Inclusion Compounds. XVIII.[1] The Catalysis of the Fission of Pyrophosphates by Cyclodextrin. A Model Reaction for the Mechanism of Enzymes

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The hydrolysis of the diaryl pyrophosphates in the presence of calcium ions is catalyzed by cyclodextrins. During this reaction a transfer of the phosphate residue to the cyclodextrin in a diluted aqueous solvent (10^{-3} M) takes place. Inside the inclusion compound dextrin–pyrophosphate OH groups of the sugar are in close proximity with the phosphate thus they can take over a phosphate residue. It has been proved that the internal phosphate residue of the inclusion compound is transferred to the dextrin. Further parallels between enzyme catalysis and inclusion catalysis are discussed.

The "lock and key" hypothesis of Fischer[2] for the description of enzyme specificity as well as the later hypotheses like the "template mechanism"[3] or "induced fit"[4] all require a hollow space in which the substrate is embedded. This hollow space may be rigid or adaptable to the substrate molecule.

As soon as the substrate molecule finds itself in the "hole" it is in the right position with respect to the active centers and, as in the case of hydrolysis, it will be cleaved.

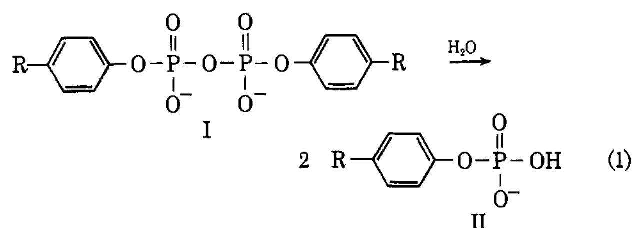
In the preceeding communication[1] we have shown with the aid of the decarboxylation reaction the analogy between enzyme catalysis and inclusion catalysis by cyclodextrin. These considerations will now be continued according to the example of the cleavage of the pyrophosphate bond.

Symmetrical diesters of pyrophosphate with phenols are very stable in neutral and alkaline solution.[5] However, in the presence of divalent metal ions they are slowly cleaved at reasonable rates.[6-9] We now find that this cleavage is accelerated catalytically by cyclodextrin to a considerable extent.[10,11] If a noncyclic glycoside (α-methylglucoside) is added instead of cyclodextrin, no catalysis is observed; on the contrary the reaction rate diminishes.

(1) (a) Part XVII: F. Cramer and W. Kampe, *J. Am. Chem. Soc.*, 87, 1115 (1965); (b) this work was supported by Deutsche Forschungsgemeinschaft, Bad Godesberg; Rockefeller Foundation, New York, N. Y.; Verband der Chemischen Industrie, Düsseldorf; and Research Corporation, New York, N. Y.
(2) E. Fischer, *Chem. Ber.*, 27, 2985 (1894); *Z. Physiol. Chem.*, **26**, 60 (1898).
(3) D. E. Koshland, Jr., in "The Enzymes," Vol. I, P. D. Boyer, H. Lardy, and K. Myrback, Ed., Academic Press Inc., New York, N. Y., 1959, p. 305.
(4) J. A. Thoma and D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U. S.*, **44**, 98 (1958); *J. Am. Chem. Soc.*, **82**, 3329 (1960).
(5) See F. Cramer and R. Wittmann, *Chem. Ber.*, **94**, 322 (1961).
(6) H. Trapmann, *Arzneimittel-Forsch.*, **9**, 341, 403 (1959).
(7) E. Bamann and M. Meisenheimer, *Chem. Ber.*, 71, 2233 (1938).
(8) E. Cherbuliez, J.-P. Leber, and P. Stucki, *Helv. Chim. Acta*, **36**, 537 (1953).
(9) J. K. Lowenstein, *Biochem. J.*, **70**, 222 (1958).
(10) F. Cramer, *Angew. Chem.*, 73, 49 (1961), first experiments; W. Dietsche, Thesis, University of Heidelberg, 1958.
(11) N. Hennrich and F. Cramer, *Chem. Ind.* (London), 1224 (1961).

Methods

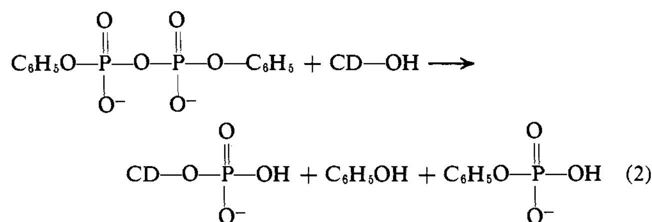
(1) Course of the Reaction. Reaction 1 can be



followed qualitatively by paper chromatography and quantitatively by titration. The phosphate formed from pyrophosphate during hydrolysis can be titrated acidimetrically. The presence of cyclodextrins has virtually no influence on the titration as long as its concentration is of the same order as that of the substrate and product, respectively. The error of this method is about 10%. The reaction usually is carried out for several days and is therefore performed in a closed vessel in order to avoid CO_2 absorption. Samples are taken at intervals.

A typical reaction is shown in Figure 1. According to eq. 1 two molecules of alkali are needed to break one pyrophosphate bond. Chromatographic investigations of the reaction products show the following results. The noncatalyzed reaction strictly follows eq. 1 and monophenyl phosphate is the only reaction product.

The reaction catalyzed by cyclodextrin (CD–OH) has an altogether different course (eq. 2). On cleavage one obtains from one molecule of diphenyl pyrophosphate only one molecule of monophenyl phosphate, one molecule of phenol, and one molecule of a substance which is a monophosphorylated cyclodextrin, according to its chemical properties, chromatographic characteristics, and elementary analysis.



In the over-all reaction of eq. 2, two molecules of acid are formed, as in the noncatalyzed reaction; thus the titration gives comparable values. In the presence of α-methylglucoside the reaction runs according to eq. 1. It must be stated that catalyzed reactions run according to different mechanisms. Before these mechanisms are discussed in detail, substrate specificity

Table I. Reaction Rate Constants of Pyrophosphate Cleavage[a,b]

Substituent R in I	Ca²⁺ ion alone	Ca²⁺ ion With 1 mole of α-dextrin	With 1 mole of β-dextrin	With 1 mole of γ-dextrin	With 7 moles of α-methylglucoside
H	7.4	11.5 (1.5)	32.9 (4.4)	18.0 (2.4)	3.4 (0.45)
p-CH_3	4.8	5.5 (1.1)	44.4 (9.2)	28.7 (6.0)	
p-Cl	<0.5	15.1 (>30)	100.0 (>200)	30.8 (>60)	

[a] $k \times 10^{-2}$; the figures given in parentheses are catalytic acceleration factors. [b] Concentration of substrate, calcium chloride, and cyclodextrin = 2.5×10^{-3} M; pH 12.0; 40°.

and other characteristics of the reaction will be considered.

(2) *Substrate Specificity of Catalysis.* Previous chromatographical investigations had shown that zinc(II) is about equally effective as Ca^{2+}. Magnesium(II) and manganese(II) ions are a little less effective. In the quantitative titration, Ca^{2+} gives the least trouble, the solution remains clear, and the end point of the titration is precise. Therefore Ca^{2+} was used as cocatalyst in all experiments.

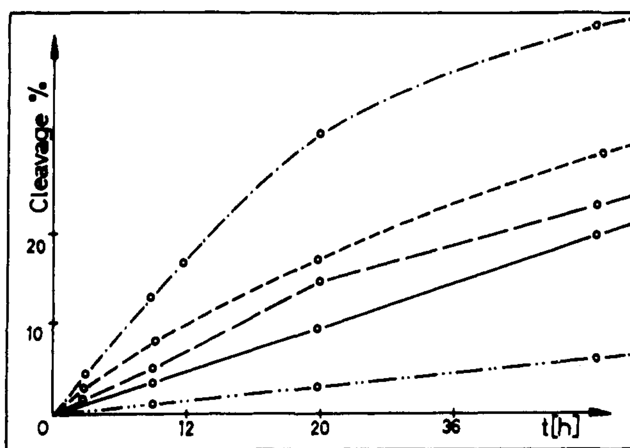


Figure 1. Cleavage of diphenyl pyrophosphate (I, R = H, 0.3123 $\times 10^{-2}$ M) at pH 12 and 40°: curve 1, —, in the presence of 0.3125 $\times 10^{-2}$ M Ca^{2+} only; curve 2, – –, with additional 0.3125 $\times 10^{-2}$ M α-dextrin; curve 3, - · - · - ·, with additional 0.3125 $\times 10^{-2}$ M β-dextrin; curve 4, - - -, with additional 0.3125 $\times 10^{-2}$ M γ-dextrin; curve 5, - · · · · · · · ·, with additional 2.19 $\times 10^{-2}$ M α-methylglucoside.

Catalyzed and noncatalyzed reactions are, under the reaction conditions employed, both of first order or rather pseudo-monomolecular, at least at the beginning of the reaction. The deviations from the first order which occur during the reaction will be discussed later. In Table I the comparable k values for 10% cleavage ($k_{10\%}$ value) are given. A typical reaction curve is shown in Figure 1. In all cases β-dextrin (ϕ = 8 Å.) is the best catalyst, α-dextrin (ϕ = 6 Å.) is the least effective, and γ-dextrin (ϕ = 10 Å.) has a middle position. Observations on the Stuart–Calotte models show that an axial[12] inclusion of a phenyl group or of a substituted phenyl group is possible for all three cyclodextrins, but an equatorial inclusion with α-dextrin (hollow space, ϕ = 6 Å.) is difficult even in the case of nonsubstituted diphenyl pyrophosphate. It must of course always be assumed that only *one* phenyl residue is enveloped by *one* cyclodextrin, so that the pyrophosphate is enclosed on one side only. The substrate dependency of the catalysis is especially evident looking at p-tolyl pyrophosphate. Its cleavage by α-dextrin can hardly be measured. The weak effect of γ-dextrin can be explained by the fact that in a too large cavity of γ-dextrin, the fixation is not sufficiently defined. Thus the OH groups of the cyclodextrin, which must be in suitable positions, cannot become effective catalytically (*cf.* section 5). Especially powerful is the effect on 4-chlorophenyl pyrophosphate. We know from earlier observations that halogen-substituted compounds are very easily included.[13,14] The formation of the inclusion compounds, though necessary, is not a sufficient condition for the catalysis. This fact becomes evident by the example of p-chlorophenyl pyrophosphate. The calcium salt of this acid is rather insoluble in water. In the standard mixture (see Experimental section), 60% of the substance is dissolved at pH 12; on adding an equimolar quantity of α-dextrin, the solubility rises to 77% through formation of the inclusion compounds; with β-dextrin it rises to 85% and with γ-dextrin to 93%. However, γ-dextrin is still far less effective than β-dextrin (see Table I), *therefore the catalysis cannot be explained only by the solvation by cyclodextrin.* α-Methylglucoside as noncyclical saccharide reduced the reaction rate because of the known complex formation of calcium ions with the hydroxyl groups of the sugars.[15]

(3) *Concentration Dependency of the Catalysis. Variation of Reaction Conditions.* In the experiments

Table II. Reaction Rate Constants of Diphenyl Pyrophosphate Cleavage[a,b]

Ca^{2+} $\times 10^{-3}$ M	Ca^{2+}: dextrin	Without dextrin[b]	Ca^{2+} With α-dextrin[b]	With β-dextrin[b]
3.125	1 : 1	7.4	11.5 (1.5)	32.9 (4.4)
1.56	0.5:1	1.5	8.6 (5.7)	17.1 (12.0)
1.04	0.33:1	<0.5	5.1 (>10)	13.7 (>27)
0.78	0.25:1	0	4.2 (∞)	10.8 (∞)
0.625	0.20:1	0	4.9 (∞)	9.8 (∞)
0.312	0.1:1	0	2.4 (∞)	5.4 (∞)

[a] Concentration of pyrophosphate and cyclodextrin = 3.125 $\times 10^{-3}$ M, of Ca^{2+} according to first column, pH 12, 40°. [b] $k_{10\%}$ $\times 10^{-2}$; the figures given in parentheses are catalytic acceleration factors.

(12) W. Kampe, Thesis, University of Heidelberg, 1961.

(13) F. Cramer, "Einschlussverbindungen," Springer-Verlag, Heidelberg, 1954.
(14) F. Cramer and F. M. Henglein, *Chem. Ber.*, **90**, 2561 (1957).
(15) Compare "Methoden der organischen Chemie," Vol. I/1, Houben-Weyl, Ed., 4th Ed., Georg Thieme Verlag, Stuttgart, 1958, p. 427.

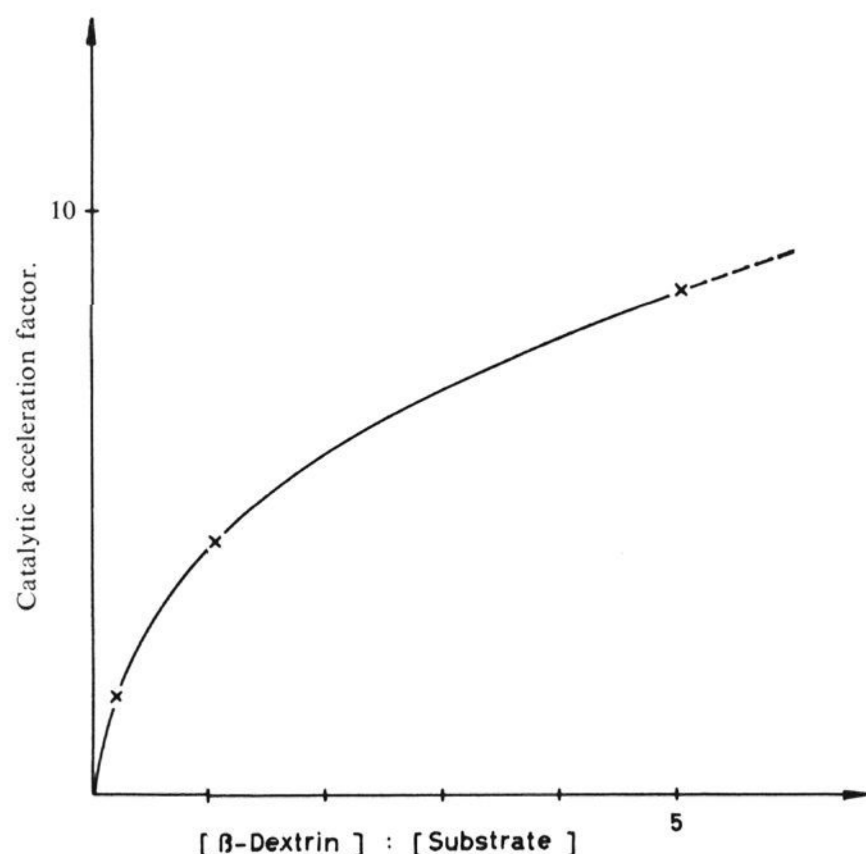


Figure 2. Concentration of pyrophosphate = $[CaCl_2]$ = 3.125 × 10^{-3} *M*, pH 12, 40°; catalytic acceleration factors in dependency of cyclodextrin excess.

described so far, we have used substrate (pyrophosphate), co-catalyst (Ca^{2+}), and microheterogeneous catalyst (cyclodextrin) in an equimolar proportion. In this system now, one component was varied, while the remaining two were kept constant. In Table II the results of the variation of the calcium concentration are shown. Below 0.25 *M* Ca concentration, there is no cleavage of pyrophosphate without cyclodextrin. Here, we find for the first time an example of "absolute catalysis" by inclusion compounds. From Figure 2, one can see that the catalytic effect of β-dextrin does not increase proportionally to its concentration. When all substrate molecules have occupied the active centers, *i.e.*, the inclusion cavity (this is the case with a 10-fold dextrin excess[16]), a further addition of catalyst can have no more effect. At low cyclodextrin concentration, its catalytic activity decreases rapidly in the course of the reaction,[17] because cyclodextrin is phosphorylated according to eq. 2 whereby catalyst is withdrawn from the reaction. Also the reaction product, monophenyl phosphate,

Table III. Product Inhibition of Inclusion Catalysis by β-Dextrin[a]

Diphenyl pyrophosphate, %	Monophenyl phosphate, %	Relative splitting rate[a,b]
100	0	4.4
90	10	3.4
60	40	1.7
50	50	1.5

[a] Concentration of β-dextrin and calcium chloride = 3.125 × 10^{-3} *M*, of diphenyl pyrophosphate in the first line also, in lines 2–4 accordingly less; pH 12, 40°. [b] Related to the noncatalyzed reaction is one.

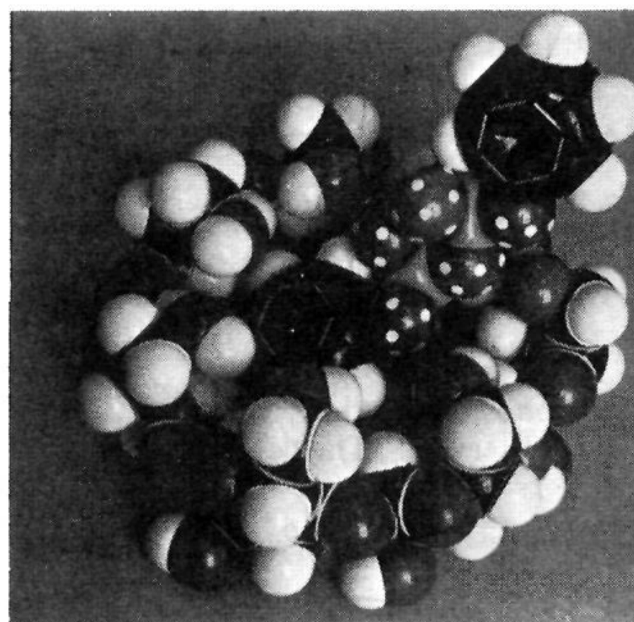


Figure 3. Model of the inclusion compound β-dextrin–diphenyl pyrophosphate. The oxygen atoms of the pyrophosphate bear white dots for better differentiation.

is an inhibitor because it forms an inclusion compound as well, and it therefore occupies "active centers," as shown in Table III. Therefore our catalysis shows, as the enzyme catalysis, the phenomenon of product inhibition.

For the determination of the activation energy of the catalysis, the temperature dependency of the reaction was measured. Results are shown in Table IV. From these data the following values for ΔH resulted (in kcal.): calcium alone, 14.5; with α-dextrin, 14.8; and with β-dextrin, 12.0. The reduction of activation energy by catalysis is not very significant.

Table IV. Temperature Dependency of Diphenyl Pyrophosphate Cleavage[a,b]

Temp., °C.	Ca^{2+} Alone	Ca^{2+} With α-dextrin	Ca^{2+} With β-dextrin
20	(1.1)[c]	4.0	8.4
30	5.0	7.2	14.8
40	7.4	11.5	32.4
50	15.3	25.6	51.2
70	64.0	96.0	165.0

[a] $k_{10\%} \times 10^{-2}$. [b] Concentration of substrate, calcium chloride, and dextrin = 3.125 × 10^{-3} *M*; pH 12. [c] This value is not accurate because of the slowness of the reaction, and it was not considered for the determination of ΔH.

(*4*) *Reaction Mechanism.* The reaction catalyzed by cyclodextrin proceeds according to eq. 2 as demonstrated. Because of the proximity of an OH group of dextrin in the inclusion compound, one-half of the pyrophosphate is transferred to dextrin. The Stuart model (Figure 3) shows this clearly. During reaction, first an ester of the monophenylphosphoric acid with cyclodextrin (III) must be formed. This ester now turns, under splitting off of phenol, into a cyclophosphate (IV), which is immediately hydrolyzed (eq. 3) into a simple phosphate ester (V) at pH 12. This is analogous to many examples from the nucleotide chemistry.[18] This second sequence of reaction steps is faster than

(16) Because of the limited solubility of the β-dextrin, only up to fivefold dextrin excess can be measured.
(17) This is the reason for the limitation on the $k_{10\%}$ values.
(18) Compare for instance, D. M. Brown and A. R. Todd, *J. Chem. Soc.*, 52 (1952).

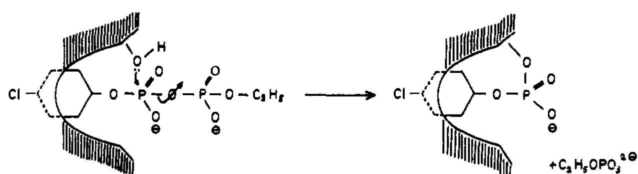


Figure 4. Effect of cyclodextrin catalyst (schematic illustration).

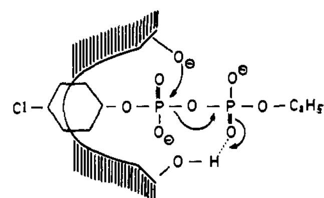
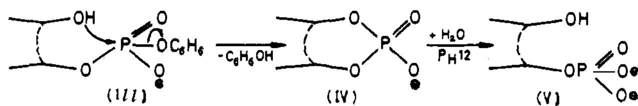


Figure 5. Schematic illustration of a push–pull mechanism of the cyclodextrin catalysis by hydroxy groups on one side and hydrogen bondings on the other side.

the primary phosphorylation; therefore, only the end product V is observed. The catalytic step, the transfer



of the phosphate residue to the dextrin, is the primary reaction. For this reaction there are the following possibilities.

(1) The inside phosphate group, which is adjacent to the enclosed phenyl group, phosphorylates the dextrin; the outside one leaves as a phosphate ion.

(2) The outside phosphate group, which is adjacent to the nonenclosed phenyl group phosphorylates, and the inner one is split off as phosphate ion.

For each of these two versions there are again two possibilities with respect to the influence of cyclodextrin on the substrate: (a) SN2 reaction of an CH_2OH group of dextrin on the phosphate group (Ca^{2+} ion lowers the resonance energy of the pyrophosphate ion, the phosphorylation of dextrin is simply a neighboring group reaction (Figure 4), and also, transition states might be considered of the sort as indicated in Figure 5); (b) influence of dextrin on the substrate molecule by hydrogen bonding which polarize the electronic system of pyrophosphate in one direction and render the compound unstable (Figure 5).

Mechanism 1 can be proved as follows. Instead of a symmetrical substituted pyrophosphate, the unsymmetrical P^1-chlorophenyl P^2-ethyl phosphate (dilithium salt[19]) was used. It can be assumed that in the cavity of cyclodextrin, only the aromatic ring and not the ethyl group is enclosed since its affinity to the cavity is certainly greater. During the cleavage reaction, the following different products should be observed depending on whether the inner or outer phosphate ester is attacked by the dextrin.

case no. 1	phosphorylated dextrin ethyl phosphate 4-chlorophenol
case no. 2	phosphorylated dextrin ethanol 4-chlorophenyl phosphate

The run was carried out under the usual conditions and was followed chromatographically. It was compared with a reaction carried out with phosphate alone.

The Ca^{2+}-catalyzed reaction (without cyclodextrin) showed as products 4-chlorophenyl phosphate and ethyl phosphate in equal proportions, while the reaction catalyzed with dextrin gave ethyl phosphate only, 4-chlorophenol, and phosphorylated dextrin. This proves that the inner part of the pyrophosphate, namely the 4-chlorophenyl phosphate part (according to Figure 3), is transferred to cyclodextrin.

As to the further questions whether dextrin works only by a neighboring group or additionally by polarization, we can so far only speculate. Cyclodextrins can, however, change the dissociation constants of the included phosphoric acids by hydrogen bonds. The p*K* value of the first dissociation of monophenyl phosphoric acid falls by 2 units in the presence of cyclodextrin.[20] Since the OH groups of cyclodextrin dissociate from pH 12 on, one might discuss here a push–pull reaction as indicated in Figure 5. This speculation, however, leaves the role of Ca^{2+} ion unconsidered. An alternative possibility has already been discussed elsewhere.[11]

Experimental

A. Materials and Methods. (*a*) *The pyrophosphates* were prepared according to Cramer and Winter[21]; the *cyclodextrins* were obtained from starch by *Bac. macerans* amylase according to Cramer and Henglein.[22] All runs were followed chromatographically; the solvent used for phosphates and pyrophosphates was 1-propanol–NH_3–H_2O (6:3:1). Paper No. 2261 (stamped) was obtained from Machery, Nagel, and Co. For preparative chromatography, cardboard paper no. 2827 of the same firm was used. The chromatograms ran for 15 hr. at 22°. Development was accomplished with Hanes–Isherwood spray reagent. R_f values are given in Table V.

Table V. R_f Values

Diphenyl pyrophosphate	0.70–0.73
Ditolyl pyrophosphate	0.78[a]
Di(*p*-chlorophenyl) pyrophosphate	0.84
Monophenyl phosphate	0.39–0.41
Monotolyl phosphate	0.46–0.48
Mono(*p*-chlorophenyl) phosphate	0.54–0.55[a]
Monoethyl phosphate	0.26
Monophenyl pyrophosphate	0.30–0.32
Inorganic trimetaphosphate	0.25–0.26
Inorganic phosphate	0.15–0.17
Inorganic pyrophosphate	0.10–0.11

[a] Visible in ultraviolet.

(*b*) *Quantitative Determinations.* A standard mixture (2 ml.) (3.125×10^{-3} *M*) was used. After chromatography, the ultraviolet absorbing spots with R_f

(19) Prepared by M. Winter, Darmstadt; compare F. Cramer and M. Winter, *Chem. Ber.*, **94**, 989 (1961).

(20) F. M. Henglein, Thesis, University of Heidelberg, 1957.

(21) F. Cramer and M. Winter, *Chem. Ber.*, **92**, 2761 (1959).

(22) F. Cramer and F. M. Henglein, *ibid.*, **91**, 308 (1958).

Table VI. Reaction with Cyclodextrins with Varying Ca^{2+} Concentrations[a]

Time,	0.3125 × 10^{-3} *M*			0.625 × 10^{-3} *M*			0.78 × 10^{-3} *M*			1.04 × 10^{-3} *M*			1.56 × 10^{-3} *M*			3.125 × 10^{-3} *M*				
hr.	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	D	E
3	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	2.25	4.8	3.85	..
9	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	4.5	5.8	13.8	8.95	0.7
15	..	..	..	..	5.4	6.7	..	..	..	..	..	..	..	..	..	..	..	..	..	..
18	..	..	..	..	..	..	..	5.45	8.65	..	6.1	10.9	..	8.65	15.4	..	..	..	..	..
24	..	3.5	6.1	..	..	..	..	..	..	..	..	..	..	..	..	9.6	15.5	32.0	17.0	3.8
42	..	..	..	..	9.6	17.3	..	..	..	..	..	..	..	..	..	..	..	..	..	..
45	..	..	..	..	..	..	..	8.33	18.2	..	9.6	23.4	..	13.1	27.5	..	..	..	..	..
48	0.7	5,75	10.9	0.7	..	..	..	..	..	1.3	..	..	..	..	..	..	..	..	..	..
49	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	20.2	24.0	43.5	29.4	6.4
65	1.3	..	..	..	..	..	0.3	..	..	..	..	..	4.5	..	..	..	..	..	..	..
69	..	..	..	..	..	..	..	11.5	24.6	..	13.1	31.4	..	18.2	36.2	..	..	..	..	..
72	..	8.65	14.4	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
87	..	..	..	..	14.7	28.8	..	..	..	..	..	..	..	..	..	..	..	..	..	..
93	..	..	..	..	..	..	..	13.7	29.8	..	17.3	36.5	..	24.3	41.5	..	..	..	..	..
96	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	38.4	35.6	55.8	..	12.8
102	..	..	..	..	..	..	..	..	..	2.6	..	..	..	..	..	..	..	..	..	..
113	1.9	..	..	1.9	..	..	1.0	..	..	..	..	..	6.4	..	..	..	..	..	..	..
120	..	11.5	19.8	..	15.7	32.0	..	..	..	..	..	..	..	..	..	..	..	..	..	..
143	..	..	..	..	18.5	36.5	..	..	..	..	..	..	..	..	..	..	..	..	..	..
161	0	..	..	0.7	..	..	..	..	..	4.5	..	..	..	..	..	..	..	..	..	..
167	..	..	24.0	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
168	..	..	..	..	..	..	..	..	..	..	..	..	11.2	..	..	..	..	..	..	..
184	..	..	..	..	20.8	39.4	..	..	..	..	..	..	..	..	..	..	..	..	..	..
208	..	..	..	..	..	..	..	..	..	..	..	..	..	28.2	49.0	..	..	..	..	..
215	..	14.1	27.2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..
216	0.7	..	..	1.0	..	..	1.9	..	..	5.75	..	..	15.3	..	..	..	..	..	..	..

[a] Percentage of cleavage, conditions as described under method B: A, reaction with Ca^{2+} alone; B, with Ca^{2+} and α-dextrin; C, with Ca^{2+} and β-dextrin; D, with Ca^{2+} and γ-dextrin; and E, with Ca^{2+} and α-methylglucoside.

Table VII[a]

			$CaCl_2$	
PP	O.D. = 0.412	75.3% PP	Relating to 24.7% cleavage	Proportion PP split to MP formed, 1.2:1
MP	O.D. = 0.358	21.0% MP	Relating to 21.0% cleavage product	
			$CaCl_2$ + β-Dextrin	
PP	O.D. = 0.326	59.5% PP	Relating to 40.5% cleavage	Proportion PP split to MP formed, 1.8:1
MP	O.D. = 0.373	22.2% MP	Relating to 22.2% cleavage product	
			$CaCl_2$ + α-Methylglucoside	
PP	O.D. = 0.417	76.5% PP	Relating to 23.5% cleavage	Proportion PP split to MP formed, 0.8:1
MP	O.D. = 0.488	29.0% MP	Relating to 29.0% cleavage product	

[a] Abbreviations used: PP = diphenyl pyrophosphate, MP = monophenyl phosphate.

0.7 and 0.33 were eluted for 20 hr. with 5 ml. of 0.1 *N* NH_3, then filled up to 10 ml., and determined colorimetrically at 260 mμ. The solvent for the cyclodextrins and sugar[23] was *sec*-butyl alcohol–pyridine–water (1:1:1). Chromatograms ran for 15 hr. at 22°, ascending, using as spray: for α-dextrin, 0.2% iodine in 85% aqueous ethanol; for β- and γ-dextrin, 0.01% crystal violet in 85% aqueous ethanol; and for sugars, 1.66 g. of phthalic acid and 0.93 g. of aniline in 100 ml. of butanol saturated with water. The following R_f values were obtained: α-dextrin, 0.59–0.62; β-dextrin, 0.58–0.61; γ-dextrin, 0.47–0.50; phosphoryl-β-dextrin, 0.21–0.25; glucose, 0.75; and maltose, 0.70.

Preparative Paper Chromatography. From a run (6.25 × 10^{-3} *M*) 100 ml. was spotted on a starting line after 48-hr. reaction. After chromatography, the spot was cut out over the complete width of the paper. The substance was eluted with water for 24 hr. and the solution evaporated to dryness at 40°.

pH Measurements and Potentiometric Titrations. The pH measurements were carried out with a commercial pH meter; titrations using an automatic titrator of Radiometer Co., Copenhagen, Type TTT-1-a.

(23) Compare F. Cramer and D. Steinle, *Ann. Chem.*, **595**, 81 (1955).

B. Fission of Diphenyl Pyrophosphate, Course of Reaction, and Standard Conditions of Hydrolysis (Figure 1). A solution (40 ml.) containing 3.125 × 10^{-3} mole $l.^{-1}$ of diphenyl pyrophosphate dilithium salt, calcium chloride, and cyclodextrin was brought to pH 12.0 ± 0.1 and kept at a temperature of 40 ± 0.5°. At intervals 5 ml. was removed and titrated to pH 7 with 0.01 *N* HCl. The result is shown in Figure 1. The *k* values were calculated from the rates up to 10% fission (see Table VI) ($k_{10\%}$ value). For the reaction with α-methylglucoside, 7 × 3.125 × 10^{-3} *M* solution was used (equal weight of carbohydrate and β-dextrin).

C. Reaction Products. Mechanism of the Reaction. (*a*) *Phosphoryl-β-dextrin.* When taking samples from a cyclodextrin-catalyzed reaction mixture for a chromatogram, one can observe, apart from monophenyl phosphate, a spot with R_f 0.21–0.25 which contains both cyclodextrin and phosphate. In a larger run with β-dextrin, this substance was isolated by paper

chromatography (compare section A (b): white powder, infrared spectrum nearly identical with β-dextrin, no phenyl absorption at 1510 cm.$^{-1}$.

Anal. Calcd. for $C_{42}H_{69}O_{35} \cdot PO_3Na_2$ (mol. wt., 1258): C, 40.0; H, 5.62; P, 2.48. Found: C, 39, 39; H, 4.92; P, 2.36.

(*b*) *Quantitative Determination of the Reaction Products.* A standard run with β-dextrin was stopped after 24 hr. (32% cleavage) and the reaction products were determined by the following *phenol* method. Standard solutions were prepared by diluting 10^{-3} *M* phenol solution 2-fold, 5-fold, and 20-fold. Diazotized sulfanilic acid (0.1%) was added and the color determined photometrically (425 mμ, Perkin-Elmer 4000). The standard curve thus obtained was used. A 32% cleavage of pyrophosphate gave 29% phenol. A run without β-dextrin (interrupted after 20% cleavage) showed 1.5% phenol.

(*c*) *Determination of Diphenyl Pyrophosphate and Monophenyl Phosphate from the Chromatogram. Determination of the Proportion of Diphenyl Pyrophosphate to Monophenyl Phosphate on Cleavage.* Ultraviolet absorption of diphenyl pyrophosphate showed λ_{max} 260 mμ (ϵ 8.75 × 10^2); monophenyl phosphate showed λ_{max} 266 mμ (ϵ 6.75 × 10^2). The chromatograms of the run (6.25 × 10^{-4} *M*) were eluted quantitatively according to section A(b). The solutions of the eluate would be on complete cleavage 2 × 6.25 × 10^{-4} mole of monophenyl phosphate (O.D. = 1.68, *d* 2.0 relating to 100%); without cleavage, 6.25 × 10^{-4} mole of diphenyl pyrophosphate (O.D. = 0.546, *d* = 1.0, relating to 100%). The values found are shown in Table VII.

A Highly Reactive Colored Reagent with Selectivity for the Tryptophan Residue in Proteins. 2-Hydroxy-5-nitrobenzyl Bromide[1]

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The environmentally sensitive protein reagent, 2-hydroxy-5-nitrobenzyl bromide, was shown to react rapidly with tryptophan in aqueous solutions over a wide range of pH. In acidic or neutral solutions it was highly specific in its reaction; the only other amino acid which was modified by the reagent was cysteine, whose reactivity was no more than one-fifth that of tryptophan. In alkali, tyrosine and cysteine were found to react to almost the same extent as tryptophan. Methionine did not form a stable derivative with the reagent in aqueous media, although it did catalyze the tryptophan reaction. The high order of reactivity of 2-hydroxy-5-nitrobenzyl bromide and its specificity for tryptophan are properties which contrast with those of 2-methoxy-5-nitrobenzyl bromide or unsubstituted benzyl bromide, and apparently involve the participation of the o-hydroxyl substituent in activation of the reagent. Environmental sensitivity of its absorption spectrum, ease of reaction with amino acids under mild conditions, and specificity for tryptophan contribute to the usefulness of 2-hydroxy-5-nitrobenzyl bromide as a protein-modification reagent.

One of the most important tools in the correlation of protein structure with function is the protein reagent. In general, the more specific the reagent the more valuable it is, although "broad spectrum" reagents are useful also. For the delineation of the role of tryptophan a number of reagents which fit in the latter category are known. Weil and co-workers have used photooxidation which modifies tryptophan, histidine, methionine, cysteine, and tyrosine.[2] Witkop and co-workers[3] have described the valuable reagent N-bromosuccinimide which modifies tryptophan, histidine, tyrosine, SH, and probably methionine.[4] Iodination under appropriate circumstances modifies tryptophan and also tyrosine, methionine, and cysteine.[5,6]

In a previous publication the synthesis and reactivity of a new reagent, 2-hydroxy-5-nitrobenzyl bromide, hereafter designated as φ′ Br,[7] were described.[8] It was found that in acid and neutral solutions this reagent reacted readily with tryptophan residues of chymotrypsin, and did not react with any other of the amino acids. The reagent absorbs in a region of the spectrum in which the protein is transparent and its spectral absorption is sensitive to environment. Although the reagent was not absolutely specific (it showed a sluggish

(1) Research was carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

(2) L. Weil, S. James, and A. R. Buchert, *Arch. Biochem. Biophys.*, **46**, 266 (1953).

(3) T. Viswanatha, W. B. Lawson, and B. Witkop, *Biochim. Biophys. Acta*, **40**, 216 (1960); T. Viswanatha and W. B. Lawson, *Arch. Biochem. Biophys.*, **93**, 128 (1961).

(4) Although it has not been reported that methionine is oxidized by N-bromosuccinimide, it is known that most oxidizing agents, including ICl, do oxidize this residue. Since it has been demonstrated [W. J. Ray, Jr., and D. E. Koshland, Jr., *J. Biol. Chem.*, **237**, 2493 (1962)] that methionine is regenerated from methionine sulfoxide during acid hydrolysis, it seems probable that the oxidation of this residue has not been observed in the past because of the acid treatments prior to analysis.

(5) L. K. Ramachandran, *Chem. Rev.*, **56**, 199 (1956).

(6) M. E. Koshland, F. M. Englberger, M. J. Erwin, and S. M. Gaddone, *J. Biol. Chem.*, **238**, 1343 (1963).

(7) Abbreviations: φ′ Br, 2-hydroxy-5-nitrobenzyl bromide; φ′OH, 2-hydroxy-5-nitrobenzyl alcohol; φ′-Try, 2-hydroxy-5-nitrobenzyl derivative of tryptophan, precise structure unknown; φ′-Cys, 2-hydroxy-5-nitrobenzyl derivative of cysteine.

(8) D. E. Koshland, Jr., Y. D. Karkhanis, and H. G. Latham, *J. Am. Chem. Soc.*, **86**, 1448 (1964). By raising the temperature of bromomethylation it has been possible to obtain the bisbromomethyl derivative (m.p. 155–156.5). Control of the temperature at 70° is, therefore, desirable when high yields of the monobromomethyl derivative are desired.