[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

The DL-2,3-Dihydroxyisobutyric Acid Monophosphates

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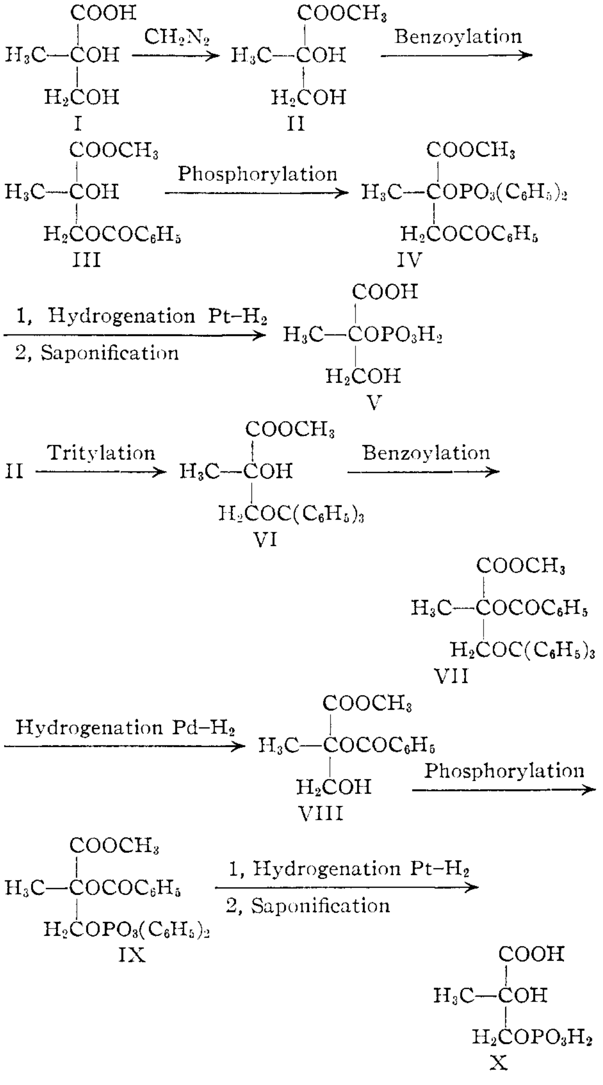
The syntheses of DL-2,3-dihydroxyisobutyric acid 2- and 3 phosphate are described. The compounds have been studied as substrate analogs in the *enolase* and *glyceric acid phosphate mutase* reaction and have also been used in obtaining data on the relative rates of hydrolysis of various phosphate esters in neutral and acid solutions. The 2-phosphate is hydrolyzed with remarkable ease between *p*H 3 and 7, a result related to the fact that it is a phosphate ester of a tertiary alcohol group located adjacent to an ionized carboxyl group.

In connection with the study of substrate specificity of the enzymes *enolase* and *glyceric acid phosphate mutase*, the 2-phosphate (V) and 3-phosphate (X) of DL-2,3-dihydroxyisobutyric acid have been synthesized. This paper describes the synthesis and some properties of the two phosphate esters.

DL-2,3-Dihydroxyisobutyric acid (I) was prepared from chloropropanone according to the method of Glattfeld and Sherman[2] with certain modifications as described, and the phosphate esters were obtained from this intermediate through a series of reaction steps similar to those used for the synthesis of the D-glyceric acid phosphates[3] and the D-*erythro*-2,3-dihydroxybutyric acid phosphates.[4] DL-2,3-Dihydroxyisobutyric acid was esterified (II) and the primary alcohol was blocked with the benzoyl group (III). The tertiary alcohol was phosphorylated with diphenylphosphorochloridate to give IV, which could subsequently be converted to V by reduction and saponification. For synthesis of the other isomer, II was tritylated (VI) and benzoylated VII, followed by reductive detritylation to give VIII, which was phosphorylated to IX. Reduction and saponification gave X. Both esters were obtained as crystalline cyclohexylammonium salts.

The free acids of 2,3-dihydroxyisobutyric acid 2- and 3-phosphate consumed 3 equivalents of potassium hydroxide. Biochemical studies showed that the two dihydroxyisobutyric acid phosphates are not substrates for either *enolase* or *glyceric acid phosphate mutase*.[5] The 2-phosphate inhibits *enolase* competitively, but the affinity of the enzyme for this compound is only about one tenth the affinity for the natural substrate, D-glyceric acid 2-phosphate. The 3-phosphate has no effect on the enolase reaction. Both the 2- and the 3-phosphate inhibit competitively *glyceric acid phosphate mutase*.[5]

A characteristic feature which distinguished the two esters was the ease with which the phosphate group was hydrolyzed. The rates of hydrolysis of the two dihydroxyisobutyric acid phosphates and of D-glyceric acid 2-phosphate were studied under various conditions in order to compare the phosphate esters of primary, secondary and tertiary alcohols. The results are shown in Fig. 1. At neutral *p*H (imidazole buffer,[6] *p*H 7.1 at 100°) the hydrolysis of the ester of the tertiary alcohol is much faster than that of the secondary alcohol, which again is faster than that of the primary alcohol. Under acid conditions (1 *N* sulfuric acid at 100°) however, the rates of hydrolysis of the primary and tertiary esters are approximately identical and much lower than at *p*H 7. The fact that the hydrolysis rates are identical in acid is in agreement with the observation of Kiessling[7] on the rates of hydrolysis of glyceric acid 2- and 3-phosphate and has been explained[3] in terms of phosphate migration.[3,4]



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[2] J. W. E. Glattfeld and L. P. Sherman, THIS JOURNAL, **47**, 1742 (1925).

[3] C. E. Ballou and H. O. L. Fischer, *ibid.*, **76**, 3188 (1954).

[4] C. E. Ballou, *ibid.*, **79**, 984 (1957).

[5] L. I. Pizer and C. E. Ballou, unpublished.

[6] The rates of hydrolysis are approximately the same in a bicarbonate buffer, and in non-buffered media the initial rates are comparable to those in the buffered media.

[7] W. Kiessling, *Ber.*, **68**, 243 (1935).

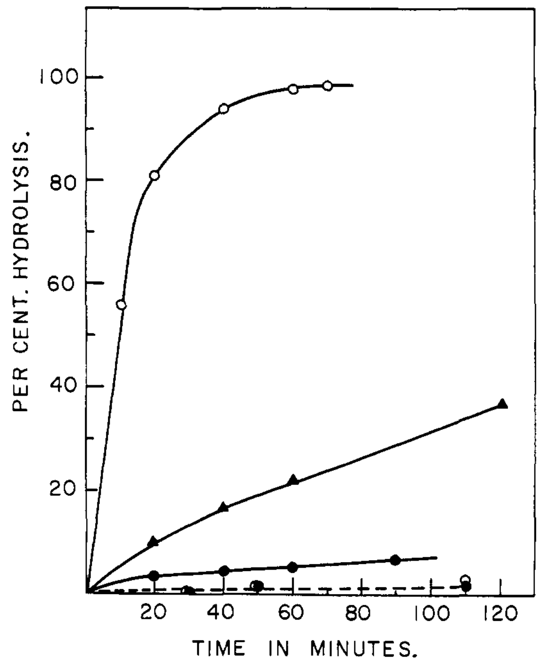


Fig. 1.—The rate of hydrolysis of the 2-phosphate (○) and 3-phosphate (●) of 2,3-dihydroxyisobutyrate, and of glyceric acid 2-phosphate (▲). Solid lines signify hydrolysis at 100° in 0.1 *M* imidazole buffer, *p*H 7.1; and the dotted line, hydrolysis at 100° in 1 *N* sulfuric acid.

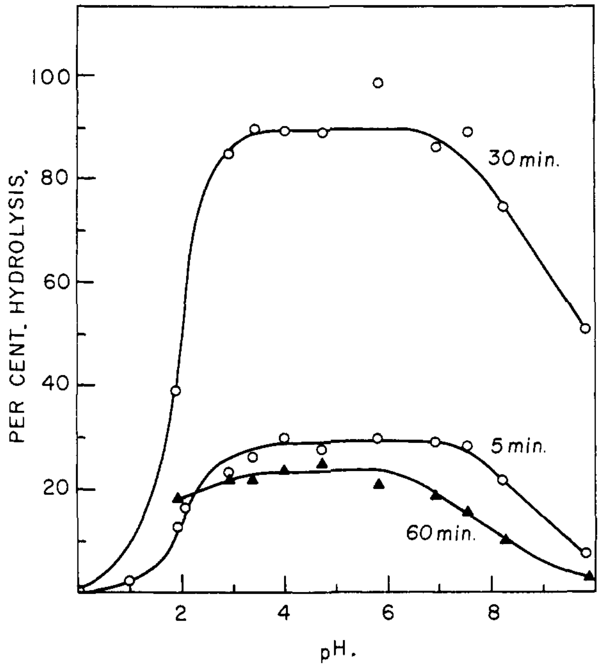
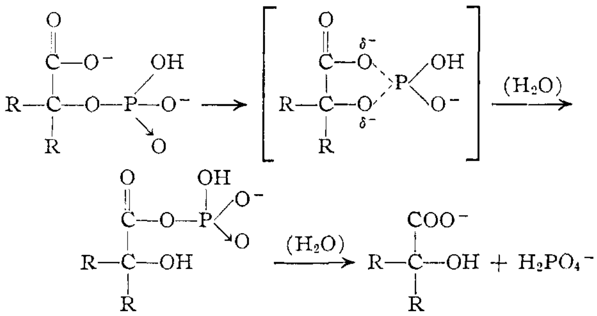


Fig. 2.—The effect of *p*H on the rate of hydrolysis of 2,3-dihydroxyisobutyric acid 2-phosphate (○) and glyceric acid 2-phosphate (▲) at 100°. The hydrolysis times are given on the figure. *p*H 0, 1 and 2 represent 1, 0.1 and 0.01 *N* sulfuric acid, and the other *p*H values were obtained with 0.1 *M* acetate–veronal buffer and represent the initial *p*H values determined with the *p*H-meter at 26°.

The effect of *p*H on the rate of hydrolysis of 2,3-dihydroxyisobutyric acid 2-phosphate and glyceric acid 2-phosphate is shown in Fig. 2 and is in agreement with the *p*H dependent hydrolysis rates reported for a variety of aliphatic and aromatic phosphate esters,[8] for thiophosphate esters[9] and for aromatic phosphonamides.[10] In the earlier studies evidence has been presented that the rapid hydrolysis of the monoionic phosphates (*p*H 2–6) is due to breaking the P–O bond (O^{18} from water goes to the orthophosphate, and no racemization of optically active esters takes place).[8f] In acid and alkali, however, breaking of the C–O bond by an SN2 type reaction mechanism has been proposed (O^{18} from water goes to the alcohol moiety, and racemization of optically active esters is observed).[8f] The rate of hydrolysis of the esters increases very rapidly with increasing hydrogen ion concentration below *p*H 1,[8d,e,f] and in compounds where SN2 and SN1 reactions are favored (*e.g.*, mono- and dibenzyl phosphates)[8e] the hydrolysis rate in acid is very high, actually extending into the *p*H range 3–5 where P–O bond breaking normally is predominant. When a carboxyl group is introduced into the molecule the hydrolysis rate *versus* *p*H curve indicates that the diion is the most labile form of the ester.[8c,d] In the mechanism proposed by Chanley and Gindler[8c] for the hydrolysis of *o*-carboxynaphthyl phosphates, the diion is the reactive species, and such a mechanism is in agreement with the findings reported here



The acid-catalyzed hydrolysis at low *p*H is not apparent in Fig. 2. In view of the migration of the phosphate group under acid conditions, however, this is not surprising, and the complete picture of the hydrolysis mechanism of polyhydroxy acid phosphates may well be different from the one discussed above.

Experimental

DL-2,3-Dihydroxyisobutyric Acid (I).—The calcium salt was prepared according to the method of Glattfeld and Sherman[2] with minor modifications. The reactants (1 mole of freshly distilled chloro-2-propanone, 1 mole of finely powdered and dried sodium formate and 0.1 mole of redistilled formic acid) were refluxed for 20 hr. rather than the 2 hr. used previously.[2] After treatment with hydrocyanic acid and hydrolysis, the calcium salt pentahydrate was obtained in an over-all yield of 20–25%. Thirty-five grams of the calcium salt dissolved in water was converted to the free acid by the use of 300 ml. of Amberlite IR-120 (H^+) ion-

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[8] (a) M. C. Bailly, *Bull. soc. chim.*, [5] **9**, 421 (1942); (b) A. Desjobert, *Compt. rend.*, **224**, 575 (1947); (c) J. D. Chanley and E. M. Gindler, THIS JOURNAL, **75**, 4035 (1953); (d) J. D. Chanley and E. Feageson, *ibid.*, **77**, 4002 (1955); (e) J. Kumamato and F. H. Westheimer, *ibid.*, **77**, 2415 (1955); (f) W. W. Butcher and F. H. Westheimer, *ibid.*, **77**, 2420 (1955).

[9] E. B. Herr, Jr., and D. E. Koshland, Jr., *Biochem. Biophys. Acta*, **25**, 219 (1957).

[10] J. D. Chanley and E. Feageson, THIS JOURNAL, **80**, 2686 (1958).

exchange resin. The eluate was concentrated *in vacuo*, giving an amber sirup that weighed 22 g. This was crystallized from hot ethyl acetate, giving 18 g. with m.p. 103–104° (95% yield from the calcium salt). The reported m.p. is 104°.[2]

Methyl DL-2,3-Dihydroxyisobutyrate (II).—Seven grams of the free acid I was dissolved in ether and the solution was treated with aliquots of an ether solution containing an excess of diazomethane. The reaction took place instantaneously, and when the yellow color of unreacted diazomethane remained, the reaction mixture was concentrated *in vacuo*, and the last traces of solvent were removed in high vacuum. The resulting slightly amber sirup weighed 8 g. (100%).

Methyl 3-*o*-Benzoyl-DL-2,3-dihydroxyisobutyrate (III).—The methyl ester II, 5.4 g., was dissolved in 25 ml. of dry pyridine, and a slight excess of benzoyl chloride (5 ml.) was added slowly to the reaction at ice-bath temperature. Reaction took place instantaneously as evidenced from the formation of pyridine hydrochloride. After 10 hours at room temperature, a few drops of water were added to destroy excess reagent, and the reaction mixture was dissolved in 75 ml. of chloroform. The chloroform solution was washed with 50-ml. samples of water (1×), 1 *N* hydrochloric acid (6×), 1 *M* potassium bicarbonate (4×) and water (4×), all in the cold. The final pyridine free, neutral solution was dried over anhydrous sodium sulfate, filtered and concentrated. When the last trace of solvent was removed, the compound was crystallized. The weight of the crystals was 8.7 g. (90%). The compound was recrystallized from hot benzene by addition of heptane to turbidity, giving crystals which melted at 66.5–68°.

Anal. Calcd. for $C_{12}H_{14}O_5$ (238): C, 60.5; H, 5.88; OCH_3, 13.00. Found: C, 59.6; H, 6.0; OCH_3, 13.3. Saponification equivalent of three individually weighed samples: 0.350 equiv. (calcd. 0.349); 0.315 equiv. (calcd. 0.316): 0.440 equiv. (calcd. 0.452).

DL-2,3-Dihydroxyisobutyric Acid 2-Phosphate.—The phosphorylation of the tertiary alcohol group did not take place very readily, and success in this step depended on using an excess of phosphorylating reagent and running the reaction at an elevated temperature. To 2 g. of methyl 3-*o*-benzoyl-DL-2,3-dihydroxyisobutyrate in 9 ml. of dry pyridine was added 2 equivalents of diphenylphosphorochloridate (4.5 g.), the flask was stoppered with a drying tube containing calcium chloride, and the reaction mixture was kept at 70° for 8 hours. After cooling it to room temperature, a few drops of water were added to destroy excess phosphorylating reagent, and the reaction mixture was dissolved in 50 ml. of chloroform and washed with water, 1 *N* hydrochloric acid, 1 *M* potassium bicarbonate and water as described above. After drying the solution and removing the chloroform by evaporation, the resulting light brown sirup weighed 3.2 g. (80%). The sirup was dissolved in absolute ethanol and the solution was treated with activated carbon. Then the phenyl groups were removed by reductive cleavage with platinum oxide (300 mg.) and hydrogen at atmospheric pressure. The hydrogen uptake (1500 ml.) was complete in less than 3 hr. The catalyst was removed by centrifugation, and 25 ml. of 1 *M* sodium hydroxide was added to the supernatant. After removal of most of the alcohol *in vacuo*, the reaction mixture was left at room temperature for several hours, to saponify. The solution was chilled in an ice-bath and passed through a small column containing Dowex-50 (H^+). The resulting acid solution was extracted several times with ether and finally was filtered through Filter-cel to remove the last traces of turbidity. The filtrate was brought to *p*H 9 with cyclohexylamine, and the water was removed *in vacuo*. The residue, 1.8 g. (55%), was redissolved in a small volume of water, and acetone was added to slight turbidity. Crystals of cyclohexylammonium DL-2,3-dihydroxyisobutyric acid 2-phosphate (V) formed in the cold. This was recrystallized from an acetone–water mixture.

The initial analysis of the product indicated that the 2-phosphate crystallized as the dicyclohexylammonium salt with two moles of water. This was confirmed by titration of an aqueous solution of the crystalline product with cyclohexylamine, in which the product consumed one equivalent of the amine. The weight loss resulting from drying over phosphorous pentoxide *in vacuo* at room temperature for 12 hr. corresponded to the loss of two moles of water.

Anal. Calcd. for dicyclohexylammonium salt dihydrate $C_{16}H_{35}O_7N_2P \cdot 2H_2O$ (434): N, 6.45; P, 7.15. Found: N, 6.52; P, 7.06 (air dried crystals). Calcd. for dicyclohexylammonium salt. $C_{16}H_{35}O_7N_2P$ (398): N, 7.05; P, 7.80. Found: N, 7.2; P, 7.85 (after drying as described above).

Methyl 3-O-Trityl-DL-2,3-dihydroxyisobutyrate.—Four grams of the methyl ester II was dissolved in 15 ml. of dry pyridine, and a slight excess of chlorotriphenylmethane (8.5 g.) was added in the cold. The reaction mixture was shaken for 2 hr. to effect complete solution of the trityl chloride, and it was then left at room temperature for 10 hr. At this time the mixture, which was almost solid with crystals of pyridine hydrochloride, was dissolved in 60 ml. of chloroform. The chloroform solution was worked up as described in the benzoylation step above. The product VI crystallized upon removal of the last trace of solvent. The brown crystalline mass (12 g.) was dissolved in a small volume of ether, and after treatment of the solution with activated carbon, petroleum ether was added to turbidity. Left at −20° overnight, the substance crystallized, and 9 g. (80%) of colorless crystals was collected. Recrystallization from hot, absolute ethanol gave crystals melting at 106–110°.

Anal. Calcd. for $C_{24}H_{24}O_4$ (376): C, 76.6; H, 6.4; OCH_3, 8.25. Found: C, 76.7; H, 6.5; OCH_3, 8.3.

DL-2,3-Dihydroxyisobutyric Acid 3-Phosphate.—Three grams of methyl 3-O-trityl-2,3-dihydroxyisobutyrate was benzoylated with a slight excess of benzoyl chloride (1.3 g.) in 6 ml. of dry pyridine. The reaction was left at room temperature overnight and worked up as described above for the tritylation step. The solvent free, amber colored sirup weighed 3.8 g. (100%). The trityl group was removed from this compound (methyl 2-O-benzoyl-3-O-trityl-2,3-dihydroxyisobutyrate) by catalytic hydrogenation with palladium-on-carbon and hydrogen in absolute ethanol. The theoretical hydrogen uptake (180 ml.) was completed in about 4 hr. The catalyst was removed by filtration and the filtrate was concentrated *in vacuo*. The last trace of alcohol was removed in a high vacuum. The resulting mixture of methyl 2-O-benzoyl-2,3-dihydroxyisobutyrate and triphenylmethane was dissolved in dry pyridine and treated with a slight excess (2 g.) of diphenylphosphorochloridate. After 12 hr. at room temperature, the reaction mixture was worked up as above, and the sirup (3.2 g.) was reduced with platinum and hydrogen in absolute ethanol. The hydrogen uptake was 940 ml. (approximately 75% of theory) in several hours. After removal of the catalyst, the filtrate was worked up as described for the 2-phosphate. The final crystalline tricyclohexylammonium salt weighed 2 g. corresponding to a 50% over-all yield from the trityl ether. The compound was recrystallized from a water–acetone mixture.

Anal. Calcd. for $C_{22}H_{48}O_7N_3P$ (497): N, 8.47; P, 6.24. Found: N, 8.6; P, 6.3.

Test of Biological Activity.—The inhibitory effect of the two phosphate esters in the enolase reaction was determined according to the procedure described previously.[11] The initial rates of the enolase reaction in the standard medium[10] were determined for the substrate (D-glyceric acid 2-phosphate) range 0.8–1.5 × 10^{-4} *M* in the absence and in the presence of 3 × 10^{-3} *M* DL-2,3-dihydroxyisobutyric acid 2- and 3-phosphate. No inhibition was found for the 3-phosphate. From the inverse plot of Lineweaver–Burk,[12] the K_s was found to be 2 × 10^{-4} moles per liter and the K_I for 2,3-dihydroxyisobutyric acid 2-phosphate 1 × 10^{-3} moles per liter, with the characteristics of a competitive inhibitor. In calculating K_I it was assumed that only the D-isomer was active.

Hydrolysis of the Phosphate Esters.—All the hydrolysis experiments were carried out on a boiling water-bath using 0.01 *M* solutions of the esters. The controls kept at room temperature showed no increase in inorganic phosphate. Inorganic phosphate was determined by the method of Fiske and SubbaRow.[13] Aliquots of the esters, dissolved in the proper acid or buffer solutions, were added to colorimeter tubes equipped with air condensers. At zero time the tubes were put in the boiling water-bath and then removed after

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(11) F. Wold and C. E. Ballou, *J. Biol. Chem.*, **227**, 313 (1957).

(12) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

(13) C. H. Fiske and Y. SubbaRow, *J. Biol. Chem.*, **81**, 629 (1929).

various time intervals. The color was developed directly in the colorimeter tube and was read in a Coleman Junior Spectrophotometer at 660 mμ. The pH values given in the figures are those determined at room temperature with a Beckman Model G pH meter.

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Solvent Effects in the Reaction of p-Substituted-α-chlorotoluenes with Thiosulfate. The Relationship of Rho and Dielectric Constant

BY RICHARD FUCHS AND ALEX NISBET

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The rates of reaction of α-chloro-p-nitro-, α,p-dichloro-, α-chloro-, and α-chloro-p-isopropyltoluenes with thiosulfate have been measured in eleven partially aqueous solvents of dielectric constant (D) 25 to 101. For reactions in seven of the solvents the Hammett rho value is a linear function of $1/D$. The conditions under which this relationship can be expected to hold are discussed. Although the rate for a given compound is not a simple function of the dielectric constant of the solvent, the activation energies show a decided tendency to rise with increasing D.

The observation[1] that the rates of reaction of four variously substituted α-chlorotoluenes with thiosulfate are unequally affected by a change of solvent evoked an interest in a more general study of solvent effects in these reactions. Extensive studies of solvent effects in solvolyses have been reported,[2–5] and attempts have been made to correlate the rates or entropies and enthalpies of activation with solvent composition or solvent properties such as dielectric constant. Ideally, one might prefer a relationship between rates and independently determined properties of the solvent or solution, but attempts in this direction[4] have met with limited success.[6] To date more useful results have been obtained with empirical relationships such as those of Swain[7] and of Winstein,[8] which employ parameters derived from kinetic data.

Non-solvolytic displacement reactions by anions on neutral molecules, although an extremely important mechanistic type, have received relatively little attention in solvent studies. This has resulted in part from the limited effect of solvent variation, due to the rather indirect participation of solvent in these reactions. To a considerable extent, however, the limited data available[2,9] have followed the generalization[10] that (with the degree of ion-pair dissociation taken into account) anion–neutral molecule reactions proceed faster in solvents of low dielectric constant. The present work presents evidence (Table I) in apparent disagreement with such a statement. Second-order rate constants are presented for the reactions of α-chloro-p-nitro-, α,p-dichloro-, α-chloro- and α-chloro-p-isopropyltoluenes with sodium thiosulfate taking place in nine mixtures of 40% water–60% organic solvent and in two 50–50 mixtures. Energies of activation (Table III) have been calculated for the reaction of one or more of the α-chlorotoluenes in each of seven solvents, making use of rate measurements at 10, 20, and 30°, or 20, 30 and 40° (Table II).

TABLE I

RATES OF REACTION[a] OF p-SUBSTITUTED-α-CHLOROTOLUENES WITH THIOSULFATE AT 30°

Solvent[b]	D[c]	p-NO$_2$	p-Cl	p-H	p-i-Pr
Dioxane	25	9.95	5.20	3.48	3.84
Dioxane[d]		17.2	...	5.29	6.12
Dioxane[e]	34.5	14.5	8.54	6.35	7.24
1,2-Dimethoxyethane	37.4	13.8	6.57	4.12	4.07
Diglyme[f,g]	38	26.0	11.9	7.10	7.95
2-Ethoxyethanol	41	13.7	8.19	5.38	6.87
Ethanol[g]	45	9.62	7.32	5.35	8.35[h]
Acetone	45.5	11.9	6.59	4.82	5.64
Acetone[d]		18.3	...	6.47	8.94
Acetone[e]	71.2	13.7	9.35	6.99	8.22
Acetonitrile	52.3	4.12	3.17	2.88	3.24
Butyrolactone	58.5	16.0	11.4	8.69	11.3
N-Methylacetamide	101	...	...	39.3	...

[a] Second-order rate constants (l. mole^{-1} sec.$^{-1}$) × 10^3. All values are averages of two to four determinations. At time of mixing, ($S_2O_3^=$) = 0.04 M, (RCl) = 0.0 25 M; stated temperature regulated to ± 0.02°. [b] 40% water + sufficient solvent as indicated to make up the total volume. [c] Dielectric constant measured with a Sargent Oscillometer at 23 ± 1°. [d] At time of mixing ($S_2O_3^=$) = 0.008 M, (RCl) = 0.005 M. [e] 50% water + sufficient solvent to make up the total volume. [f] Bis-(2-methoxyethyl) ether. [g] Ref. 1. [h] 9.55 with initial ($S_2O_3^=$) = 0.02 M, (RCl) = 0.013 M.

Discussion

The following statements can be made on the basis of the above data: (1) The Hammett ρ-value is a linear function of $1/D$ [or $(D-1)/(2D+1)$] for the majority of the solvents (Fig. 1). (2) There is no simple relationship between absolute reaction rates and D, nor is there any particular tendency for the reaction to proceed faster in solvents of relatively low dielectric constant or of low water content. (3) The rate constant for the

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(1) R. Fuchs, THIS JOURNAL, **79**, 6531 (1957).

(2) A. Streitwieser, Jr., *Chem. Revs.*, **56**, 571 (1956).

(3) S. Winstein and A. H. Fainberg, THIS JOURNAL, **79**, 5937 (1957).

(4) G. S. Hammond, C. E. Reeder, F. T. Fang and J. K. Kochi, *ibid.*, **80**, 568 (1958).

(5) J. B. Hyne and R. E. Robertson, *Can. J. Chem.*, **34**, 863 (1956); **34**, 931 (1956).

(6) However, for a correlation of solvolysis rates with spectral data see E. M. Kosower, THIS JOURNAL, **80**, 3267 (1958).

(7) C. G. Swain, R. B. Mosely and D. E. Bown, *ibid.*, **77**, 3731 (1955); C. G. Swain, D. C. Dittmer and L. E. Kaiser, *ibid.*, **77**, 3737 (1955).

(8) See, for example, E. Grunwald and S. Winstein, *ibid.*, **70**, 846 (1948).

(9) Reference 1, footnote 6.

(10) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953, p. 347.