175–177°), which was identified by a mixture melting point with an authentic sample; λ_{max} 239 mμ, ε 9,100 (methanol). An intractable gum was eluted with 9:1 benzene–petroleum ether. Benzene and benzene containing increasing concentrations of methanol up to 1% removed material which, after three crystallizations from ether–petroleum ether, weighed 137 mg. and melted at 159–160°. It was identified by mixture melting point and its infrared spectrum (nujol) as 3β-acetoxy-16α-methoxy-Δ⁵-pregnen-20-one,[5] $[\alpha]_D$ −29 ± 2°, at 239 mμ ε = 25 (methanol).

Acknowledgment.—I am grateful to Dr. E. C. Kendall and Dr. H. L. Mason for numerous helpful suggestions during the course of this investigation and to Mrs. Grace Dews for determining and Dr. H. L. Mason for interpreting the infrared spectra. The chromatographic separations were performed by Barbara Towey.

ROCHESTER, MINNESOTA

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, THE MOUNT SINAI HOSPITAL]

The Mechanism of the Hydrolysis of Salicyl Phosphate. I[1]

BY J. D. CHANLEY, E. M. GINDLER AND HARRY SOBOTKA

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The hydrolysis of salicyl phosphate shows an unusual *p*H dependency, the compound being most unstable at *p*H 5.3. The kinetics of this hydrolysis have been investigated. These studies and comparison with related cases lead to the conclusion that it is the ionized carboxyl group which brings about the rapid scission of the O–P bond. The *p*H dependency is discussed and a mechanism for the reaction is suggested involving the formation of a cyclic transition state. The energy and entropy of activation have been evaluated.

The rate of hydrolysis of salicyl phosphate (A) to salicylic and phosphoric acids varies with *p*H in an unexpected manner[2]; hydrolysis is most rapid at *p*H 5.3, while the compound is very stable in the extreme acid region and completely stable in strong alkali. A similar behavior between *p*H 2 and 8 has been described by Desjobert[3] for ethyl phosphate and glycol phosphate. However, the maximum rate of hydrolysis of these phosphates around *p*H 3–5 at 100°, is about 10^5 times slower than that of salicyl phosphate extrapolated to the same temperature. Salicyl phosphate has a half-life period of 2.1 hours at *p*H 5.3 and 37°. The observed ease of hydrolysis is in contrast to that of phenyl phosphate,[4a,b] *m*- and *p*-carboxyphenyl phosphate[2] and salicylaldehyde phosphate[2b] which remain virtually unchanged under the extremely mild conditions that effect complete hydrolysis of salicyl phosphate. Moreover, the maximum of the *p*H *vs.* hydrolysis rate curve is in contrast to the usual hydrogen ion and hydroxyl ion catalysis, operative in the hydrolysis of carboxyl acid esters, and to the very acid conditions usually necessary to effect the rapid hydrolysis of phosphoric acid esters.[3,4b]

We have observed the same type of *p*H dependency in the hydrolysis of 3-carboxynaphthyl-2-phosphate.[5] The aim of this investigation was to explain the *p*H dependency, and suggest a mechanism for this hydrolysis.

pH **Dependency.**—We have determined, by analysis for liberated phosphoric acid, the rate of hydrolysis of salicyl phosphate at three temperatures (37.2°, 42.0°, 47.4°) over the *p*H range 2–10. The same *p*H dependency was observed at all temperatures and in each instance first order kinetics obtained throughout the course of this reaction (Fig. 1).

$$-d[SP]/dt = d[P]/dt \quad (1)$$

$$-d[SP]/dt = k_{obsd}[SP] \quad (2)$$

where SP stands for salicyl phosphate, P for phosphoric acid and *k* for the observed rate constant.

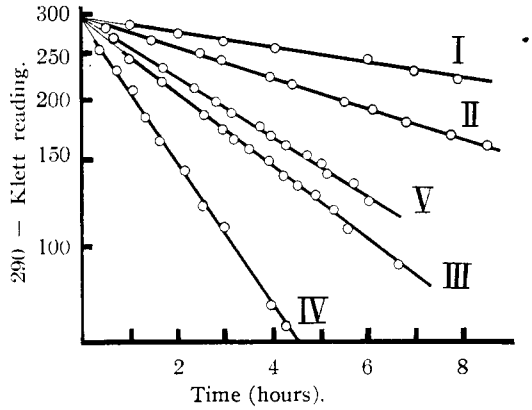
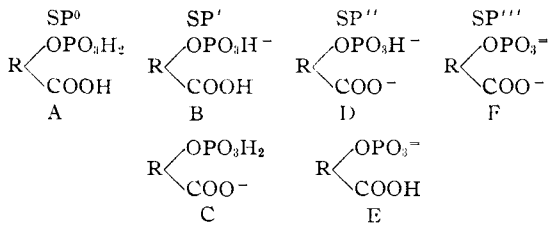


Fig. 1.—Hydrolysis of 0.00130 molar salicyl phosphate at 37° at various *p*H values: I, *p*H 2.32; II, *p*H 2.99; III, *p*H 3.76; IV, *p*H 5.67 (experiment at *p*H 4.92 gave substantially the same figure as (IV)); V, *p*H 6.93 (experiment at *p*H 7.67 gave substantially the same figure as I).

In the elucidation of the *p*H dependency the following considerations are necessary. Salicyl phosphate at *p*H values 2–10 exists predominantly in one or more of these ionic species



(1) This work was supported in part by a grant from the National Science Foundation. This material was presented at the Annual Meeting of the American Association for the Advancement of Science, Section C (Chemistry) on December 26, 1951, at Philadelphia, Penna.

(2) (a) C. Manaka, *J. Biochem.*, **14**, 191 (1931); (b) **14**, 481 (1932); (c) J. Arai, *ibid.*, **20**, 465 (1934).

(3) A. Desjobert, *Compt. rend.*, **224**, 575 (1945); *Bull. soc. chim.*, 809 (1947).

(4) (a) Unpublished observations; (b) *cf.* R. H. Plimmer and W. Burch, *J. Chem. Soc.*, 290 (1929); R. H. Plimmer, *Biochem. J.*, **7**, 72 (1913).

(5) To be reported in a subsequent communication.

TABLE I

SUMMARY OF EXPERIMENTAL RATE RUNS[a]

pH	μ	Ionization constants pK1	pK2	pK3	Mole fractions M_0	M_1	M_2	M_3	37.2° $k_{obsd.}$	k_{calcd}	42.0° $k_{obsd.}$	$k_{calcd.}$	47.4° $k_{obsd.}$	$k_{calcd.}$
2.32	0.16	1.35	3.87	6.74	0.094	0.88	0.025	..	1.04	(1.04)[b]	1.83	(1.83)[b]	4.03	(4.03)[b]
2.99	.16	1.35	3.87	6.74	.019	.89	.12	..	1.90	2.00	3.42	3.45	6.75	8.10
3.76	.16	..	3.87	6.74	...	.56	.44	..	4.70	5.00	9.28	8.92	17.2	20.8
4.92	.22	..	3.80	6.66	...	.07	.91	0.02	9.11	9.30	16.2	16.7	36.1	39.3
5.67	.27	..	3.75	6.60	...	.01	.89	.10	9.11	(9.11)[b]	16.2	(16.2)[b]	38.0	(38.0)[b]
6.93	.20	..	3.82	6.68	...	..	.36	.64	3.97	3.67	9.50	7.30	18.6	15.4
7.70	.19	..	3.82	6.70	...	..	.097	.90	1.04	1.00	2.06	1.55	5.68	3.64

[a] All rate constants in sec.$^{-1}$ × 10^5. [b] Computations based on figures in parentheses.

Experimentally, the four ionic groups [SP°], [SP′], [SP″] and [SP‴] are easily distinguishable and need be considered for an analysis of the rates.

$$[SP] = [SP^0] + [SP'] + [SP''] + [SP'''] \quad (3)$$

$$\frac{-d[SP]}{dt} = \frac{-d[SP^0]}{dt} + \frac{-d[SP']}{dt} + \frac{-d[SP'']}{dt} + \frac{-d[SP''']}{dt} \quad (4)$$

The observed rate of hydrolysis is the sum of the rates of hydrolysis of the groups. We assume, for the general case, that each of them forms salicylic and phosphoric acids following first order kinetics. We may therefore rewrite the latter equation as

$$k_{obsd}[SP] = k_0[SP^0] + k_1[SP'] + k_2[SP''] + k_3[SP'''] \quad (5)$$

where k_0, k_1, k_2 and k_3 are the specific rate constants for the hydrolysis of their respective group.

The terms $-d[SP^0]/dt$, etc., are strictly the sums of two terms; one referring to the disappearance of the species due to hydrolysis, the other to the change in species concentration by association or dissociation of protons to keep equilibrium between the species while hydrolysis proceeds. The sum of the latter terms is zero, since the total concentration of salicyl phosphate, [SP], is not affected by redistribution reactions.

Salicyl phosphate is completely stable at alkaline *p*H's above 8.5 at the temperatures investigated. Since it exists in this region entirely in the triionic form, its rate of hydrolysis equals zero; $k_3 = 0$. The rate of hydrolysis of undissociated salicyl phosphate, [SP°], as measured by its slow cleavage at *p*H values below zero, is small. Owing to the fact that its concentration in the region of *p*H 2–10 is less than 10%, the term $k_0[SP^0]$ may be neglected. Therefore

$$k_{obsd}[SP] = k_1[SP'] + k_2[SP''] \quad (6)$$

$$k_{obsd} = k_1\frac{[SP']}{[SP]} + k_2\frac{[SP'']}{[SP]} \quad (7a)$$

$$k_{obsd} = k_1[M_1] + k_2[M_2] \quad (7b)$$

where M_1 and M_2 stand for the mole fractions of the monoionic and diionic groups, respectively. At a given *p*H the mole fractions of the various ionic species will be constant, if we make the reasonable assumption that the rate at which equilibrium between the species is re-established is very much faster than the rate at which it is disturbed by the hydrolysis of any of the ionic species. The values of the specific rate constants k_1 and k_2 may then be determined by measurements of the rate of hydrolysis of salicyl phosphate in a buffered medium and evaluation of M_1 and M_2 at the various *p*H's (see Table I). These latter quantities would vary with ionic strength and were therefore measured at the same ionic strength at which the hydrolyses were run. (See Experimental section for a discussion of the constancy of M_1 and M_2 with temperatures.)

The specific rate constant k_2, at each of the three temperatures investigated, was derived at *p*H 5.67. At this *p*H the concentration of the diionic form is almost at a maximum ($M_2 = 0.89$) and the contribution of the term $k_1[M_1]$ is negligible, $M_1 = 0.01$. For example $k_{obsd} = 0.329$ hour^{-1} at 37.2° and *p*H 5.67. Substitution into equation 7b yields

$$0.329 = k_2[0.89];\ k_2 = 0.370 \text{ hour}^{-1}$$

The specific rate constant k_1, at all temperatures, was derived at *p*H 2.32. The relevant quantities M_1, M_2 and k_2 were substituted in equation 7b.

$$k_{obsd} = 0.0381 \text{ hour}^{-1} \text{ at } 37.2°$$

Thus

$$0.0381 = k_1[0.89] + 0.37[0.025];\ k_1 = 0.0327 \text{ hour}^{-1}$$

(see Table II)

The evaluation of k_1, k_2, M_1 and M_2 enabled us to draw the theoretical *p*H–hydrolysis rate curve at the three temperatures investigated. The calculated and observed values were in good agreement at 37.2 and 42.0° for all *p*H's investigated (2.99, 3.76, 4.92, 6.93, 7.70) (see Fig. 2 and Table I). We attribute the deviations, at the higher temperature 47.4°, and especially at *p*H's 6.94 and 7.70, to the changes in the ionization constants of salicyl phosphate. Slight changes in the latter constants effect a considerable percentage change in the concentration of the diionic group [SP″] at these high *p*H's (see discussion in Experimental section.)

The maximum in the *p*H–hydrolysis rate curve results from the fact that only two of the main groups [SP′] and [SP″] are hydrolyzed at reasonable speeds. The sharpness of the maximum results from the fact that the specific rate constant k_2 is more than ten times that of k_1.

Mechanism.—It is most reasonable to assume that the observed ease of hydrolysis of salicyl phosphate is best accounted for by assuming the participation of the ortho-carboxyl group in the hydrolysis; Winstein and co-workers[6,7] in their comprehensive investigations, have given many ex-

(6) (a) S. Winstein, C. Hansen and E. Grunwald, THIS JOURNAL, **70**, 812 (1948); (b) S. Winstein, E. Grunwald, R. Buckles and C. Hansen, *ibid.*, **70**, 816 (1948); (c) S. Winstein, E. Grunwald and L. Ingraham, *ibid.*, **70**, 821 (1948); (d) S. Winstein and E. Grunwald, *ibid.*, **70**, 828 (1948).

(7) E. Grunwald and S. Winstein, *ibid.*, **70**, 841 (1948).

amples which demonstrate that a neighboring group may not only participate in the removal (solvolysis) of the neighbor, but by such participation accelerates its removal. A secondary factor which may enhance the ease of this hydrolysis is the fact that phosphorus is capable of expanding its valence shell,[8] and thus, in a sense, becomes more susceptible to attack by a nucleophilic reagent. We postulate the attack of the oxygen of the carboxyl group upon the phosphorus with the formation of a cyclic transition state and its subsequent decomposition to salicylic and phosphoric acids.

The question arises as to whether both or only one of the forms (B, C; D, E) is reactive in their respective groups [SP′] and [SP″].

In the group [SP″] the distribution between D and E cannot be ascertained directly. A fair approximation of this distribution may be obtained from the ratio of the ionization constant of *o*-methoxybenzoic acid, pK 4.1,[9] and the second ionization constant of phenylphosphate, pK 6.0.[10] The concentration of D would be at least a hundred times that of E.

In the case of the group [SP′] the distribution between form C and B may be approximated by a comparison of the first ionization constant of phenylphosphoric acid, pK *ca.* 1.5[11] with that of *o*-methoxybenzoic acid. We conclude that the ratio of C to B is approximately 1 to 100.

For the following reasons we believe that the forms C and D are the principal contributors to the rate of hydrolysis of salicyl phosphate: (1) Salicylaldehyde phosphate does not hydrolyze.[2b] (2) Carboxylate ion is more nucleophilic than carboxyl. (3) In ethyl phosphate the diionic form hydrolyzes much slower (if at all) than the monoionic form.[3] These ions correspond amongst the species of salicyl phosphate to the monoionic form B and the diionic form E, respectively, both with intact carboxyl group. Thus, if one ascribed the tremendous enhancement of this hydrolysis in salicyl phosphate to the participation of the undissociated carboxyl group, one would reasonably have to expect faster hydrolysis of the monoionic group than of the diionic group in salicyl phosphate. Moreover, from an estimate of their relative abundance, one should expect an even greater difference in favor of the preponderant monoionic form B, since the concentration of form E is small compared to that of the total diionic group. In other words, a maximum of scission at pH 2 to 3 rather than at pH 5.2 would result. Since this is not the case, we ascribe the specific rate constant k_2 to form D. Because of the scarcity of form C, the real value for the rate constant k_1 may be 100 times greater than the specific rate constant obtained for the hydrolysis of the group [SP′]; however, no quantitative evaluation of this figure appears possible at present.

We assume, essentially, the same mechanism for the hydrolysis of [SP′] and [SP″]. Two schemes (I and II), kinetically indistinguishable, and deemed reasonable in the light of the preceding discussion are considered. The only reasonable assumption to satisfy scheme I and to account for *both* the first order kinetics and the observed pH dependency is that the formation of salicoyl phosphate (Z), step (a), is rate determining. The following considerations necessitate this view: (1) Neither benzoyl phosphate[12] nor acetyl phosphate,[13] in their hydrolysis, exhibit the type of pH dependency observed with salicyl phosphate. (2) In our method of determining phosphate a molybdate reagent was employed. Acyl phosphates are hydrolyzed extremely rapidly in the presence of this reagent.[14] Therefore, our determination of phosphoric acid is a measure of free phosphoric acid and the phosphoric acid available from salicoyl phosphate. However,

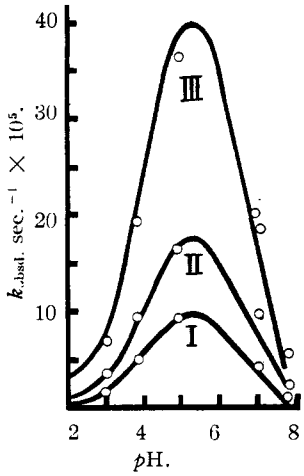
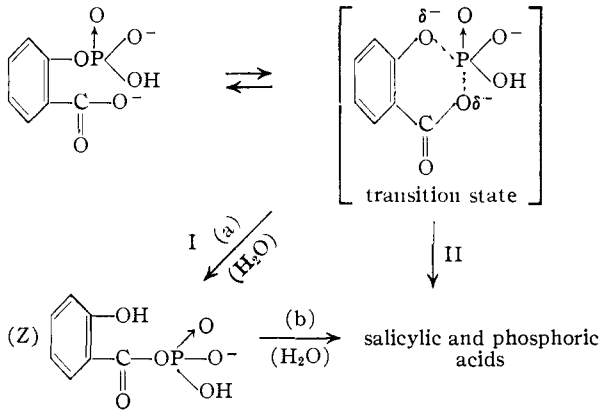


Fig. 2.—Observed rates of hydrolysis of salicyl phosphate at 37.2° (I), 42.0° (II), 47.4° (III) at various pH values is indicated by O. The solid curve is derived from the values of k_1 and k_2 obtained at pH 2.32 and 5.67, respectively.



we have ascertained that the amount of salicylic acid determined by our analytical procedure was the same as that actually isolated from the reaction mixture when acidified in the cold and in the absence of molybdate. This could not be the case, if there was an accumulation of salicoyl phosphate (Z) resulting from step (a) of scheme I being the fast stage and step (b) the slow one. If scheme I is the correct interpretation, the intermediate salicoyl phosphate (Z) is hydrolyzed extremely rapidly. However, the fact that both acetyl

(8) A. F. Wells, "Structural Inorganic Chemistry," Clarendon Press, Oxford, England, 1945, p. 410.

(9) W. Ostwald, *Z. physik. Chem.*, **3**, 266 (1899). We have not employed salicylic acid in this approximation, since it is well known that the latter is inordinately strong, pK 3, due to hydrogen bonding, which presumably does not obtain in the case of salicyl phosphate.

(10) (a) Our determination; (b) *cf.* C. Morton, *Chemist and Druggist*, **113**, 138 (1930).

(11) *Cf.* P. Oesper, "The Chemistry and Thermodynamics of Phosphate Bonds" in "A Symposium on Phosphorus Metabolism," ed. by W. McElroy and B. Glass, The Johns Hopkins Press, 1951, p. 534, *cf.* 10b.

(12) H. Chantrenne, *Compt. rend., Carlsberg*, **26**, 297 (1948); *Biophys. Biochim. Acta*, **2**, 286 (1948).

(13) D. E. Koshland, Jr., THIS JOURNAL, **73**, 4103 (1951).

(14) F. Lipman and L. C. Tuttle, *J. Biol. Chem.*, **159**, 21 (1945), *cf.* (12); this of course implies that, irrespective of which of the two steps in scheme I is the slow one, our observed rate would be a measure only of the formation of salicoyl phosphate.

TABLE II

CALCULATED SPECIFIC RATE CONSTANTS; ENTROPY AND HEAT OF ACTIVATION

Temp., °C.	k_1, sec.$^{-1}$ × 10^5	k_2, sec.$^{-1}$ × 10^5
26.6	...	2.46
31.5	0.439	5.00
37.2	0.890	10.2
42.0	1.56	18.2
47.4	3.35	42.9
$\Delta H_1^{\ddagger}$, kcal./mole	22.4 ± 1.0	...
$\Delta H_2^{\ddagger}$, kcal./mole	.....	23.5 ± 0.8
$\Delta S_2^{\ddagger}$, e.u.	.....	−1.2 ± 2.5

phosphate[13,15,17] and benzoyl phosphate[16] have half-life periods, which are roughly the same as that of salicyl phosphate, may be considered as evidence against the formation of salicoyl phosphate as an intermediate (scheme I). As a less likely alternative to scheme I we suggest that in the transition stage a water molecule is intimately bound to the phosphorus and the activated complex decomposes directly to salicylic and phosphoric acid according to scheme II.

We have observed that in the methanolysis of salicyl phosphate, in the presence of two equivalents of pyridine, neither free phosphoric acid nor methyl salicylate was formed, but methyl phosphate and salicylic acid were the products in practically quantitative yield. The methanolysis experiment implies that the O–P bond is split whether the course of reaction follows scheme I or scheme II. The fission at the O–P bond in the assumed hydrolysis of salicoyl phosphate (scheme I) is compatible with the work of Koshland[17] who has presented convincing evidence that in the hydrolysis of acetyl phosphate over the *p*H range 3–9, it is the P–O bond which is split.

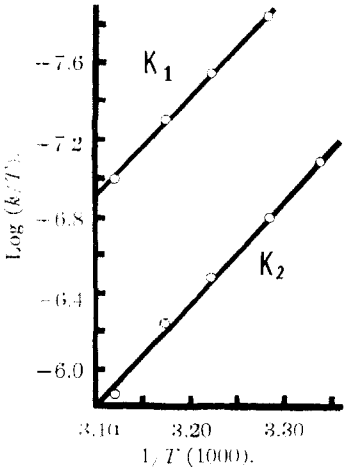


Fig. 3. Plot of log (k/T) *vs.* $1/T$; k_1 and k_2 refer to the specific rate constants for the groups [SP′] and [SP″], respectively.

Any decision as to the correctness of either of the two schemes awaits the study of the hydrolysis of salicoyl phosphate. We are investigating the synthesis of this compound.

We have determined the rates of hydrolysis of salicyl phosphate at *p*H 2.32 at 31.5° and *p*H 5.67 at 31.5° and 26.6°. The values of the heat of activation $\Delta H_1^{\ddagger}$ and $\Delta H_2^{\ddagger}$ and the entropy of activation $\Delta S_2^{\ddagger}$ were calculated by the least squares method from the Eyring equation[18] (see Table II).

$$k = \frac{kT}{h} e^{\Delta S^{\ddagger}/R} e^{-\Delta H^{\ddagger}/RT}$$

In Fig. 3 are the plots of log $[k]/[T]$ *versus* $1/T$ from which the quantities $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were obtained. Since the real value of the specific rate constant of monoion [SP′] is in doubt, the true entropy of activation $\Delta S_1^{\ddagger}$ for the hydrolysis of this anion cannot be calculated. The small negative value for the entropy change $\Delta S_2^{\ddagger}$ (−1.2 e.u.) associated with the hydrolysis of the [SP″] group is in contrast to the larger negative entropy values (−8 to −14 e.u.) encountered in the kinetically first-order Claisen type rearrangements, a reaction involving a cyclic transition state,[19] but is in agreement with the entropy change (*ca.* −4 e.u.) reported by Winstein[6c] for acetolysis reactions involving a neighboring group in the cyclic intermediates. An examination of a Fisher–Taylor–Hirschfelder model of salicyl phosphate (using sulfur in place of phosphorus) reveals that there is no steric hindrance to the approach of the oxygen to the phosphorus atom and indicates that the carboxyl and phosphate groupings are hindered in their rotation by each other. This would suggest that in the postulated transition state there would not be as great a relative loss in degrees of freedom as encountered in the Claisen type rearrangements and consequently not as great a negative entropy change.

We have found that at constant *p*H a change in type of buffer or ionic strength made no perceptible change in the rate of hydrolysis. The rates of hydrolysis of salicyl phosphate at 37.2°, *p*H 2.99, in phthalate buffer of ionic strength $\sqrt{\mu} = 0.20$; at *p*H 4.92 with both phthalate buffer of ionic strength $\sqrt{\mu} = 0.28$ and acetate buffer of ionic strength $\sqrt{\mu} = 0.22$ and $\sqrt{\mu} = 0.28$ was substantially the same as the rates listed in Table I for the corresponding *p*H values. In the solvolysis of α-bromopropionate,[7] a reaction involving a cyclic intermediate, increase in ionic strength from 0.06 to 1 *M* gave only a 6% increase in rates. In our hydrolysis the change in ionic strength was so small that no effect would be anticipated.

As previously indicated, the proposed cyclic

(15) F. Lynen, *Ber.*, **73**, 367 (1940), reports the half-life period to be 3.1 hours at 38° and *p*H 7.4; D. E. Koshland, Jr., reports a half-life period of 3.4 hours at 39° and *p*H 6.9; this rate of hydrolysis is practically constant over the *p*H range 3–9, while a pronounced hydrogen ion and hydroxyl ion catalysis is observed in the extreme *p*H regions; *cf.* ref. (13) and (17).

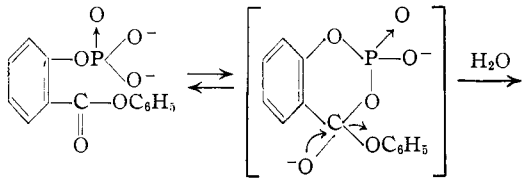
(16) H. Chantrenne reports a half-life period of 4.6 hours at 37° and *p*H 7.4 for this compound; *cf.* ref. (12).

(17) D. E. Koshland, Jr., "The Mechanism of the Hydrolysis of Acetyl Phosphate and its Relation to Some Enzymatic Reactions" in "A Symposium on Phosphorus Metabolism," The Johns Hopkins Press, Baltimore, Md., 1951, p. 536.

(18) S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., Inc., New York, N. Y., 1941; $\Delta H^{\ddagger}$ is almost exactly equal to $Ea - RT$, where Ea is the Arrhenius activation energy.

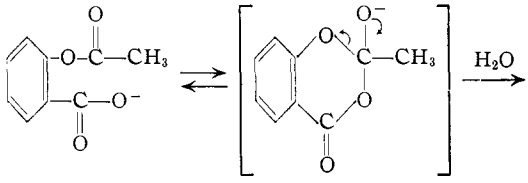
(19) E. G. Foster, A. C. Cope and F. Daniels, THIS JOURNAL, **69**, 1893 (1947); F. W. Schuler and G. W. Murphy, *ibid.*, **72**, 3155 (1950); A. T. Blades and G. W. Murphy, *ibid.*, **74**, 1039 (1952); L. Stein and G. W. Murphy, *ibid.*, **74**, 1041 (1952); J. F. Kincaid and D. Tarbell, *ibid.*, **61**, 3085 (1939).

mechanism parallels the numerous examples of displacements involving neighboring groups. The following reactions, from the literature, are closely related to the hydrolysis of salicyl phosphate. They may, we believe, best be explained by a similar cyclic mechanism: (1) The reversibility of the reaction O-acetylsalicylamide ⇌ N-acetylsalicylamide.[20] (2) The extreme ease of breakdown at *p*H 5–9 of salol phosphate.[2c,21] The phenol is completely liberated at these *p*H's at 37°, in 10 minutes with salicyl phosphate as the second product; whereas in acid solution (hydrolysis at 100°) the products are mainly salol and phosphoric acid.[21] (3) The *p*H–hydrolysis rate curve of acetyl



SP + C_6H_5OH

salicylate, aspirin,[22] besides exhibiting typical hydrogen and hydroxyl ion catalysis with a minimum at *p*H around 2, shows a high flat, kinetically first order region, in the *p*H range 5–8. The rate of hydrolysis in this range is much higher than would be expected for the spontaneous hydrolysis of the anion and indicates involvement of the carboxylate ion in the mechanism of hydrolysis in this *p*H region. We picture it as



Salicylic and acetic acids

The stability of salicyl phosphate in alkaline medium at *p*H values above 8.5 at temperatures at least up to 55° may be attributed to the enormous hindrance by the large negative charge surrounding the phosphorus atom in the triionic form. Thus, the approach of the carboxylate group is completely repressed. Since the splitting of the O–P bond in phenyl phosphate in alkaline medium at temperatures at least up to 55° is negligible, no hydrolysis is observed with the triionic form of salicyl phosphate.

Experimental

Preparation of Salicyl Phosphate.—The method of Anschuetz[23] for the preparation of salicyl phosphate is not satisfactory. The following procedure gave a pure product without difficulty and in high yield. A mixture of phosphorus pentachloride (54.8 g., 0.27 mole) and salicylic acid (38.5 g., 0.28 mole) was allowed to react at room temperature in a one-liter flask protected by a calcium chloride tube. The mixture liquefied within half an hour and the reaction was brought to completion by warming on a water-bath (60–65°) for one-half hour. To the cooled reaction mixture (ice-bath) dissolved in 100 ml. of acetone and 100 ml. of benzene, 14.0 ml. (0.78 mole) of water was then added slowly with swirling; after standing for half-hour (ice-bath) 200 ml. more of benzene was added and the mixture was allowed to stand overnight at room temperature. The filtered precipitate was washed with small portions of benzene, dried overnight *in vacuo* over potassium hydroxide (48 g., 80% yield), and recrystallized by dissolving in a boiling solution of 430 ml. of acetone containing 2.5 ml. of water. Upon addition of 650 ml. of benzene to the hot solution, small needles were obtained; yield of pure product 36 g., 60%; m.p. 162.5–163°; reported range, 140–150°.[2a,23] The product is extremely soluble in water, gives no ferric chloride test and no test for free phosphoric acid.

Anal. Calcd. for $C_7H_7O_6P$: P, 14.20; neut. equiv., 72.7. Found: P, 14.46; neut. equiv., 73.0, 73.1.

Determination of Mole Fractions: (M_0, M_1, M_2, M_3).[24]—Our measurements of acidity are substantially activity measurements.[25] Since concentrations of the particular ionic groups are the quantities in the rate equations 6 and 7, we have defined the three ionization constants of salicyl phosphate in the semi-classical manner; where [SP⁰], etc., are

$$pK_1 = p_aH + \log \frac{[SP^0]}{[SP']}; \quad pK_2 = p_aH + \log \frac{[SP']}{[SP'']}; \quad pK_3 = p_aH + \log \frac{[SP'']}{[SP''']}$$

concentration terms.[26] These semi-classical ionization constants will vary with ionic strength. They were obtained by measuring the p_aH at the three successive half neutralization points of salicyl phosphate, employing the modified Henderson equation in the calculations.[27] By dilution with water at these three points we could ascertain the change in *pK* values with ionic strength. From a plot of *pK versus* $\sqrt{\mu}$ we determined the *pK*'s for the three ionic strengths at which our hydrolyses were carried out (Table I). Our values are in substantial agreement with those reported in the literature[2a,28] and were obtained at room temperature 27 ± 2°. The equations for the mole fractions of the three main groupings of any tribasic acid are given by Smith.[29]

$$M_0 = \frac{[SP^0]}{[SP]} = \frac{(H^+)^3}{W}; \quad M_1 = \frac{[SP']}{[SP]} = \frac{(H^+)^2K_1}{W}; \quad M_2 = \frac{[SP'']}{[SP]} = \frac{(H^+)K_1K_2}{W}; \quad M_3 = \frac{[SP''']}{[SP]} = \frac{K_1K_2K_3}{W}$$

$$W = (H^+)^3 + (H^+)^2K_1 + (H^+)K_1K_2 + K_1K_2K_3;$$

( ) = activity; [ ] = concn.

The mole fractions M_0, M_1, M_2, M_3 were evaluated using the ionization constants determined at 27° (Table I). We have assumed in all our calculations that the mole fractions remain constant throughout the temperature range investigated. This assumption is valid if the following conditions are fulfilled: neither the *p*H of the buffer nor the *p*K's of the salicyl phosphate can vary in the relevant temperature region. The phthalate buffer (employed at all *p*H's, except at 6.93 and 7.67) changes only 0.05 *p*H unit from 12–45°.[29a] Calculations show that as great a change in pK_2 and pK_3 as ±0.2 unit effects only a very small change (*ca.* 2%) in M_2 at *p*H 5.67 (also 4.92). Therefore our evaluation of this specific rate constant k_2 at 5.67, assuming the constancy of M_2 is substantially correct. However, a change in pK_2 of ±0.1 unit shows a much greater change (*ca.* 15%) in M_2

(20) K. Auwers, *Ber.*, **40**, 3506 (1907); B. Anschuetz, *Ann.*, **442**, 20 (1920).

(21) A. Michaelis and W. Kerkhof, *Ber.*, **31**, 2174 (1898).

(22) L. J. Edwards, *Trans. Faraday Soc.*, **49**, 723 (1950).

(23) R. Auschuetz and W. O. Emery, *Ann.*, **228**, 308 (1885).

(24) The *p*H determinations were obtained with the use of a Cambridge *p*H meter which is claimed to have an accuracy of plus or minus 0.02 *p*H unit. The instrument was set with potassium acid phthalate buffer solution (0.05 *M*) of p_aH 4.01 ± 0.01 at 25° as recommended by the National Bureau of Standards in Research Paper RP 1495.

(25) R. G. Gates, *Chem. Revs.*, **42**, 1 (1948).

(26) The thermodynamic ionization constants are related to the semi-classical constants in the following manner: $pK_1 = -\log \left[K_1' \frac{f_{SP^0}}{f_{SP'}}\right]$ etc., where K_1', K_2', etc., are the thermodynamic ionization constant and f_{SP^0}, etc., are the relevant activity coefficients for the particular ionic strength.

(27) S. Glasstone, "Text Book of Physical Chemistry," D. Van Nostrand Co., Inc., New York, N. Y., 1943, p. 982.

(28) P. G. Walker and E. J. King, *Biochem. J.*, **47**, 93 (1950).

(29) T. B. Smith, "Analytical Processes," Edward Arnold and Co., London, 1946, p. 187.

(29) (a) W. M. Clark, "Topics in Physical Chemistry," Williams and Wilkins Co., Baltimore, Md., 1948, pp. 284, 490.

when the latter is evaluated at pH 6.93. The same considerations apply to the evaluation of k_1 at pH 2.32. However, since k_2 enters into the calculation for k_1 and at this pH (2.32) changes in pK_1 and pK_2 have a marked effect on M_2, k_1 is not considered as accurately evaluated as k_2. A detailed analysis of the change in mole fractions resulting from changes in pK's is given by Smith.[29]

Reagents. Molybdate Reagent.—A mixture of 139 ml. of concd. H_2SO_4 (Baker C.P. analyzed, 96.7%) and 25.0 g. of ammonium molybdate $(NH_4)_6(Mo_7O_{24})\cdot 4H_2O$ (Merck C.P.), diluted with distilled water to give one liter of solution.

1,2,4-Aminonaphtholsulfonic Acid Reagent (ANS).—One gram of Na_2SO_3 (Merck, C.P.) and 0.5 g. of the purified 1,2,4-aminonaphtholsulfonic acid[30] was diluted to 50 ml. with distilled water and a solution of 29.2 g. of $NaHSO_3$ (Merck, C.P.) in 150 ml. of distilled water was added to the latter. This solution was allowed to stand overnight in the ice-box and filtered from any precipitate. The reagent, when kept in the ice-box, was found to be stable for at least 4 months.

Buffers.—pH 2–4 prepared acc. to W. M. Clark from $M/5$ KH-phthalate and $M/5$ HCl; pH 4–6 prepared acc. to W. M. Clark from $M/5$ KH-phthalate and $M/5$ NaOH; pH 7–9 prepared acc. to L. Michaelis from sodium veronal and $M/10$ HCl; pH 10 prepared acc. to W. M. Clark from $M/5$ H_3BO_3, $M/5$ NaOH and $M/5$ KCl.

Analytical Method.[31]—A standard solution of KH_2PO_4 (Analytical Grade), containing 10 μg. of phosphorus per ml. of solution, was prepared. To each of 5 test-tubes, calibrated to 10.0 ml., and containing exactly 0, 1, 2, 3, 4 and 5 ml. of the standard solution, 1.0 ml. of the molybdate reagent and 0.4 ml. of the ANS reagent was added, diluted with distilled water to the mark, mixed and placed in the water-bath at 25 ± 1° for 20 minutes. The intensity of the blue color was determined with a Klett–Summerson photoelectric colorimeter employing a 660 mμ filter. The instrument was set to zero with the blank. Dilution with buffers (as in the experiments) or water, made no difference in the readings. A straight line was obtained for this range of concentrations when the reading was plotted against the concentration of phosphorus. A reading of 72 corresponded to 10 μg. of phosphorus. Insignificant changes in the readings were observed when the reading was taken after 25 minutes. It is, however, necessary to develop the color always at the same temperature, since it was observed that, *e.g.*, at 30° after 20 minutes 10 μg. of phosphorus was equivalent to a reading of 78 instead of 72.

As an orienting experiment the above procedure was employed with a quantity of salicyl phosphate sufficient to give 40 μg. of phosphorus per ml. after complete hydrolysis by boiling in water for several hours; calcd., 288; found, 290.

Determination of Rates of Hydrolysis.—The same procedure was employed at all pH's and temperatures. Test-tubes calibrated to 25.0 ml. and containing 12.5 ml. of the appropriate buffer were placed in a constant temperature bath (±0.05°) for at least 10 minutes. To the buffer solution 5 ml. of a solution of salicyl phosphate (1.4 mg./ml.) was delivered with a volumetric pipet and water was added to mark (final concentration 0.0013 mole/liter). After mixing, a sufficient sample of solution was immediately taken for a pH determination. After 10 minutes in the bath, 1 ml. of solution (containing maximally 40 μg. of available phosphorus as phosphate) was removed with a volumetric pipet and drained into a calibrated 10.0-ml. test-tube containing 1 ml. of molybdate reagent, previously cooled in an ice-bath. This effectively stops the reaction and the tube may be kept at ice temperature for 3–4 hours without any change in subsequent readings. Four-tenths ml. of ANS Reagent was added, followed by distilled water to mark, the tube inverted, and placed in a water-bath at 25° for 20 minutes. The reading was then taken. At appropriate intervals of time, 1.0-ml. samples were withdrawn and the same procedure followed. The final readings were obtained by allowing the tubes to remain in the bath until the reading remained constant. Invariably, the final reading was 290 ± 2. The pH determined after hydrolysis never varied more than 0.03 pH unit from the original reading. All runs were done at least in duplicate. The probable error was estimated to be less than 3%.[31] A plot of log (290 − Klett reading) *vs.* temperature gave a straight line. The rate constants were evaluated from the slope of the least squares straight lines (see Fig. 1).

Isolation of Salicylic Acid.—A solution of salicyl phosphate (1.0906 g.) (0.005 mole) in 10.0 ml. of NaOH (1.000 N) was prepared. After removal of 4.0 ml. and 0.1 ml. of this solution for pH (4.8) and phosphate determination (negligible), respectively, the mixture was incubated at 37.2 for 2.1 hours. Two ml. of ice-cold 5 N sulfuric acid was added to the ice-cold reaction mixture after removal of 0.10 ml. and subsequent dilution for phosphate determination, reading 54. The precipitate was filtered and dried overnight *in vacuo;* yield 210 mg., m.p. 158° with no depression of m.p. on admixture with salicylic acid; m.p. 158°. The amount of salicylic acid corresponding to the observed phosphate reading of 54, after 2.1 hours, is 212 mg. The value calculated for a half-life period of 2.1 hours is 56.

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(30) 1,2,4-Aminonaphtholsulfonic acid was purified according to Dr. S. Natelson (private communication). A mixture of 15 g. of the sulfonic acid (Eastman Kodak, White Label), 150 g. of $NaHSO_3$ and 5 g. of Na_2SO_3 (anhydrous) in one liter of water was warmed to approximately 90°, shaken until practically all the solid had dissolved, and filtered hot. Ten ml. of concd. HCl was added to the cooled filtrate. The precipitate was collected, washed with 95% ethanol, until the washings were colorless, dried *in vacuo* over calcium chloride and kept in a dark colored bottle, preferably in the cold.

(31) The method employed for phosphate determination was essentially the Fiske-SubbaRow method with the micro-modification of A. E. Sobel, *Ind. Eng. Chem., Anal. Ed.*, **17**, 242 (1945).