 97 (1935).

(11) This material was obtained from the Wyandotte Chemicals Corp., Wyandotte, Michigan, through the Michigan State College, Department of Horticulture.

(12) E. H. Lucas, private communication.

(13) R. A. Steinberg, *J. Agr. Research*, **78**, 733 (1949).