sure of 0.05 mm. The flask was slowly lowered into a Wood metal bath as the temperature of the bath was slowly raised from 170° to 195°. After five minutes, the temperature was 195°. The bath was held at 195–200° for 15 minutes with the bulb portion of the flask totally immersed. The solid acid melted with vigorous frothing and bubbling for the first ten minutes of heating. After 15 minutes the gas evolution had practically ceased. On cooling the material solidified. The substance was dissolved in boiling methanol, filtered, and cooled. The product was collected, dried and weighed. The material (0.49 g., 40%) melted at 155–160°. Several crystallizations from methanol raised the melting point to 167–169°.

Anal. Calcd. for $C_{29}H_{46}O_3$: C, 78.68; H, 10.48. Found: C, 78.43; H, 10.47; λ_{max}^{cal} 238 mμ, E_m 13,300; $[\alpha]_D^{25}$ − 103.5° (1% chloroform).

Methyl 7-Keto-3β-cholesterylacetate (VIb).—The filtrate from the first crop isolation of the 7-keto-3β-cholesterylacetic acid described above was diluted with methanol to a volume of 80 ml. The solution was poured into a nitrogen-filled flask and three small drops of sulfuric acid added. After standing for 16 hours at room temperature, the solution was heated under reflux for 5 hours, cooled, and poured into water. The suspension was extracted with benzene. The combined benzene extracts were washed with water and dried over anhydrous sodium sulfate. The benzene solution (120 ml.) was poured into a silica gel (50 g.) chromatography column. Elution with 9:1 benzene:ethyl acetate gave 0.64 g. of material in the only elution peak. Trituration of this material with methanol gave 0.53 g. of solid which melted at 102–104°. Crystallization from methanol gave large puffs of cotton-like material. This compound melted at 103.5–105°.

Anal. Calcd. for $C_{30}H_{48}O_3$: C, 78.90; H, 10.59. Found: C, 78.92; H, 10.56; λ_{max}^{alc} 238 mμ, E_m 14,100; $[\alpha]^{25}D$ −87° (1% in acetone).

CHICAGO 80, ILLINOIS

[CONTRIBUTION FROM THE MEDICINAL CHEMISTRY BRANCH, CHEMICAL CORPS MEDICAL LABORATORIES]

Model Reactions of Phosphorus-Containing Enzyme Inactivators. III.[1] Interaction of Imidazole, Pyridine and Some of their Derivatives with Dialkyl Halogeno-phosphates

BY T. WAGNER-JAUREGG AND B. E. HACKLEY, JR.

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The hydrolysis of diisopropyl fluorophosphate and of diethyl fluorophosphate can be accelerated by imidazole, histidine, pyridine and by certain of their derivatives. A comparison of their activity is made and the possibility is discussed that the catalytic function of the investigated amines might be explained by the formation of an intermediary quaternary complex between the tertiary nitrogen and the dialkyl fluorophosphates. N-Diisopropylphosphoryl imidazole has been synthesized and its spontaneous hydrolysis studied. A hypothesis is presented that two different essential centers may be involved in the reaction of DFP with enzymes: one group which functions as a phosphorylation catalyst, perhaps of the imidazole type, and an ultimate acceptor for the phosphoryl group.

In the inactivation of chymotrypsin by diisopropyl fluorophosphate (DFP), a diisopropylphosphoryl–chymotrypsin is formed by replacement of one reactive hydrogen atom of the enzyme by the $PO(OR)_2$ group[2]

$$\text{chymotrypsin} + FPO(OR)_2 = \text{chymotrypsin} - PO(OR)_2 + HF$$

After treatment of the phosphorylated chymotrypsin with 2 *N* hydrochloric acid at 100° for several hours, phosphorus of the bound DFP has been isolated as serine phosphate.[3] It cannot be considered definitely established that the phosphoryl group reacts directly with the OH group of serine during the inactivation process; the isolated serine phosphate could be an artefact, produced during the process of hydrolysis. The study of model reactions with halogeno-phosphates might be helpful for a better understanding of the reaction mechanism.

We have never been able to isolate phosphorylated products from serine or its methyl ester after treatment with DFP. In chloroform solution, serine methyl ester is phosphorylated on the nitrogen atom by diisopropyl chlorophosphate (DClP).[4a,b] The possibility remains that there may be specific catalytic centers present in proteins which induce the phosphorylation of groups which, in an isolated form, are not reactive with DFP.

In order to study this problem, the manometric method of measuring the amount of CO_2 produced by interaction of DFP and a substrate in a bicarbonate–CO_2 buffer was used. This technique had been applied previously in an investigation of the reactivity of phenolic substances with DFP.[5] A phosphorylation reaction is characterized by the evolution of one mole of CO_2 per mole of disappearing dialkyl fluorophosphate, according to equation A. Two moles of CO_2 is expected in the case of hydrolysis of DFP (equation B)

$$(RO)_2POF + R'OH + NaHCO_3 = (RO)_2POOR' + NaF + H_2O + CO_2 \quad (A)$$

$$(RO)_2POF + 2NaHCO_3 = (RO)_2POONa + NaF + H_2O + 2CO_2 \quad (B)$$

$$2(RO)_2POF + 2NaHCO_3 = (RO)_2OPOPO(OR)_2 + 2NaF + H_2O + 2CO_2 \quad (C)$$

The formation of tetraalkyl pyrophosphate would be characterized by the liberation of one mole of CO_2 per one mole of dialkyl fluorophosphate (equation C). This possibility certainly can be excluded when diethyl fluorophosphate is used since the

(1) Some of the results of this paper were presented at the Meeting of the American Society of Biological Chemists, at New York, N. Y., April, 1952; abstract, *Federation Proc.*, **11**, 224 (1952). For two preceding publications see footnotes 4a and 5.

(2) E. F. Jansen, M. D. F. Nutting, R. Jang and A. K. Balls, *J. Biol. Chem.*, **179**, 189, 201 (1949).

(3) N. K. Schaffer, S. C. May, Jr., and W. H. Summerson, *Federation Proc.*, **11**, 282 (1952).

(4) (a) T. Wagner-Jauregg, J. J. O'Neill and W. H. Summerson, THIS JOURNAL, **73**, 5202, I (1951). (b) Unpublished experiments with Dr. R. Plapinger.

(5) B. J. Jandorf, T. Wagner-Jauregg, J. J. O'Neill and M. Stolberg, THIS JOURNAL, **74**, 1521 (1952).

expected tetraethyl pyrophosphate is easily hydrolyzed in aqueous solution.

No increased rate of CO_2 production (*i.e.*, no increased rate of decomposition of DFP over the rate of spontaneous hydrolysis) was detectable in bicarbonate buffer at *p*H 7.6 with DFP in the presence of such amino acids as serine, serine methyl ester, tryptophan, lysine, arginine, proline, creatine, or creatinine. However, it was found that either histidine or imidazole accelerate the rate of hydrolysis of diisopropyl and diethyl fluorophosphate (DFP and DEFP).

In Fig. 1 the initial rate of acid production from DFP in the presence of a 5-fold and 7.5-fold (molar) excess of imidazole is recorded. A corresponding curve for the phosphorylation of phenol with DFP is given for comparison.

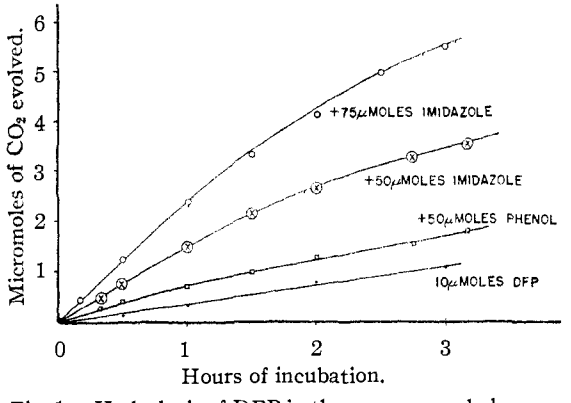


Fig. 1.—Hydrolysis of DFP in the presence and absence of imidazole at *p*H 7.6 and 38°, compared with the phosphorylation of phenol with DFP; reaction volume 2.2 ml.

The influence of two different concentrations of histidine on the initial rates of hydrolysis of DFP is illustrated by Table I.

TABLE I

HYDROLYSIS OF DFP AT *p*H 7.6 AND 38° IN THE PRESENCE AND ABSENCE OF L-(+)-HISTIDINE

After, hours:	1	2	3	4
		CO_2, mm.[a]		
0.00455 *M* DFP	10	20	30	37
+ .0114 *M* histidine	28	48	66	84
+ .0227 *M* histidine	35	61	86	102

With the figures of Table I and Fig. 1 the ratios catalyzed:uncatalyzed hydrolysis of DFP were calculated as listed in Table II.

TABLE II

COMPARISON OF THE RATES OF DFP-HYDROLYSIS IN THE PRESENCE OF IMIDAZOLE AND HISTIDINE

	Molar ratio, DFP:	Ratios catalyzed:uncatalyzed DFP hydrolysis After, hours: 1	2	3
a	Histidine = 1:2.5	2.8	2.4	2.3
b	Histidine = 1:5	3.5	3.0	2.9
c	Imidazole = 1:5	3.8	3.3	3.1
d	Imidazole = 1:7.5	6.0	5.2	5.0

There is a slight difference between the corresponding values for the DFP hydrolysis catalyzed by histidine and by imidazole in favor of the latter (b and c).

In the case of diethyl fluorophosphate (DEFP), the reaction was followed to completion in order to obtain the time values for 50% decomposition (Fig. 2). These half-times for spontaneous and histidine- or imidazole- accelerated hydrolysis were approximately 8.0, 1.1 and 1.0 hours, respectively. In the presence of imidazole or histidine only 72 and 78%, respectively, of the theoretical 2 moles of CO_2 are evolved. This fact can be explained by CO_2 retention, caused by the buffer capacity of imidazole and its derivatives. In order to demonstrate this phenomenon, diethyl fluorophosphate previously hydrolyzed was added separately to imidazole and to histidine in bicarbonate buffer under the experimental conditions of Fig. 2. The amount of CO_2 evolved was 70 and 87%, respectively, in the presence of imidazole or histidine as compared with 100% in the absence of the organic bases (Table VIII).

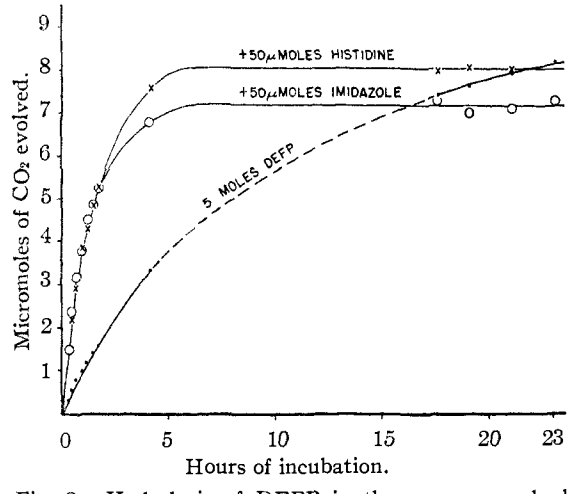


Fig. 2.—Hydrolysis of DEFP in the presence and absence of imidazole and histidine at *p*H 7.6 and 38°; reaction volume 2.2 ml.

That the function of histidine as an accelerator of the hydrolysis of DFP is a catalytic one could be demonstrated by colorimetric determination of the amino acid; no histidine disappears during the reaction. Also, the action of imidazole is a true catalytic effect, since it is not exhausted after fluorophosphate has been hydrolyzed in the presence of an equimolecular amount of the base; when more fluorophosphate is added to the reaction mixture it is hydrolyzed at the same rate as the first portion.

N-Methylimidazole, 4-hydroxymethylimidazole, histidine methyl ester, histamine and carnosine produce about the same increased rate of acid production in the hydrolysis of dialkyl fluorophosphates as the unsubstituted imidazole or histidine, respectively; benzimidazole and 3,5-dimethylpyrazole are less effective, tetrazole, morpholine and 2-methyloxazoline have only a very slight activity, while 2-methylimidazole, 2-mercaptoimidazol, ergothioneine, pyrazine and 2-methyloxazolidine do not increase the rate of acid production in the hydrolysis of the fluorophosphates which have been tested.

Concerning the mechanism of the observed reaction, the fact is important that there is no difference in activity between imidazole and N-methyl- or N-ethylimidazole. Since the latter substances

have no reactive hydrogen atoms a phosphorylated imidazole in this case can be excluded as an intermediate of the catalyzed DFP hydrolysis. Therefore, the tertiary nitrogen of imidazole has to be taken into consideration as a catalytic center.

In order to obtain more experimental evidence, other tertiary amines have been tested for their catalytic activity. Triethylamine had no effect on the hydrolysis of dialkyl fluorophosphates. However, pyridine was found to be effective, although to a lower degree than imidazole.

From the figures given in Table V the half-times were estimated and compared (Table III).

TABLE III

COMPARISON OF THE RATE OF HYDROLYSIS OF DIALKYL FLUOROPHOSPHATES IN THE PRESENCE OF IMIDAZOLE, PYRIDINE, METHYL- AND AMINOPYRIDINES

	Molar ratio	Half-time of hydrolysis (approximate values)
DFP		50 hr.
DFP + imidazole	1:7.5	2.5 hr.
DFP + pyridine	1:7.5	>6 hr.
DEFP + imidazole		50 min.
DEFP + pyridine		80–100 min.
DEFP + α-picoline		84 min.
DEFP + β-picoline	1:10	62 min.
DEFP + γ-picoline	1:10	51 min.
DEFP + 2-aminopyridine	1:10	54 min.
DEFP + 3-aminopyridine	1:10	53 min.
DEFP + 4-aminopyridine	1:10	174 min.
DEFP		8 hr.

The figures demonstrate that the hydrolysis of DFP in the presence of imidazole was more than 2.4 times faster, in terms of half-time, than in the presence of pyridine. The corresponding ratio in the case of the hydrolysis of DEFP was approximately 1.8.

α-Methylpyridine (α-picoline) has the same activity as a catalyst of the hydrolysis of DEFP as pyridine. β-Picoline is somewhat more active and γ-picoline is about equal to imidazole. The same is true for 2- and 3-aminopyridine while 4-aminopyridine is much less active than pyridine. The activation effect of methyl and amino groups in the proper position is remarkable.

2-Hydroxypyridine had almost no effect in our experiments, whereas 3-hydroxypyridine gave a rather high rate of CO_2 production in the presence of DEFP. The half-time of the reaction (8 minutes) is identical with that of catechol (Fig. 3). 0.71 mole of CO_2 per mole of DEFP is evolved at the end; considering the CO_2 retention of about 23.5% in the presence of 3-hydroxypyridine (Table VIII) only about one mole of CO_2 per mole DEFP is obtained, instead of 2 moles as in the case of DEFP hydrolysis. This fact indicates a phosphorylation reaction, which obviously takes place with the phenolic OH-group.

The rate of reaction between DFP and 3-hydroxypyridine under conditions as in Fig. 3 is lower than that of the phosphorylation of catechol by DFP (half-times 47 and 29 minutes). However, the velocity of the reaction was found to be much higher than that of DFP in the presence of a corresponding equimolecular mixture of phenol and pyridine. Therefore the assumption seems to be justified that in the molecule of 3-hydroxypyridine the simultaneous presence of a catalytic center (tertiary nitrogen) in addition to an acceptor for the phosphoryl group (phenolic OH) in proper position strongly enhances the reactivity with dialkylfluorophosphates.

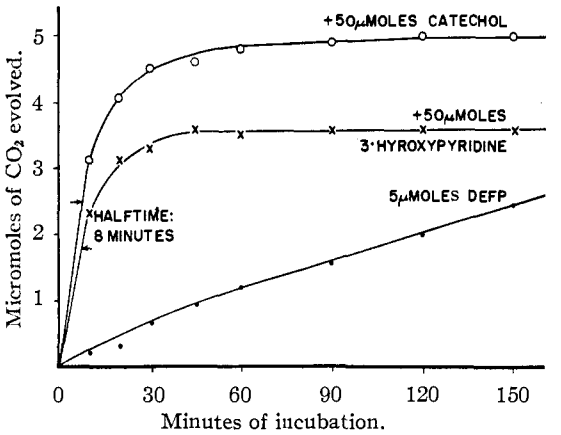


Fig. 3.—Comparison of the reaction of DEPF with 3-hydroxypyridine and with catechol at *p*H 7.6 and 38°; reaction volume 2.2 ml.

Pyridoxal and pyridoxamine, which are derivatives of 3-hydroxypyridine with substituents in position 2, 4 and 5 react much slower with DFP and DEFP than 3-hydroxypyridine. Steric hindrance by the substituents adjacent to the reactive group might be partly responsible for the decrease of reactivity.

Concerning the mechanism of the acceleration of the hydrolysis of dialkyl fluorophosphates by imidazole or pyridine it seems possible that the first step of the reaction consists in the formation of a quaternary complex between DFP and the tertiary ring nitrogen (I).[6] Eventually a hydrated form of DFP might be involved in the formation of a reactive intermediate.

An explanation involving a quaternary complex was also given for the catalytic action of tertiary amines in the synthesis of tetraalkyl dithionopyrophosphates from dialkyl thionochlorophosphates.[7] The poor activity of triethylamine has been explained by steric hindrance, the three ethyl groups around the nitrogen atom being unfavorable for the formation of an addition complex. Pyridine has been shown also to catalyze the hydrolysis of acetyl phosphate in neutral solution.[8]

The higher catalytic activity of imidazole as compared with pyridine may be visualized on a steric basis by the fact that the outer angle around the tertiary nitrogen atom in a five-membered ring is larger than in the case of a six-membered ring. Therefore, more space is provided for the addition of a dialkyl fluorophosphate molecule. This factor will be of greater importance if the alkyl group is the relatively large diisopropyl group, as compared with the less space-consuming ethyl group. There-

(6) Concerning the formation of addition compounds between tertiary amines and acid chlorides see: K. Freudenberg and D. Peters, *Ber.*, **52**, 1463 (1919).

(7) A. D. F. Toy, THIS JOURNAL, **73**, 4670 (1951).

(8) D. E. Koshland, Jr., *ibid.*, **74**, 2286 (1952).

fore, one would expect imidazole to be relatively more effective than pyridine against DFP, whereas for DEFP the difference between imidazole and pyridine should be smaller, as was actually found experimentally.

A steric factor also would seem to be the reason for the ineffectiveness of the imidazoles substituted in 2-position as catalysts for the hydrolysis of dialkyl fluorophosphates. The tertiary nitrogen atom is blocked by the presence of a substituent on the adjacent carbon atom. These derivatives likewise are unable to bind hemin, while 1- or 5-substituted imidazoles form parahematins.[9,10]

The fact that α-methylpyridine is as good a catalyst for the hydrolysis of DEFP as pyridine itself seems to deny the importance of steric factors. However, since 3- and 4-picoline both are more active than pyridine one might speculate that a methyl group in 2-position primarily also has an increasing influence on the catalytic activity of the tertiary nitrogen atom, but that this effect is compensated by steric hindrance. It should be emphasized that all steric considerations concerning the activity of the studied catalysts constitute only one possible approach to the problem. Another important factor probably is the basicity of the amines used.

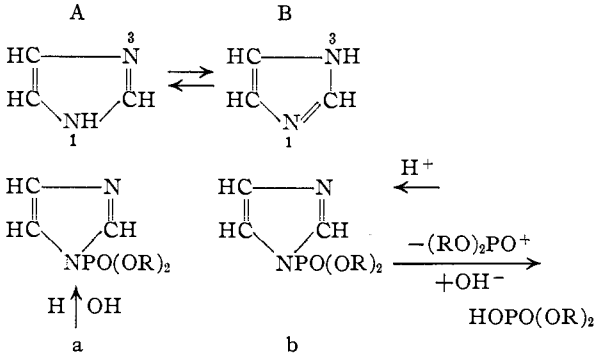
When equimolar amounts of DFP and imidazole are mixed together no visible reaction occurs. By heating the mixture in a high vacuum a lower boiling fraction of DFP and a higher boiling fraction containing imidazole are obtained. In order to perform this distillation process the temperature of the heating bath has to be kept 100–120° above the boiling point of the distillate. We consider this fact to be in favor of the assumption that a nonvolatile addition complex is formed, which only on heating at higher temperatures suffers a breakdown into the components.

Quite different is the reaction of imidazole with diisopropyl chlorophosphate (DClP) in chloroform. In this case we have been able to isolate diisopropylphosphorylimidazole (DPI) as an oil, which can be distilled at low pressure. This substance is easily hydrolyzed at room temperature with formation of the diisopropyl phosphate of imidazole (m.p. 48–50°). The instability of DPI in water is comparable to that of N-benzoylimidazole, which also is very readily hydrolyzed with formation of imidazole benzoate.[11]

DPI is as unstable against water as some of the known energy-rich phosphate compounds. Acetylphosphoric acid, for instance, has a half-life of 3 hours at 38° and *p*H 7.4.[12] In a 0.0023 *M* solution of DPI in bicarbonate buffer approximately 50% was saponified at *p*H 7.6 and 30° after 2.7 hours as measured by CO_2 production; about 13 hours was required for total hydrolysis. N-Diisopropylphosphorylserine methyl ester produced only a negligible amount of acid during 3 hours in a similar experiment. The rate of spontaneous hydrolysis of DFP under comparable conditions is 20–25 times slower than that of DPI.

(9) W. Langenbeck, *Angew. Chem.*, **64**, 167 (1952).
(10) *Cf.* R. W. Cowgill and W. M. Clark, *J. Biol. Chem.*, **198**, 32 (1952).
(11) O. Gerngross, *Ber.*, **46**, 1909 (1913).
(12) F. Lynen, *ibid.*, **73**, 367 (1940).

N-Acylated imidazoles are perhaps easily hydrolyzable because of the particular chemical structure of imidazole. A resonance phenomenon exists between the two formulas A and B (see scheme below). Therefore, the saponification of DPI might occur either by hydrolysis at the N–P linkage (a), or by attachment of a proton at position 3, with expulsion of the phosphoryl group (b). Thus, two different reaction mechanisms appear to be simultaneously possible, the latter (which could be either a "push button" mechanism or a "push–pull" mechanism) presumably not influenced by steric hindrance



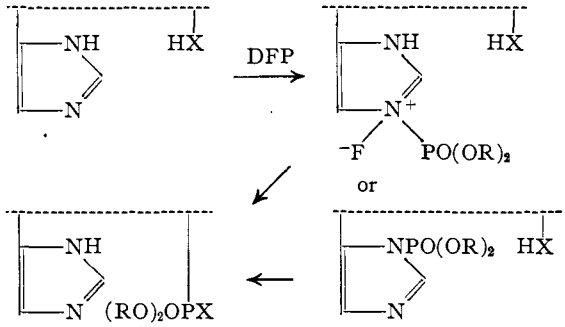
Conclusion

From measurements of the ionization constant of cholinesterase Wilson and Bergmann[13] have assumed that imidazole could be a possible functional group of this enzyme. Furthermore, the possibility has been discussed by Doherty and Vaslow,[14] that in the formation of a chymotrypsin–substrate complex the binding might be ascribed to ionized imidazole groups. The importance of an imidazole ring as a constituent of the histidine group for the enzymatic activity of chymotrypsin gained some support from a recent investigation by Weil and Buchert.[15] These authors have found that during the process of photoöxidation of this enzyme in the presence of methylene blue, complete inactivation is reached when 1 mole of histidine out of a total of 2, besides 2.4 moles of tryptophan out of a total of 6, were photoöxidized. No changes in solubility and viscosity of the protein occurred during this process.

Since DFP is a potent inactivator of cholinesterase, and evidence has been presented in this paper for intermediary reaction of dialkyl halogeno-phosphates with imidazole, one might postulate that such a group could be the first spot of the DFP attack in an enzyme. Just as the DFP molecule activated by imidazole reacts with water, so in a protein other phosphorus acceptors containing a reactive hydrogen atom may be present in a favored position, to take over the diisopropylphosphoryl group from the imidazole nitrogen. The following scheme, where the broken line symbolizes an enzyme surface, might illustrate this hypothesis.

To account for the high reaction rate between the enzyme and DFP, one has to assume that the catalytic centers and the phosphorus acceptor as well as their interactivity are highly activated by

(13) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **186**, 683 (1950).
(14) D. G. Doherty and F. Vaslow, THIS JOURNAL, **74**, 931 (1952).
(15) L. Weil and A. R. Buchert, *Federation Proc.*, **11**, 307 (1952).



the particular structural surroundings in the protein molecule.

The nature of the group XH in the scheme given above does not necessarily have to be the OH group of serine. It well might be that another group is the acceptor of the dialkyl phosphoryl group and that only during the process of acid hydrolysis the phosphoryl group shifts over to the aliphatic hydroxyl group. Different examples are known for N → O acyl migration in acid solution.[16] The isolation of serine phosphate from casein might be subject to the same objection.

From a study of the effects of ketene, nitrous acid, phenyl isocyanate, formaldehyde, iodine, oxidants and reductants upon chymotrypsin Sizer[17] has concluded that primary amino, sulfhydryl or disulfide groups are not required for the enzymatic activity, while tyrosine constitutes an essential group. The imidazole and the tyrosine group have the same reactivity toward certain reagents; for instance, substitution by iodine takes place with tyrosine and histidine residues, and both give the Pauly reaction with freshly diazotized sulfanilic or similar acids. Therefore, it should be taken into consideration that some inactivation reactions of enzymes originally attributed to the blocking of tyrosine in protein molecules also might be caused, at least partly, by the reactivity of imidazole groups.

Experimental

Materials Used.—The DFP (diisopropyl fluorophosphate) was a product of Merck and Co., Inc., Rahway, N. J., DEFP (diethyl fluorophosphate) was prepared according to methods described in the literature. Some samples of the fluorophosphates contained a small amount of an easily hydrolyzable substance which caused a rapid production of a few cu. mm.[3] of carbon dioxide during the first minutes of the hydrolysis experiments; this initial extra acid production can be eliminated in the figures by extrapolation of the slope of the hydrolysis curve to zero time.

Imidazole and most of the derivatives studied were prepared by Mr. R. Proper in this Laboratory. The carnosine was a sample from Prof. V. du Vigneaud, the α-hydroxypyridine a sample by Prof. W. A. Mosher, kindly given us by Dr. W. H. Summerson. For the tetrazole we are indebted to Mr. Ronald A. Henry of the U. S. Ordnance Test Station, Inyokern, China Lake, California. Pyrazine was furnished by Mr. K. W. Saunders of the American Cyanamid Co., Stamford, Conn.

3-Hydroxypyridine was prepared by Dr. Robert L. Sublett in this Laboratory from nicotinamide, *via* 3-aminopyridine, *Org. Syntheses*, **30**, 3 (1950) by treating the latter with nitrous acid in diluted sulfuric acid.

(16) Recent papers; A. P. Phillips and R. Baltzly, THIS JOURNAL, **69**, 200 (1947); G. Fodor, *J. Org. Chem.*, **14**, 337 (1949); *Nature*, **164**, 917 (1949); THIS JOURNAL, **72**, 3495 (1950); L. Anderson and H. A. Lardy, *ibid.*, **72**, 3141 (1950); G. E. McCasland, *ibid.*, **73**, 2295 (1951); D. F. Elliott, *Biochem. J.*, **50**, 542 (1952).

(17) I. W. Sizer, *J. Biol. Chem.*, **160**, 547 (1945).

Manometric Determinations of the Rates of Hydrolysis.—The method described in an earlier paper[5] was used to determine the CO_2 production. The reaction medium was 0.025 *M* sodium bicarbonate, saturated with 5% CO_2 in nitrogen. This buffer mixture has a *p*H 7.58 at 30°, and a *p*H 7.63 at 38°.[18] The final volume of reaction in all the experiments was 2.2 ml.

The values resulting from our experiments with DFP and with DEFP in the presence and absence of imidazole or histidine at *p*H 7.6 and 38° are evident from Figs. 1 and 2. These values have been obtained by subtracting the appropriate control blanks. The same is true for all the following tables, unless the contrary is mentioned. Some figures for experiments at 30° are given in Table IV.

TABLE IV

HYDROLYSIS OF DIALKYL FLUOROPHOSPHATES IN THE PRESENCE AND ABSENCE OF IMIDAZOLE AND L-(+)-HISTIDINE AT *p*H 7.6 AND 30°

Mole	After, hours: 2	4	8
	CO_2, mm.[3]		
5 × 10^{-6} DFP	15	23	30
+50 × 10^{-6} histidine	27	47	73
+50 × 10^{-6} imidazole	36	61	91
5 × 10^{-6} DEFP	36	50	81
+50 × 10^{-6} imidazole	86	114	136
+50 × 10^{-6} histidine	87	128	158

The final volume of evolved CO_2 expected at the end of the hydrolysis of the dialkyl fluorophosphate in the absence of a base is 224 mm.[3]. All the figures of CO_2 production in Table IV are obtained without the corresponding control blanks for the incubation of imidazole or histidine alone, since these blanks, in other runs, were found to be small.

TABLE V

COMPARISON OF IMIDAZOLE, PYRIDINE, METHYL- AND AMINOPYRIDINES IN THEIR ACTION ON THE HYDROLYSIS OF DFP AND DEFP AT *p*H 7.6 AND 38°

a.

Mole	After, hours, 1	2.5	4.5	21.5
	CO_2, mm.[3]			
10 × 10^{-6} DFP	6	14	25	109
+75 × 10^{-6} imidazole	54	112	149	217
+75 × 10^{-6} pyridine	43	82	125	271

b.

After, hours,	1	2.5	3.5	8	22.5
5 × 10^{-6} DEFP	23	48	61	109	163
+50 × 10^{-6} imidazole	81	130	140	149	153
+50 × 10^{-6} pyridine	64	120	141	180	196

c.

After, hours,	1	2	3	4	20
5 × 10^{-6} DEFP	26	43	62	76	165
+50 × 10^{-6} pyridine	73	117	140	156	181
+50 × 10^{-6} α-picoline	75	114	143	157	187
+50 × 10^{-6} β-picoline	88	130	150	162	179
+50 × 10^{-6} γ-picoline	103	141	159	168	186

d.

After, hours,	1	2	3	19
5 × 10^{-6} DEFP	25	45	63	178
+50 × 10^{-6} 4-aminopyridine	39	67	90	175
+50 × 10^{-6} imidazole	83	119	133	141
+50 × 10^{-6} 2-aminopyridine	95	136	148	176
+50 × 10^{-6} 3-aminopyridine	104	150	170	191

The initial CO_2 production is not very much faster with imidazole than with pyridine, but since the final CO_2 values are much higher in the latter case (due to lack of CO_2 retention) the half-time is strongly in favor of the imidazole.

(18) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques and Tissue Metabolism," Second edition, Burgess Publishing Co., Minneapolis, Minn., 1949.

TABLE VI

COMPARISON OF IMIDAZOLE AND 1-ALKYL IMIDAZOLES WITH REGARD TO THEIR EFFECT ON HYDROLYSIS OF DFP AT *p*H 7.6 AND 38°

Mole	After, hours: 1	2	3
	CO_2, mm.³		
10×10^{-6} DFP	21	34	46
$+50 \times 10^{-6}$ imidazole	45	74	100
$+50 \times 10^{-6}$ 1-methylimidazole	36	70	94
$+50 \times 10^{-6}$ 1-ethylimidazole	36	68	

The values for the spontaneous hydrolysis of DFP are not corrected for the bicarbonate blanks while the imidazole and 1-methylimidazole values are corrected.

TABLE VII

ACID PRODUCTION IN THE PRESENCE OF DFP AND 3-AMINOPYRIDINE, RESPECTIVELY, 3-HYDROXYPYRIDINE AT *p*H 7.6 AND 38°

Mole	After, hours: ½	1	1.5	2.5	3	4	24
	CO_2, mm.³						
10×10^{-6} DFP	10	14	20	30	33	41	147
$+50 \times 10^{-6}$ 3-aminopyridine	32	61	87	128	146	175	343
$+50 \times 10^{-6}$ 3-hydroxypyridine	53	91	110	132	139	145	

As can be seen from the data given in the preceding tables and in the figures, there is some fluctuation in the rate of acceleration of the hydrolysis of dialkyl fluorophosphates by a catalyst. This might be caused partly by the presence of traces of certain heavy metals, which in certain cases influence the speed of the reaction. Data concerning the influence of metals on the described catalytic reactions will be given in a later paper. The effect of imidazole was diminished a little, but not abolished, by the presence of sodium sulfide or the powerful complexing agent versene (tetrasodium salt of ethylenediaminetetraacetic acid). This demonstrates that traces of heavy metals are not needed for the reaction. The reproducibility of the curves for the uncatalyzed hydrolysis of DFP and DEFP was within the limits of about ±20% deviation from the average values.

Retention Controls.—In order to determine the amount of CO_2 which is retained in the presence of imidazole or pyridine and their derivatives, the following experiments have been performed. DEFP was hydrolyzed in distilled water at 38° for 72 hours in a glass-stoppered test-tube, coated with paraffin. Measured amounts of this solution containing an equimolar mixture of HF and diethylphosphoric acid were reacted with the listed bases, dissolved in bicarbonate buffer at *p*H 7.6.

TABLE VIII

CO_2-RETENTION IN BICARBONATE BUFFER AT *p*H 7.6 AND 38° IN THE PRESENCE OF HYDROLYZED DEFP AND DIFFERENT BASES

Mole	Found: CO_2, mm.³	CO_2, %	CO_2 retention, %
5 × 10^{-6} hydrolyzed DEFP	226	100	..
$+50 \times 10^{-6}$ pyridine	228	100	0
$+50 \times 10^{-6}$ 3-aminopyridine	218	96.5	3.5
$+50 \times 10^{-6}$ histidine	195	86.9	13.6
$+50 \times 10^{-6}$ 3-hydroxypyridine	173	76.6	23.4
$+50 \times 10^{-6}$ imidazole	158	70.0	29.6
$+50 \times 10^{-6}$ 1-methylimidazole	146	64.6	35.4

Stability of Histidine in the Presence of DFP. (a).—52.4 mg. of histidine (250 μmoles) and 0.00875 ml. of DFP (50 μmoles), dissolved and made up to 11 ml. with $NaHCO_3$-CO_2 buffer, *p*H 7.6, was maintained at room temperature (27°). Macpherson's modification of the Pauly reaction[19] was used for the determination of histidine with the aid of a Klett-Summerson photoelectric colorimeter. Found: 52.6 mg. histidine after 67 hours, 52.5 mg. histidine after 168 hours.

(b).—Similar conditions to above, but 250 μmoles of DFP per 250 μmoles (52.4 mg.) of histidine. Found: 52.0 mg. histidine after 0.5 hour, 52.7 mg. histidine after 90 hours.

Diisopropylphosphorylimidazole (DPI).—To a solution of 3.73 g. of imidazole in 10 ml. of dry chloroform a mixture of 4.5 ml. of diisopropyl chlorophosphate and 5.5 ml. of dry chloroform was added, in portions. A crystalline product separated out with the evolution of heat; after washing with dry ether, it was observed to decompose at 155–161°. After adding the washing ether to the filtrate, the solution was kept overnight at −20° in order to crystallize the remainder; the total yield was 2.2 g. For identification the crude hydrochloride was treated with dry ammonia in chloroform; imidazole could be obtained in good yield.

The concentrated filtrate, on distillation in a high vacuum, gave two fractions, about 1.5 ml. boiling at 95–106.5° (0.3 mm.), and 2.2 ml. boiling at 107° (0.3 mm.). Both fractions were soluble in water and were not miscible with petroleum ether. With aqueous $CuSO_4$ they gave the same violet-blue color as does imidazole. The higher boiling fraction, which had an $n^{26}D$ 1.4503, was analyzed (this sample was prepared by Dr. R. Plapinger).

Anal. Calcd. for $C_9H_{17}O_3N_2P$ (232.2): N, 12.1; P, 13.3. Found: N, 12.4; P, 13.3.

Hydrolysis.—When an aqueous solution of DPI is kept at room temperature for 24 hours the original *p*H drops from about 8.5 to *p*H 6.7. The rate of hydrolysis was measured manometrically in a sodium bicarbonate buffer. Only about half a mole of CO_2 was liberated per mole of diisopropylphosphorylimidazole, at the end of the hydrolysis, owing to CO_2 retention due to buffer capacity of the imidazole–diisopropylphosphate.

TABLE IX

HYDROLYSIS OF 5 × 10^{-6} MOLE DIISOPROPYLPHOSPHORYLIMIDAZOLE IN 2.2 ML. BICARBONATE BUFFER AT *p*H 7.6 AND 30°

Time, min.	30	60	90	185	210	1260
CO_2 evolved, mm.³	15	16	23	37	39	70

In order to isolate the product of hydrolysis undistilled diisopropylphosphorylimidazole was kept in an open flask for several days; the sirupy mass was extracted several times with petroleum ether and then kept at −20° with protection from moisture. The crystallized imidazole salt of diisopropylphosphoric acid melted at 48–50°. It was very hygroscopic.

Anal. Calcd. for $C_9H_{19}O_4N_2P$ (250.2): N, 11.2. Found: 11.3.

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(19) R. J. Block and D. Bolling, "The Amino Acid Composition of Proteins and Foods," Second Ed., Charles C Thomas, Publisher, Springfield, Ill., 1951, p. 53.