Allylic Phosphate Hydrolysis: Kinetics and Mechanism of Degradation of 3β,17α-Dihydroxy-6α-methylpregn-4-en-20-one 17-Acetate, 3-Phosphate

T. O. OESTERLING and J. H. GUSTAFSON*

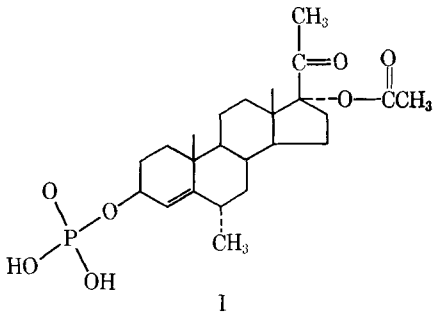
Abstract □ The kinetics and mechanism of degradation of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I) was studied as a function of pH and temperature. At low pH the least stable moiety is the allylic phosphate ester at C_3, while the most reactive function at high pH is the acyl ester at position 17. At pH 7 and 37°, the rate of phosphate ester hydrolysis is approximately 10^4 times faster than hydrolysis of the 17α-acetoxy function. Between pH 4 and 10 at 37° and between pH 2 and 6 at 4°, the rate of phosphate ester hydrolysis is first order with respect to hydrogen-ion concentration. The major products of allylic phosphate hydrolysis are an epimeric mixture of C_3 alcohols, supplying evidence that the reaction occurs principally by carbon–oxygen bond fission. The activation energy for phosphate ester hydrolysis of I is 21.5 ± 0.9 kcal./mole.

Keyphrases □ 3β,17α-Dihydoxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate degradation—kinetics, mechanism □ pH, temperature effects—allylic phosphate hydrolysis □ TLC—separation, identification □ Mass spectroscopy—identification □ NMR spectroscopy—identification

The synthesis and bioactivity of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I) have been reported by Morozowich *et al.* (1). It is a highly water-soluble progestational agent, which could be administered intravenously when rapid high levels of progestin are required such as in threatened abortion or premature labor. *In vivo*, I is enzymatically metabolized to form the corresponding C_3 alcohol (II) (1) and other potent progestational agents such as the 3-keto analog (2).

Table I—Effect of pH and Temperature on Rate of Phosphate Ester Hydrolysis of 3β,17α-Dihydroxy-6α-methylpregn-4-en-20-one 17-Acetate, 3-Phosphate (I)

pH	Buffer	Temperature	Ionic Strength	k, sec.$^{-1}$ × 10^5
2.42	3-Chloropropionate	4°	0.1	739
3.12	3-Chloropropionate	4°	0.1	131
4.08	Acetate	4°	0.1	12.8
4.97	Acetate	4°	0.1	1.61
6.12	Acetate	4°	0.1	0.124
6.10	Acetate	15°	0.1	0.582
6.10	Acetate	25°	0.1	1.23
4.00	Acetate	37°	0.1	1070
5.05	Acetate	37°	0.1	81.5
5.14	Acetate	37°	0.1	92.7
6.05	pH stat	37°	—	13.6
6.09	Acetate	37°	0.05	10.2
6.11	Acetate	37°	0.1	8.78
6.80	Maleate	37°	0.1	2.70
6.95	pH stat	37°	—	2.19
7.80	pH stat	37°	—	0.518
8.01	Borate	37°	0.1	0.286
8.90	Ammonia	37°	0.1	0.0700
9.00	Borate	37°	0.1	0.0301
9.04	Borate	37°	0.1	0.0308
9.95	Borate	37°	0.1	0.00375
6.20	Acetate	47°	0.1	19.0



I

The two moieties that are least stable from the standpoint of development of a chemically stable parenteral formulation of I are the acetoxy function at C_{17} and the allylic phosphate ester at C_3. Esters such as the C_{17}-acyl function of I are highly susceptible to general acid–general base catalysis. Turner (3) reported that the 17α-acetoxy function of 3β,17α-diacetoxyallopregnan-20-one was completely hydrolyzed after standing overnight in 0.25 *N* methanolic sodium hydroxide at room temperature. However, these type esters are usually much less reactive near neutral pH where, on the other hand, allylic phosphates such as the C_3 moiety of I are relatively unstable. For example, the half-life for phosphate hydrolysis of I at pH 8.78 and 37° is approximately 5 days (1).

Most previous studies of hydrolysis of allylic phosphate esters have been confined to those of acyclic monoterpene and sesquiterpene alcohols. The enzyme-catalyzed synthesis of squalene from mevalonic acid proceeds through acyclic terpenol phosphate and pyrophosphate intermediates such as farnesyl and geranyl pyrophosphate (4–6). These intermediates have been isolated and their properties studied to elucidate their role in the biochemical process. Such acyclic allylic phosphates are very unstable in acid and hydrolyze through carbonium-ion intermediates.

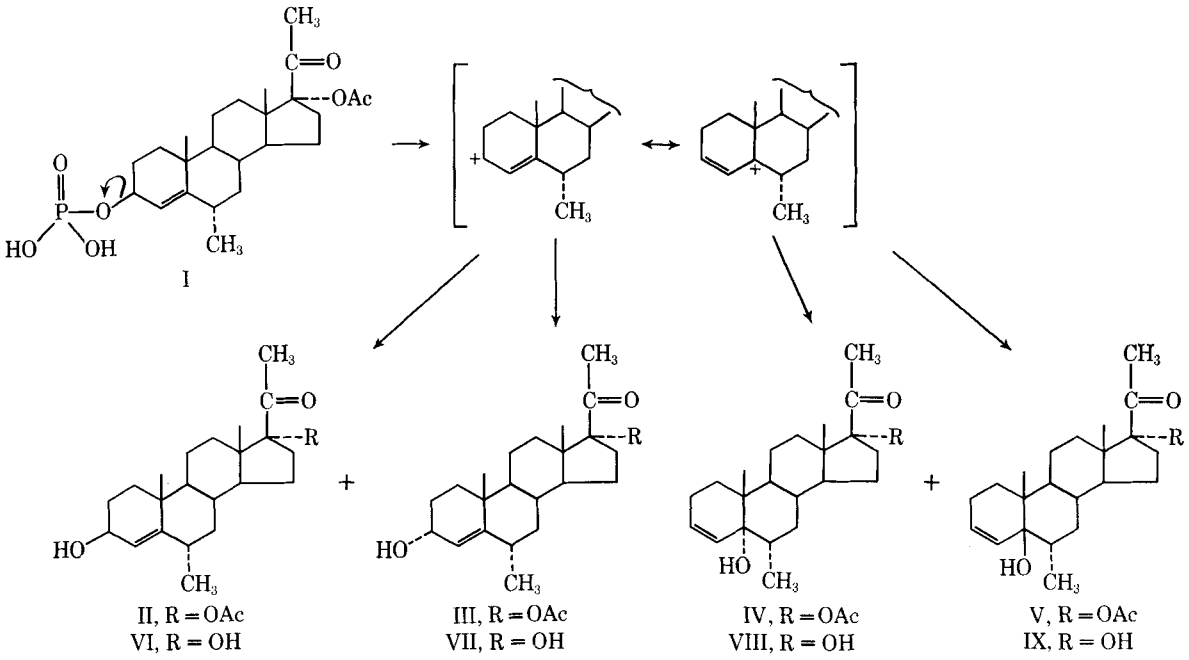
This report describes the kinetics and mechanism of degradation of I. The effects of pH and temperature on the rates of hydrolysis of the C_3-phosphate and C_{17}-acyl esters have been studied, and the major products of degradation have been identified.

EXPERIMENTAL

Materials—3β,17α-Dihydroxy-6α-methylpregn-4-en-20-one 17-acetate (II), 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I), and 17α-acetoxy-6-methylpregna-3,5-diene-20-one (XII) containing less than 2% impurities were used.[1] All other chemicals were reagent grade.

Kinetic Studies—*Phosphate Ester Hydrolysis*—The pH of reaction mixtures of I was maintained by a pH stat technique or by the

[1] Supplied by the research laboratories of The Upjohn Co.



Scheme I

buffers shown in Table I. Individual buffers were prepared from 3-chloropropionic acid, acetic acid, maleic acid, boric acid, ammonium hydroxide, and disodium carbonate and were adjusted to the desired pH at the temperature of the run by the addition of sodium hydroxide or hydrochloric acid. The ionic strength of buffered reaction mixtures was adjusted by the addition of potassium nitrate. Reactions were initiated by mixing temperature-equilibrated aqueous solutions of I with the buffer so that the final concentration was: I, 0.001 *M;* buffer, 0.05 *M;* and ionic strength, 0.1. After placing the mixture in a constant-temperature bath, 1.0-ml. samples were withdrawn at appropriate times, added to 1.0 ml. of 0.1 *M* NH_4OH, and refrigerated until assayed for inorganic phosphate. The pH of the reaction mixture was measured periodically during the run; if the pH varied by more than 0.2 unit from the initial pH, the run was discarded. All reaction mixtures in which phosphate ester hydrolysis was measured were prepared with freshly boiled deionized water.

For reaction mixtures where the half-life of phosphate ester hydrolysis was less than 1 hr., special techniques were employed. One-milliliter portions of an aqueous solution of I containing 2 mg./ml. were equilibrated at the temperature of the run. To obtain one time point, 1.0 ml. of equilibrated buffer was added with a syringe. At an appropriate time the reaction was quenched by the addition of 2.0 ml. of 0.1 *M* ammonium hydroxide with a syringe. Each portion of the reaction mixture was equilibrated for a different length of time until enough samples for kinetic analysis were obtained.

Kinetic runs in which pH was maintained with a pH stat were carried out as follows. Electrodes from the pH stat (Radiometer TTT1C titrator and SBR2C titragraph) and the titrant delivery tube were immersed in 39.0 ml. of freshly boiled deionized water in a stoppered jacketed vessel maintained at 37 ± 0.1°. After thermal equilibrium was attained, 40 mg. of I in 1.0 ml. of deionized water was added, and the reaction was initiated by adjusting the pH to the desired value by the addition of 1 *N* HCl or 1 *N* NaOH. Drift in pH during the run was corrected by the addition of 1 *N* HCl or 1 *N* NaOH. At appropriate times, 1.0-ml. aliquots were withdrawn and added to 1.0 ml. of deionized water; the sample was assayed immediately for inorganic phosphate content by the following procedure. Corrections for the volume of titrant added prior to sample withdrawal were incorporated into the assay results.

Table II—TLC Analysis of 3β,17α-Dihydroxy-6α-methylpregn-4-en-20-one 17-Acetate, 3-Phosphate (I) Reaction Mixtures

Zone Number	R_f	Percent	Molecular Ion
1	0.10	<1	388
2	0.18	5	346
3	0.31	57	388
4	0.39	3	346
5	0.48	32	388
6	0.55	<1	—[a]
7	0.63	<1	—[a]
8	0.70	<1	—[a]

[a] Insufficient sample.

The amount of inorganic phosphate in each reaction mixture aliquot was determined by Mokrasch's (7) assay. Because of the instability of the mixed reagent used in this assay, a standard was assayed after every three unknowns. Rates of phosphate ester hydrolysis were determined by least-squares analysis of log ($P_{i,\infty} - P_{i,t}$)-time data, where $P_{i,\infty}$ is the molar concentration of inorganic phosphate present at completion of hydrolysis that is equivalent to the initial molar concentration of I, and $P_{i,t}$ is the molar concentration of inorganic phosphate present at any time.

Acyl Ester Hydrolysis—Reaction mixtures were prepared by mixing 5 ml. of a temperature-equilibrated aqueous solution of I (40 mg./ml.) with a sufficient volume of 0.1 *N* NaOH to give the final desired pH. After diluting to 10 ml. with deionized water, the solution was mixed and placed at 37°. At appropriate times, 1.0-ml. samples were withdrawn and mixed with 1.0 ml. of 0.1 *M*, pH 9 borate buffer. The amount of intact 17α-acetoxy function remaining in the reaction mixture aliquot was measured by the method of Goddu *et al.* (8) as modified by Forist and Theal (9). Second-order rate constants of acyl ester hydrolysis were obtained by least-squares analysis of ln $(a-x)/(b-x)$-time data, where a is the initial concentration of I, b is the initial concentration of sodium hydroxide, and x is the amount of each reacted at any time

Identification of Products—Degradation products were identified by mass spectral and NMR studies and by comparison of TLC R_f values with authentic samples, when available. TLC studies were carried out by spotting ethyl acetate extracts of quenched reaction mixture aliquots on silica gel G and developing with methanol–acetone–water–chloroform–cyclohexane–hexane (2:10:0.2:80:20:40). Zones were visualized by heating the plates at 100–130° after spraying with 30% ammonium sulfate.

The relative amount of each zone was estimated by scanning the thin-layer plates with a Schoeffel model SD-3000 spectrodensitometer. Samples for mass spectral and NMR analyses were obtained by preparative TLC. Mass spectra were determined with an Atlas CH4 mass spectrometer in which samples were introduced by the direct inlet technique. NMR spectra were determined with a Varian HA-100 spectrometer.

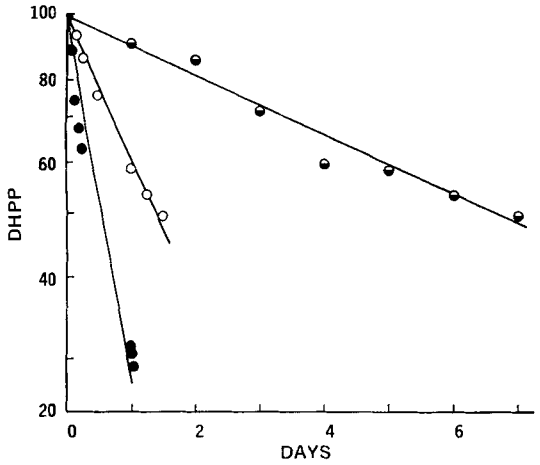


Figure 1—*Apparent first-order phosphate ester hydrolysis of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I) at pH 6.1. Key: ◒, 4°; ○, 15°; and ●, 25°.*

RESULTS AND DISCUSSION

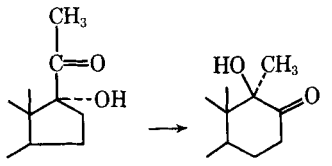
Identification of Products—Table II shows the R_f values of the products in a reaction mixture of I after about 50% phosphate hydrolysis. Also shown are the relative amounts of the zones and the number assigned as molecular ion determined by mass spectral studies of compounds isolated by preparative TLC. In general, the relative amount of each species represented in Table II was independent of pH, although at higher pH the percentages of Zones 2 and 4 tended to increase. Four of the eight degradation products of I (Table II) were present only in trace amounts; of these four, a sufficient sample for mass spectral studies could only be obtained from Zone 1.

Numerous reports have shown that allylic phosphates and pyrophosphates hydrolyze through carbonium-ion intermediates (4–6, 10). Such a mechanism is reasonable for dephosphorylation of I and is compatible with the data shown in Table II. Scheme I shows a possible mechanism by which I could hydrolyze; it involves a carbonium-ion intermediate stabilized by the allylic double bond. The three products in Table II possessing molecular weight 388, the molecular weight of the alcohol which would result from hydrolysis of the C_3-phosphate, could correspond to three of the four products shown in Scheme I (I–IV). Goodman and Popjak (4) reported that the relative amounts of secondary and tertiary alcohols resulting from pyrophosphate hydrolysis through a carbonium-ion intermediate differ from one compound to another (4). For the case of I, the secondary C_3 epimeric alcohols (II and III) are the most likely products due to the relative instability of tertiary allylic alcohols formed at C_5 and the steric hindrance involved in addition reactions at C_5. The R_f of Zone 3 is identical to the authentic sample of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate (II) which, combined with the mass spectral data in Table II, establishes the identity of the major product. The presence of a carbinol proton in the NMR spectra of Zone 5 in conjunction with a molecular weight of 388 was taken as conclusive evidence that Zone 5 represents III, the C_3 epimer of II. Mass spectra of the compounds isolated from Zones 3 and 5 provided further evidence that they are II and III. The signal at *m/e* 370 (molecular ion minus water) of Zone 3 was of lower intensity than the molecular ion signal typical of equatorial hydroxyl groups (11, 12), while the signal at *m/e* 370 of the compound from Zone 5 was of greater intensity than the molecular ion signal typical of axial hydroxyl groups (11, 12). The unsaturation in Ring A of II and III slightly distorts the configuration of the C_3 hydroxyl groups. However, probably sufficient axial (Compound III) and equatorial (Compound II) character remains in the 3-hydroxyl groups of these products to show this mass spectral response.

An epimeric mixture of C_3 alcohols could be formed in reaction mixtures of I by means other than the mechanism shown in Scheme I. For example, III could be formed by epimerization of II after phosphate hydrolysis. However, only trace amounts of III were determined by TLC when authentic II was subjected to similar conditions for comparable periods of time, providing evidence that epimerization subsequent to hydrolysis is unlikely. Since a substantial amount of III was formed in reaction mixtures of I under conditions where epimerization of II is negligible, the mechanism of hydrolysis postulated in Scheme I is quite reasonable and indicates that the reaction proceeds primarily by carbon–oxygen bond fission.

The molecular weights of the other two major products of I, Zones 2 and 4 in Table II, correspond to the alcohol that would result from hydrolysis of the acyl ester at C_{17}. Since hydrolysis of the 17α-acetoxy moiety proceeds with retention of configuration under the conditions of this study (3), Zones 2 and 4 probably represent C_3 epimers of the 17α-deacetoxy derivative of I. By analogy to the products resulting from phosphate hydrolysis, Zone 2 represents 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one (VI), and Zone 4 represents 3α,17α-dihydroxy-6α-methylpregn-4-en-20-one (VII).

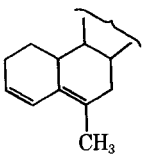
The identities of Zones 1 and 6–8 in Table II were not conclusively established. Mass spectral data suggest that Zone 1 may be IV or V, and inspection of Drieding models shows that IV is favored. However, there was an insufficient sample for further tests. Other possible products which might be represented by Zones 6–8 include those that would arise from D-homoannulation of Compounds VI–IX (Scheme II).



Scheme II

Ring expansions, such as the one depicted in Scheme II, are both acid (13) and base (14) catalyzed, and only trace amounts would be expected under the mild conditions of this study.

Secondary and tertiary allylic alcohols, such as Compounds II–V, reportedly undergo facile dehydration in acid to form conjugated dienes of the type represented by XI (15). The R_f of an authentic sample of the diene of Compounds II–V, 17α-acetoxy-6-methylpregna-3,5-dien-20-one (XII), was 0.9 in the solvent system



XI

described in the *Experimental* section, and none of this product was detected in any of the reaction mixtures in Table I. How-

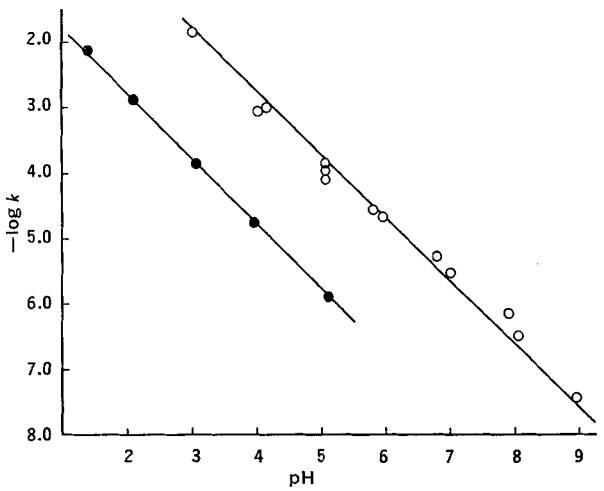


Figure 2—*pH–rate profile of phosphate hydrolysis of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I). Key: ●, 4°; and ○, 37°.*

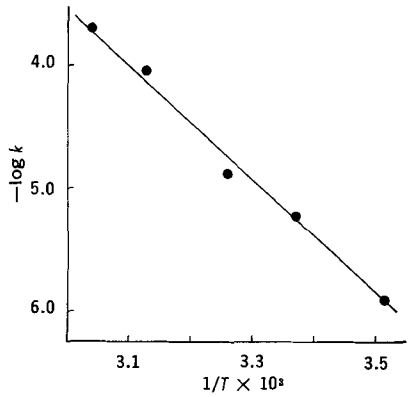


Figure 3—*Arrhenius plot of phosphate hydrolysis of 3β,17α-dihydroxy - 6α-methylpregn - 4 - en-20-one 17-acetate, 3-phosphate (I) at pH 6.1.*

ever, this diene can be detected in lower pH reaction mixtures where phosphate hydrolysis cannot be conveniently measured. For example, when I was treated with 0.25 *N* HCl at room temperature, almost complete conversion to XII occurred after 1 hr.

Rate Studies—The phosphate ester moiety of I hydrolyzed by an apparent first-order process under all experimental conditions. Some typical first-order curves of phosphate hydrolysis are shown in Fig. 1.

The effects of pH and temperature on the rate of phosphate ester hydrolysis of I are shown in Table I. In general, effects of ionic strength and buffer species on the rate constant appear minimal. However, the data at pH 6 and 37° show a slight tendency toward suppression of the rate constant with increasing ionic strength, and the pH 9 data suggest a possible buffer effect. Rate–pH profiles between pH 4 and 10 at 37° and between pH 2 and 6 at 4° are shown in Fig. 2. The slope of the curve of the 4° data is −1.00 and that of the 37° data is −0.97.

Three species of monosubstituted orthophosphate esters such as I contribute to the observed rate constant in the pH range 2–10. Although dissociation constants vary slightly with the nature of the substitution, in general the predominant species at pH 2 is the neutral or undissociated form; in the pH range 3–6, the monoanionic species predominates; and at pH 6–10, the dianion is the major species. The shape of the rate–pH profile depends on the concentration and relative reactivities of these three species. For example, in the case of 2,4-dinitrophenyl phosphate (16) where the dianion is the most reactive of the three species, the observed rate constant is greater in the pH range 6–10 where the dianion is the major species. If the monoanion is more reactive than the neutral or dianionic forms, as in the case of methyl phosphate (17), the rate–pH profile shows a maximum in the neighborhood of pH 4 where the monoanion is present at maximum concentration. The maximum in the pH–rate profile will become less pronounced as the reactivity of the neutral species increases relative to that of the monoanion. Examples of this behavior are glucose-1-phosphate (18) and lincomycin-2-phosphate (19).

Finally, the case arises where the relative reactivity of the neutral form increases to the point where the log of the rate constant shows a linear dependence on pH. The slope of the resulting line can indicate the number of species contributing to the observed rate contant; for example, a plot of log *k versus* pH for the hydrolysis of *tert*-butyl phosphate is a straight line between pH 2 and 7 (20) with a slope of −0.7. The nonunity value of the slope could be interpreted as an indication that more than one species, *i.e.*, monoanion and undissociated, of *tert*-butyl phosphate is contributing to the overall rate constant.

On the other hand, Bunton and Humeres (21) obtained a slope of −1 for the straight-line plot of pH *versus* log *k* for the hydrolysis of ribose-1-phosphate in the pH range 3.5–7.5. This behavior was attributed to the much higher reactivity of the neutral or undissociated form over the monoanionic or other negatively charged species of ribose-1-phosphate. A similar interpretation can be applied to the data shown in Fig. 2. The major reacting species for phosphate hydrolysis of I is probably the undissociated form over the entire pH range covered in this study. A rate–pH profile of allylic pyrophosphate hydrolysis based on a single-point measurement method has been reported by Goodman and Popjak (4); however, their method of measurement and the possibility of assay variation at high pH preclude direct comparison with Fig. 2.

Table III—Second-Order Rate Constants of Acyl Ester Hydrolysis of 3β,17α-Dihydroxy-6α-methylpregn-4-en-20-one 17-Acetate, 3-Phosphate (I) at 37°

NaOH, *N*	*k*, l. mole^{-1} sec.$^{-1}$ × 10^3
0.03	3.36
0.05	3.00
0.07	4.07
0.09	3.37

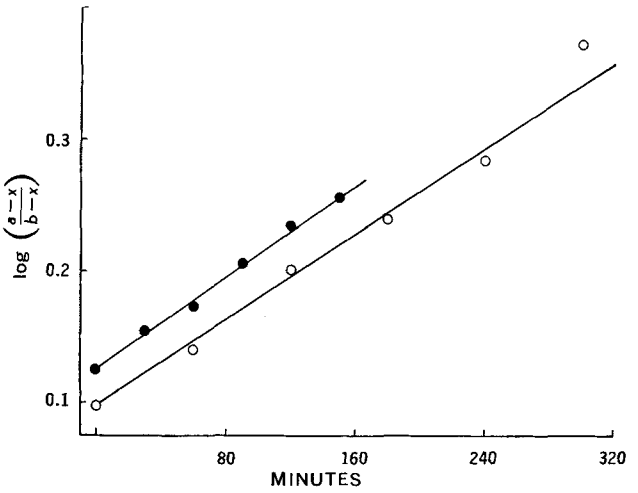


Figure 4—*Second-order plot of acyl ester hydrolysis of 3β,17α-dihydroxy-6α-methylpregn-4-en-20-one 17-acetate, 3-phosphate (I). Key:* ●, a = *0.04* M, b = *0.05* M; *and* ○, a = *0.04* M, b = *0.03* M.

The phosphate moiety of I is not sufficiently stable to allow measurement of the dissociation constant of the most acidic proton. Thus, no estimate of the specific rate constant for hydrolysis of the neutral species could be obtained. However, the data in Table I clearly show that the neutral form of I is many times more reactive than the analogous species of other types of monosubstituted-orthophosphate esters such as alkyl (17, 20), acyl (22), glycosidic (18, 21), and carbohydrate phosphates as glucose-2-phosphate (23) and lincomycin-2-phosphate (19). In fact, I is also much more reactive than acyclic allylic phosphates and pyrophosphates such as neryl, dimethylallyl, and geranyl (24, 25) phosphate and geranyl, dimethylallyl, neryl, and farnesyl pyrophosphates (4, 5). The half-lives for phosphate hydrolysis of these acyclic allylic phosphates and pyrophosphates range from 2 to 15 min. in 0.1 *N* HCl at 25°, while the extrapolated half-life (Table I) for hydrolysis of I at pH 1 and 25° is about 0.01 min.

An Arrhenius plot of phosphate ester hydrolysis (I) is shown in Fig. 3. The activation energy calculated from the slope of Fig. 3 is 21.5 ± 0.9 kcal./mole. This activation energy represents the reactivity of the neutral species, even though it was present in very low concentration in the reaction mixtures at pH 6.1.

Second-order rate constants obtained for hydrolysis of the 17α-acetoxy function of I as a function of sodium hydroxide concentration at 37° are shown in Table III. Figure 4 shows typical second-order plots of the 0.03 and 0.05 *N* NaOH data. Extrapolation of the average second-order rate constant in Table III to pH 7 (assuming minimal buffer effects) shows that phosphate ester hydrolysis is about 10^4 times faster than acyl hydrolysis at C_{17}.

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pH Effects on Salicylate Absorption from Hydrophilic Ointment

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Abstract □ Hydrophilic ointment formulations containing salicylic acid were prepared at different pH levels by inclusion of phosphate buffers. Percutaneous salicylic acid absorption was studied by measuring salicylate blood levels in rabbits at 1.5-hr. intervals from 1.5 to 7.5 hr. after ointment application. The effect of 15% dimethyl sulfoxide in the various formulations was also determined. Formulations without dimethyl sulfoxide produced maximal blood concentrations at the highest pH level (10.78) and higher concentrations at the lowest pH (2.97) than at the intermediate ones (4.48, 6.80, 9.23). Proportionally higher concentration of undissociated species appeared to account for the increased absorption observed at the most acidic pH, while increased dissolution was probably responsible for the greater blood levels recorded at the most alkaline pH. Dimethyl sulfoxide produced a more rapid rate of salicylate absorption as well as greater peak blood levels at each pH. However, its influence on rate of absorption and on peak blood levels was less pronounced at the highest pH levels. This finding seems to indicate that the positive effect of dimethyl sulfoxide on percutaneous salicylate absorption is due in part to its ability to enhance the dissolution of salicylic acid.

Keyphrases □ Salicylate absorption—hydrophilic ointment □ pH effect—salicylate absorption from hydrophilic ointments □ Dimethyl sulfoxide effect—salicylate absorption, hydrophilic ointment □ Percutaneous absorption—salicylate in hydrophilic ointment

Experimental evidence indicates that percutaneous absorption of most drugs occurs by passive diffusion of an undissociated therapeutic entity across a lipoidal barrier (1). Characteristics of an ointment base, in particular its thermodynamic activity and included solvents, influence the rate of release from the base and the rate and quantity of percutaneous penetration and absorption. Knowledge of the specific influence of each of these factors can aid in the formulation and choice of ointment bases designed to elicit a specified rate and magnitude of percutaneous drug absorption for a particular drug administered topically.

Higuchi (2) indicated that the chief driving force for diffusion and penetration is thermodynamic activity, which for a weakly acidic drug is inversely proportional to the term 10^{pH}. Bhatia and Barber (3) found changes in the local anesthetic activity of ethyl aminobenzoate incorporated into hydrophilic ointment USP buffered at nine different pH values ranging from 3.5 to 10.0. They observed maximum pharmacologic activity at pH 6 and 7 and found a marked decrease when the pH was decreased or increased from that neutral region. Bhatia and Barber (3) attributed their results to the possibility that maximum penetration and pharmacologic activity occurred at or near the pH of rat skin. Stolar *et al.* (4) theorized that the concentration of free salicylic acid present at a pH of 6.2–6.5, in either an aqueous solution of 6.95% sodium salicylate or in a sodium salicylate cream having an aqueous dispersion medium, could not account for the measurable salicylate blood levels observed in rabbits. Blank and Gould (5) observed an increase in the absorption of sodium laurate solutions in contact with excised human skin at reduced pH. They attributed the increase in absorption at the lower pH to the formation of more undissociated lauric acid.

The use of solvents to enhance percutaneous absorption was suggested by the ease with which gases, such as nitrobenzene vapor (6), can penetrate the skin (7–10), and has been confirmed by the greater enhancement of percutaneous absorption produced by the volatile