Most significant, however, is the difference in adrenal metabolism of androstenedione[3,6] and 9α-fluoroandrostenedione.

G. D. SEARLE AND COMPANY
P. O. BOX 5110
CHICAGO 80, ILLINOIS

CLARENCE G. BERGSTROM
R. M. DODSON

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SEPARATION OF STEROIDS BY GAS CHROMATOGRAPHY

Sir:

The microanalytical separation of steroids by gas chromatographic techniques has not been achieved in any practical fashion up to the present time. This is because relatively high temperatures or long retention times have been necessary to achieve even limited success.[1]

Conditions suitable for effective separations have been found through use of a silicone gum (methyl-substituted type, SE-30) coated on Chromosorb W. The table lists retention times relative to cholestane for a series of model compounds separated under two different circumstances. The separations at 260° were carried out with a column containing 7/100 SE-30 silicone on Chromosorb W, 80–100 mesh. The disadvantages of high temperature operations were evident when hydroxy compounds and acetyl esters were involved; broad peaks and multiple components suggestive of decomposition were obtained for substances of this kind. Hydrocarbons, ethers and ketones were not affected. When a column containing 2–3/100 SE-30 silicone on the same support was used, there was a major change in the observed effects. At 222° all of the compounds in the table were eluted as single components with no sign of decomposition; these included hydrocarbons, ketones, alcohols, ethers and acetyl esters. The short retention times observed at this relatively low temperature for comparatively high molecular

TABLE I

RELATIVE RETENTION TIMES[a]

Compound	Temperature 260°[b]	222°[c]
Androstane	0.17	0.11
Androstan-17-one	.30	.22
Androstan-3,17-dione	.56	.47
4-Androsten-3,17-dione	.68	.57
Pregnan-3,20-dione	.74	.67
Allopregnan-3,20-dione	.82	.74
Allopregnan-3β,20β-diol		.70
Allopregnan-3,11,20-trione	1.05	.99
Coprostane		.90
Cholestane	1.00[d]	1.00[e]
Cholestanyl methyl ether	1.58	1.78
Cholesteryl methyl ether	1.47	1.72
Cholestan-3-one	2.00	2.17
4-Cholesten-3-one	2.37	2.72
Cholestanol	1.70	1.99
Cholesterol	1.21 (broad)	1.98
Cholestanyl acetate	1.15 (v. broad)	2.84
Cholesteryl acetate	1.18 (broad)	2.81
β-Sitosterol	1.82 (v. broad)	3.26
β-Sitosteryl acetate		4.62
Stigmastane		1.65
Stigmasterol	1.62 2.29	2.84

[a] Argon ionization detector, 6 ft. × 4 mm. i.d. columns. [b] Pressure, 20 p.s.i.; 7/100 SE-30 on Chromosorb W, 80–100 mesh. [c] Pressure, 10 p.s.i.; 2–3/100 SE-30 on Chromosorb W, 80–100 mesh. [d] Time, 19.3 min. [e] Time, 17.6 min.

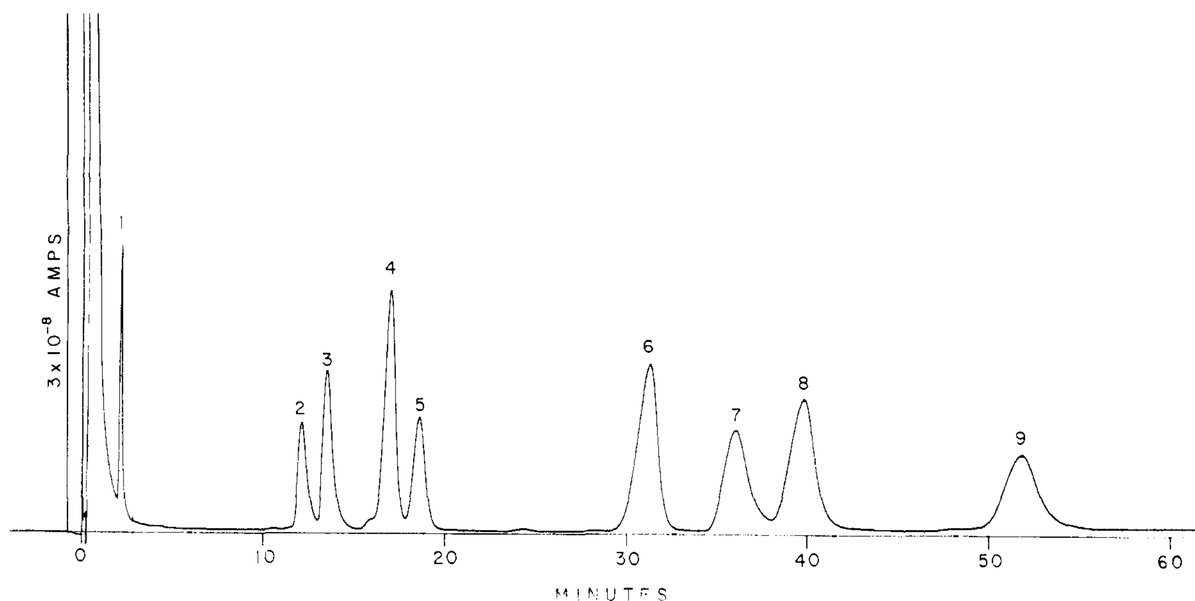

Fig. 1.—Separation of 1, androstane; 2, pregnane-3,20-dione; 3, allopregnane-3,20-dione; 4, coprostane; 5, cholestane; 6, stigmastane; 7, cholesterol; 8, cholestan-3-one; 9, stigmasterol. Conditions:—3% SE-30 silicone gum on Chromosorb W (80-100 mesh); 6 ft × 4 mm. column; 222°; argon inlet pressure, 10 psi.

(1) For example, G. Eglinton, R. J. Hamilton, R. Hodges and R. A. Raphael, *Chem. & Ind.*, 955 (1959), reported a retention time of about 4 hours for cholestanone at 220° with an Apiezon L phase (46 in. col.). An ethylene glycol-isophthalate polyester has been used at 270° for steroid separations (C. C. Sweeley and E. C. Horning, *Nature*, in press).

weight compounds may be related to the effects observed when very thin liquid films are employed[2]; for example, cholesterol required only about 35 min. for elution at 0.68 atm. inlet pressure (30 ml./min. flow rate) with a 183 cm. column at 222°.

Stereochemical change in the A/B ring junction led to different retention times for otherwise similar compounds. Hydroxy compounds were eluted before the corresponding ketones, without trailing; ketones showed some trailing. A considerable difference in retention time was observed with increasing carbon content. Both columns had about 2300 theoretical plates (cholestane).

The usefulness of this phase for other kinds of high molecular weight compounds has not been investigated. Silicone grease phases are known to have high thermal stability,[3,4] and while the present phase may be used to 250–300° its greatest value may lie in its unusual ability to resolve steroids rapidly at relatively low temperatures.[5]

(2) C. Hishta, J. P. Messerly and R. F. Reschke, Abstracts 137th A.C.S. Meeting, April, 1960, p. 29-B.

(3) J. Cason and W. T. Miller, *J. Org. Chem.*, **24**, 1814 (1959).

(4) C. Asselineau, J. Asselineau, R. Ryhage, S. Stallberg-Stenhagen and E. Stenhagen, *Acta Chem. Scand.*, **13**, 822 (1959).

(5) Sterol analysis on a silicone column, operated at 287°, has been described recently (R. K. Beerthuis and J. H. Recourt, *Nature*, **186**, 372 (1960)).

LABORATORY OF CHEMISTRY OF NATURAL PRODUCTS
NATIONAL HEART INSTITUTE
BETHESDA 14, MARYLAND
W. J. A. VANDENHEUVEL
C. C. SWEELEY
E. C. HORNING

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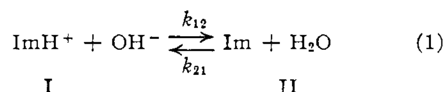
FAST REACTIONS OF IMIDAZOLE STUDIED WITH RELAXATION SPECTROMETRY

Sir:

The understanding of enzymatic reactions often is hindered because of the difficulty of isolating and studying individual steps in the mechanisms. In many enzymatic systems, it appears that imidazole is involved intimately in the catalytic process. For these reasons, studies of the kinetics of the fast reactions of imidazole with hydrogen ion, hydroxyl ion and a proton donor–acceptor system in the region of neutrality (chlor phenol red) were undertaken with relaxation techniques.

Two experimental approaches were employed: the dissociation field[1] and the temperature jump methods.[2] Both utilize the same principle: perturbation of a system at chemical equilibrium and determination of the relaxation time (or times) necessary for the reestablishment of equilibrium. The measured spectrum of relaxation times can be related to the rate constants of the chemical reactions in the system.[3] Detailed descriptions of the experimental apparatus and procedures can be found elsewhere.[1,2,4,5]

The dissociation field method was used to study the kinetics of the reaction



(1) M. Eigen and J. Schoen, *Z. Elektrochem.*, **59**, 483 (1955).

(2) G. Czerlinski and M. Eigen, *ibid.*, **63**, 652 (1959).

(3) M. Eigen, *ibid.*, **64**, 115 (1960).

(4) L. De Maeyer, *ibid.*, **64**, 65 (1960).

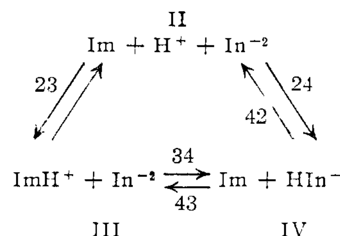
(5) H. Diebler, Ph.D. Thesis, in preparation.

in the neighborhood of pH 10. Here ImH^+ represents the imidazolium ion and Im the neutral imidazole molecule. The relaxation time, τ, in this case is

$$\tau = [k_{21} + k_{12}(C_{OH^-} + C_{ImH^+})]^{-1} \qquad (2)$$

Since the equilibrium constant is known,[6] both rate constants could be determined (at essentially zero ionic strength and 25°).

The system studied with the temperature jump consisted of imidazole, chlor phenol red (a pH indicator with pK 6.08, through which pH changes could be followed spectrophotometrically), and 0.1 M KNO_3. The measurements were carried out in the pH range of 5.5 to 6.5 at 13°. The reaction mechanism can be written as



The state II in this system differs from that in equation 1 by the presence of dissociated indicator. Furthermore, In^{-2} and HIn^- represent the forms of the indicator important in the pH range under consideration. This mechanism is characterized by two relaxation times which are complicated (but known) functions of the six rate constants and the equilibrium concentrations. Only the longer relaxation time (>10 μsec.) could be measured, although a shorter relaxation time (<5 μsec.) could be detected. By varying concentrations and using a trial and error procedure to fit the data, all six rate constants were determined. The rate constants obtained are:

M^{-1} sec.$^{-1}$	sec.$^{-1}$	%
$k_{12} = 2.3 \times 10^{10}$	$k_{21} = 2.3 \times 10^{3}$	(±15)
$k_{23} = 1.5 \times 10^{10}$	$k_{32} = 1.5 \times 10^{3}$	(±35)
$k_{24} = 3 \times 10^{10}$	$k_{42} = 2.4 \times 10^{4}$	(±50)
$k_{43} = 9 \times 10^{8}$	$k_{34} = 1.2 \times 10^{8}$ M^{-1}	(±35)

The relatively large experimental error in the rate constants determined by the temperature jump method is due to the evaluation from the complicated form of the relaxation time since individual relaxation times can be found to ±10%.

The rates of reaction of imidazolium ion and imidazole with hydroxyl and hydrogen ions, respectively (k_{12}, k_{23}), are diffusion controlled. It is interesting to note that at pH 7 and at salt concentrations usually present in physiological systems, the two rates would be about equal. The reported value of k_{12} is valid for zero ionic strength, whereas k_{23} is determined at an ionic strength of 0.1. The latter value, however, should be fairly independent of ionic strength since no net change in charge is involved. The rate of combination of chlor phenol red and a proton (k_{24}) is also diffusion controlled and is identical with the value previously found for a similar reaction of phenol red.[7] On the other hand, the rate of pro-

(6) A. H. M. Kirby and A. Neuberger, *Biochem. J.*, **32**, 1146 (1938);

(7) H. Diebler, M. Eigen and G. G. Hammes, *Z. Naturforsch.*, (b) in press (1960).