nol have also been observed to occur at low gas pressures (10^{-6}–10^{-1} torr).[3-5] It was suggested that reaction 4 involved excited ions and was in competition with collisional stabilization of the protonated dimer.[3,5]

$$C_2H_5OH_2^+ + C_2H_5OH \longrightarrow (C_2H_5OH)_2H^{+*} \quad (3')$$

$$(C_2H_5OH)_2H^{+*} \longrightarrow (C_2H_5)_2OH^+ + H_2O \quad (4')$$

$$(C_2H_5OH)_2H^{+*} + C_2H_5OH \longrightarrow (C_2H_5OH)_2H^+ + C_2H_5OH \quad (8)$$

In the present system the pressures are sufficiently high (10^2–10^3 torr) that essentially all of the initially formed dimers would be thermally equilibrated with the medium. Reaction 4 therefore represents the normal pyrolysis of the ionic species.

It may be shown by steady-state kinetics treatment that if the dimer $(C_2H_5OH)_2H^+$ were the main identity of MH^+ in the neutralization reaction 7, then ether formation should be 0.5 order. On the other hand, if the trimer were the main identity of MH^+ in reaction 7, ether formation should be -0.5 order. Experimentally, at 350° the order of the ether formation reaction was 0.7 at 100 torr and -0.5 at 1000 torr. This means that the average "solvation number" of the protons shifted from about two to three as the ethanol pressure increased from 100 to 1000 torr.

The activation energy of ether formation in the low-pressure region corresponds to $(E_4 - {}^1/_2E_7)$, and in the high-pressure region it corresponds to $(E_4 - \Delta H_6 - {}^1/_2E_7)$. The value of E_7 may be assumed to be zero. That of ΔH_6 is unknown, but as an approximation it may be taken as equal to that of the analogous reaction in water vapor, which is -22 kcal/mole.[6] This estimate is based upon information about the clustering of methanol and water molecules about protons in the gas phase.[7] Thus, $E_4 \leq E_a(\text{ether}) \leq E_4 + 22$ kcal/mole. At an ethanol concentration of 0.66 g/l. (570 torr at 350°, which is intermediate between the high- and low-pressure regions), the activation energy of the ether formation is 30 kcal/mole. Thus, $30 > E_4 > 8$ kcal/mole. However, reaction 4 is about 19 kcal/mole endothermic, so one may conclude that $30 > E_4 > 19$ kcal/mole.

Ammonia inhibits ether formation, probably through reactions such as

$$(C_2H_5OH)_2H^+ + NH_3 \longrightarrow (C_2H_5OH)_2H^+(NH_3) \quad (9)$$

$$\longrightarrow (C_2H_5OH)H^+(NH_3) + C_2H_5OH \quad (10)$$

The proton affinity of ammonia (209 ± 7 kcal/mole[8]) is greater than that of ethanol (193 ± 8 kcal/mole[9]).

$H_2CO + C_2H_5OH \rightarrow CH_3OH + CH_3CHO$

During the radiolysis of ethanol vapor (0.66 g/l., 375°), methanol was formed with a G value of 50. Mechanistic considerations indicated that the important reactions were (11) and (12). One or more ethanol molecules

$$C_2H_5OH_2^+ + CH_2O \longrightarrow CH_3CHOH^+ + CH_3OH \quad (11)$$

$$CH_3CHOH^+ + C_2H_5OH \longrightarrow CH_3CHO + C_2H_5OH_2^+ \quad (12)$$

might be clustered about the positive ions in reactions 11 and 12, but they should have little effect on the energetics of the reactions because the "solvation" energy of $C_2H_5OH_2^+$ should be approximately equal to that of CH_3CHOH^+.

Support for the proposed reactions 11 and 12 was obtained by irradiating a sample that contained 5 mole % of formaldehyde. The increases in the G values of methanol and acetaldehyde were 563 and 521, respectively, and corresponded to the conversion of 70% of the formaldehyde in the sample. The somewhat smaller increase in the acetaldehyde yield was interpreted in terms of secondary decomposition of that compound.

Reaction 11 is analogous to the reaction of alkane ions with olefins.[10]

The study of these ionic chain reactions is continuing.

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(9) L. Tal'rose and E. L. Frankevich, *J. Am. Chem. Soc.*, **80**, 2344 (1958).
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Lysozyme Catalysis. Evidence for a Carbonium Ion Intermediate and Participation of Glutamic Acid 35[1]

Sir:

A mechanism of action of lysozyme has been put forward by Phillips, *et al.*[2a] A central part of this proposal is formation of a glycosyl carbonium ion in a reaction in which glutamic acid 35 participates as an acid catalyst and aspartic acid 52 stabilizes the developing ion. Since studies of model systems[2b] have demonstrated that glycoside cleavage can be either general or specific acid catalyzed and proceed through either a covalent or a carbonium ion intermediate, it is particularly necessary to examine the chemistry of the enzymic reaction. The following data suggest that lysozyme catalysis does involve general acid catalyzed formation of a carbonium ion.

Since lysozyme catalyzes glycosyl transfer[3-5] as well as glycoside hydrolysis, there must be an intermediate that can partition between reaction with water (hydrolysis) and some other acceptor (transfer). A change in transfer rate that results from a change in acceptor chemistry should reflect the nature of the glycosyl enzyme and its path of reaction. There should be less difficulty understanding data of this kind than that derived from studies of the over-all process, in which nonproductive binding of substrate causes severe kinetic complications.[3]

In a typical transfer experiment, tri-NAG[6] was hydrolyzed by lysozyme in the presence of a small amount of radioactive acceptor (of specific activity sufficient to compete with the solvent, yet not perturb the system, *i.e.*, an acceptor concentration of less than 5%). The

(1) This work was supported by Research Grant GB-6446 from the National Science Foundation.
(2) (a) C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. Roy. Soc.* (London), **B167**, 378 (1967); D. C. Phillips, *Proc. Natl. Acad. Sci. U. S.*, **57**, 484 (1967). (b) Recent papers that cite pertinent literature are: C. A. Vernon, *Proc. Roy. Soc.* (London), **B167**, 389 (1967); T. H. Fife, *J. Am. Chem. Soc.*, **89**, 3228 (1967); D. Piszkiewicz and T. C. Bruice, *ibid.*, **89**, 6237 (1967).
(3) J. A. Rupley, *Proc. Roy. Soc.* (London), **B167**, 417 (1967); J. A. Rupley and V. Gates, *Proc. Natl. Acad. Sci. U. S.*, **57**, 496 (1967).
(4) N. Sharon and S. Seifter, *J. Biol. Chem.*, **239**, PC2398 (1964).
(5) N. A. Kravchenko and V. I. Maksimov, *Izv. Akad. Nauk SSSR, Ser. Khim.*, 584 (1964).
(6) Abbreviations: NAG, N-acetylglucosamine; tri-NAG, β-(1,4)-linked trisaccharide of NAG.

oligosaccharides produced by transfer and hydrolysis were separated on columns of either charcoal–Celite or Sephadex, the latter being used for the few cases in which transfer products did not elute from charcoal. Experimental details and calculations of the rate of transfer relative to that of hydrolysis (k_A^{rel}) are described in Table I.

Table I. Relative Acceptor Reactivity[a]

Acceptor	Concn, *M*	Method of anal	k_A^{rel} [b,c]
Benzyl alcohol	0.008–0.02	*e*	9.7
Methanol	0.25	*d*	3.7
Cyclohexanol	0.0002–0.003	*e*	2.4
1-Propanol	0.005–0.42	*d*	2.2
Ethanol	0.01–1.0	*d*	2.1
Phenol	0.01–0.02	*e*	2.1
Glycerol	0.01–0.05	*d*	1.6[g]
Water			(1.0)
2-Propanol	0.025	*d*	0.5
2-Methyl-2-propanol	0.02–0.08	*d*	<0.2
CH_3SH	0.01–0.1	*d*	<1
H_2S	0.01	*d*	<1
KCNS	1.0	*d*	<0.02
NaN_3	0.5	*f*	<2
NaF	1.0	*f*	<2

[a] Conditions of reaction: 0.048 *M* tri-NAG, 0.0033 *M* lysozyme, specified concentration of acceptor; 40°, pH 5.3, 0.1 ionic strength, 24 hr. [b] The reaction rate for an acceptor relative to water: k_A^{rel} = (transfer products)(water)/(hydrolysis products)(acceptor). For this calculation it was assumed that transfer products are hydrolyzed neither spontaneously nor in an enzyme-catalyzed reaction. In this connection, glycosides of acceptors listed in the table are stable in aqueous solution.[c] Furthermore, transfer products formed with saccharides that are better acceptors ($k_A^{rel} \sim 100$) are under similar conditions not susceptible to enzymic attack (J. A. Rupley, unpublished data). [c] J. Stanek, M. Cerny, J. Kocourek, and J. Pacak, "The Monosaccharides," Academic Press, New York, N. Y., 1963. [d] Products analyzed on charcoal columns. Concentrations of transfer products were calculated by integration over the saccharide fractions containing radioactivity, after correcting for impurities sometimes present in the acceptor. The concentration of hydrolysis products was obtained by summing with appropriate weighting of the amounts of each oligosaccharide of NAG. [e] Products separated on Sephadex columns. Calculations as in *d*, except Sephadex did not adequately resolve the NAG saccharides and the concentration of hydrolysis products was taken as the average for similar reaction mixtures that had been separated on charcoal. [f] Nonradioactive acceptors were used at high concentration. After separation of the reaction mixture on charcoal, the fractions were deacylated by alkaline hydrolysis and analyzed for amine content using ninhydrin. There were no transfer products distinguishable from NAG oligosaccharides. The limits given for k_A^{rel} correspond to the level at which a new saccharide component could have been observed. [g] Two glycoside peaks were observed. The value given is one-third of the total rate.

Saccharides that exhibit the specificity requirements of lysozyme are significantly more effective acceptors than water (NAG is 2000 times better),[3] indicating that the structure of the acceptor and presumably binding of it to the glycosyl enzyme can be important in determining k_A^{rel}. The alcohols listed in Table I do not resemble saccharides and, as expected, do not exhibit such enhanced reactivity. It is therefore significant that there is no substantial difference in k_A^{rel} between alcohols that are of similar steric requirement but greatly different p*K*.[7] Within a series of compounds having the same attacking atom, the rate of reaction as a rule follows a Brønsted relationship,[8] which the data of Table I demonstrate is not true for lysozyme. Because of the large values of k_A^{rel} for certain sugars,[3] the reaction should not be diffusion controlled. General base catalysis is then the apparent explanation of the leveling seen in Table I. Specifically, phenol is less basic than methanol and should be a poorer nucleophile, but a general base catalyst could be for phenol correspondingly more effective and bring the reactivities of the alcohols together. This explanation has been given for the nearly equal rates observed with a variety of nucleophiles in the deacylation of furoyl-chymotrypsin,[9] and a related one for the comparatively small effect of leaving-group character in the acid-catalyzed hydrolysis of glycosides.[10] The enzymic hydrolysis of the glycosidic bond is the reaction of more general interest. Assuming microscopic reversibility, reaction of the glycosyl enzyme should be along a path the reverse of that for glycoside cleavage, and thus we can conclude that general acid catalysis operates in the latter process. An alternative explanation of the data of Table I that should be considered is specific base catalysis, *i.e.*, reaction of RO^- with no involvement of a basic group of the enzyme as a catalyst. In this case there could be compensation through opposition of (1) a Brønsted effect with coefficient 1 for reactivity of the alcoholate species and (2) the dependence of the concentration of this species on alcohol p*K*. Opposing this is the evidence that in aqueous solution oxygen anions of high p*K* (such as OH^- and CH_3O^-) deviate from a Brønsted relationship that holds for reactants of lower p*K*;[8] this would be inconsistent with the data of Table I. It is of interest that if alkoxide were the species reacting with the glycosyl enzyme, then formation of the glycosyl enzyme would not be an acid-catalyzed process.

If there is a covalent glycosyl enzyme (*i.e.*, if C-1 of the intermediate is tetrahedral), sulfur acceptors should be substantially more reactive than their oxygen analogs.[11] In contrast, this may not be the case if a glycosyl carbonium ion is involved: (1) thioethoxide and thiophenoxide are not significantly more reactive than methanol in the solvolysis of tetramethylglycosyl chlorides;[13] (2) carbonyl carbon and other positive centers, such as tetrahedral phosphorus, exhibit the same or lower reactivity for sulfur compounds compared with the corresponding oxygen compounds.[8,14] Failure to observe reaction of H_2S, CH_3SH, and SCN^- (Table I) thus suggests that the glycosyl enzyme resembles more a carbonium ion than a covalent intermediate. It is necessary to note that differences in

(7) The alcohols listed in Table I range over about 4 p*K* units for dissociation of ROH_2^+ and about 6 units for dissociation of ROH; see E. M. Arnett, *Progr. Phys. Org. Chem.*, **1**, 324 (1963).

(8) For relevant review discussions of nucleophilic reactivity, see J. O. Edwards and R. G. Pearson, *J. Am. Chem. Soc.*, **84**, 16 (1962); J. F. Bunnett, *Ann. Rev. Phys. Chem.*, **14**, 271 (1963); T. C. Bruice and S. J. Benkovic, "Bioorganic Mechanisms," Vol. 1, W. A. Benjamin, Inc., New York, N. Y., 1966.

(9) P. W. Inward and W. P. Jencks, *J. Biol. Chem.*, **240**, 1986 (1965).

(10) T. E. Timell, *Can. J. Chem.*, **42**, 1456 (1964).

(11) Although reactivity correlations have usually been for the anions,[8] there is evidence that for S_N2 displacements the difference between sulfur and oxygen is even greater for thiols–alcohols than for the dissociated species. Protonation of thiophenolate and methoxide decreases reactivity against CH_3Br by 1.7×10^4 and 2×10^6, respectively.[12]

(12) R. G. Pearson, H. Sobel, and J. Songstad, *J. Am. Chem. Soc.*, **90**, 319 (1968).

(13) A. J. Rhind-Tutt and C. A. Vernon, *J. Chem. Soc.*, 4637 (1960).

(14) G. E. Lienhard and W. P. Jencks, *J. Am. Chem. Soc.*, **88**, 3982 (1966).

solvation would not explain the failure of sulfur compounds to react. Owing to the attacking atom presumably being less heavily hydrogen bonded in the enzyme cleft than in water, sulfur derivatives should be favored over oxygen analogs (more energy must be spent in breaking water-oxygen hydrogen bonds). Also, although sulfur is larger than oxygen, it is not excluded from the reaction center, inferred from the recent report of lysozyme-catalyzed hydrolysis of a thiophenyl glycoside.[15]

In summary, the enzymic properties of lysozyme support the mechanism developed from the crystal structure. This is true as well for features of the mechanism not considered in this note, *e.g.*, cleavage of the C_1–O_4 bond, retention of configuration, and distortion of the substrate.[3]

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(15) G. Lowe, G. Sheppard, M. L. Sinnott, and A. Williams, *Biochem. J.*, 104, 893 (1967).

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The Structure of Antheridiol, a Sex Hormone in *Achlya bisexualis*

Sir:

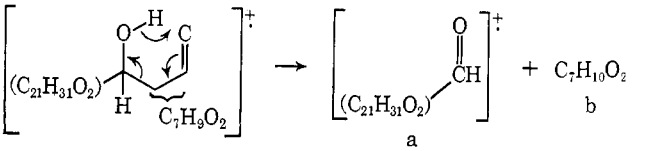
Sexual reproduction in the aquatic fungus *Achlya bisexualis* is governed by specific substances.[1] One of these, designated antheridiol, is secreted by the female plant and brings about formation of antheridial hyphae on the male plant.[2] Antheridiol, which was isolated in crystalline form, is characterized by its melting point, 250–255° dec, a broad absorption band in the uv at 220 mμ (ε 1.7×10^4), strong absorption bands in the ir at 3390, 1742, and 1672 cm^{-1}, and extremely high and specific biological activity (at a concentration as low as 2×10^{-8} mg/ml).[2]

Because of the scarcity of material and the concomitant lack of any hint concerning the chemical nature of the hormone, a high-resolution mass spectrum was taken, using a few micrograms of the substance. The data, displayed in the form of an element map,[3] showed the elemental composition to be $C_{29}H_{42}O_5$. In addition, the distribution of the elemental composition of the fragment ions was very revealing. The ions of highest mass in the C–H, C–H–O, and C–H–O_2 groups were $C_{19}H_{23}$, $C_{21}H_{30}O$, and $C_{22}H_{30}O_2$, and the species $C_{19}H_{25}O$ and $C_{19}H_{27}O_2$ were particularly abundant. Such a distribution is strongly suggestive of a steroid containing two oxygen functions in the tetracyclic system. Assuming the abundant $C_{19}H_{27}O_2$ ion to originate by simple cleavage of the C-17,C-20 bond, the steroid nucleus would have to contain two double bonds.

(1) J. R. Raper, *Am. J. Bot.*, **26**, 639 (1939); J. R. Raper and A. J. Haagen-Smit, *J. Biol. Chem.*, **143**, 311 (1942).
(2) T. C. McMorris and A. W. Barksdale, *Nature*, **215**, 320 (1967).
(3) K. Biemann, P. Bommer, and D. M. Desiderio, *Tetrahedron Letters*, 1725 (1964).

At this point a steroidlike structure was assumed as a working hypothesis with the remaining ten carbons and three oxygens representing a "side chain" at C-17. The position of the third oxygen atom was revealed by the fact that the most intense peak in the spectrum corresponded to $C_{22}H_{32}O_3$, suggesting C-22 as the point of attachment. This is further confirmed by the scarcity of C–H–O_3 ions with less than 22 carbon atoms. The changes in elemental composition of the molecular ion and the fragment ions corresponding to $C_{19}H_{27}O_2$ and $C_{22}H_{32}O_3$ in antheridiol itself upon hydrogenation (30% Pd–C, in ethanol) and acetylation (acetic anhydride–pyridine), respectively, indicated that there are two carbon–carbon double bonds (M^+ of hydrogenation product $C_{29}H_{46}O_5$), one of which is in the ring system and one in the side chain beyond C-22, and two hydroxyl groups (M^+ of acetate $C_{33}H_{46}O_7$), again one in the ring system and one at C-22. In accord with the steroid hypothesis, it follows that there should be a carbonyl group as well as a hydroxyl and a carbon–carbon double bond in the tetracyclic system, while two oxygen functions, one carbon–carbon double bond, and two more double bonds or rings have to be within the last seven carbon atoms (C-23 through C-29).

Corroborating evidence was obtained from the ir spectrum of the tetrahydro derivative, which indicated that the carbonyl bands formerly at 1672 and 1742 cm^{-1} had shifted to 1709 and 1770 cm^{-1}. This is best accommodated by the presence of an α,β-unsaturated ketone (which would be in the steroid nucleus) and an α,β-unsaturated γ-lactone (located in the side chain). The high abundance as well as the hydrogen content of the $C_{22}H_{32}O_3$ ion (fragment a) requires that it is formed in a facile cleavage process involving the rearrangement of one hydrogen atom *away* from the C_{22} unit, the lactone carbonyl or the double bond functioning as the acceptor. The absence of this rearrangement after hydrogenation (the strong peak previously at *m/e* 344, $C_{22}H_{32}O_3$, appears at *m/e* 347, $C_{22}H_{35}O_3$) suggests a 1,3 relationship of C-22 hydroxyl and carbon–carbon double bond, as shown.



The positions of the hydroxyl and carbonyl groups in the tetracyclic system appeared to be at C-3 and C-7, respectively, on the basis of the mass spectrum of the ethylene ketal of tetrahydroantheridiol (1% toluenesulfonic acid in ethylene glycol, heated to 110° in toluene for 7 hr), which exhibited abundant ions of $C_5H_7O_2$ (*m/e* 99) and $C_7H_9O_3$ (*m/e* 141) in accordance with a 3-hydroxy-7-keto steroid.[4]

Dehydration of antheridiol (refluxed in methanol–hydrochloric acid) resulted in a product (M^+ at $C_{29}H_{40}O_4$) which contained only one oxygen in the tetracyclic system (abundant ionic species $C_{19}H_{25}O$ and $C_{22}H_{30}O_2$ but lack of $C_{19}H_{27}O_2$). This evidence combined with the change in the uv spectrum to λ_{max} 215 (ε 1.29×10^4) and 278 mμ (ε 2.37×10^4) suggested

(4) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Structure Elucidation of Natural Products by Mass Spectrometry," Vol. II, Holden-Day, Inc., San Francisco, Calif., 1964, p 25.