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IMPROVED SEPARATION OF STEROID GLYCOLS EPIMERIC AT C-20 ON PAPER PRETREATED WITH BORIC ACID OR BORATE BUFFERS*

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Abstract—Thirteen epimeric pairs of steroid 20,21-glycols, 17α,20-glycols and 17α,20,21-glycerols were chromatographed on paper pretreated with boric acid or borate buffers in the pH range 7·6 to 10. Under these conditions R_f values for individual steroids are determined chiefly by the pH employed, the configuration of the hydroxyl group at C-20 and, in diols, by the site of the hydroxyl group adjacent to C-20. At the pH which affords maximal separation, R_f differences between members of epimeric pairs are notably greater than those obtained on paper treated with water only. The characteristics of a number of nuclear *vic*-glycols so chromatographed also were determined. The results have been examined in terms of conformational analysis.

INTRODUCTION

ENZYMATIC[1] or chemical[2] reduction of the C-20 carbonyl group of steroids bearing hydroxyl groups at the 17α and/or the C-21 positions usually gives both glycols epimeric at C-20, and the resolution of such mixtures by paper chromatography or other fractionating procedures is therefore of practical importance. It is evident from an examination of the literature on paper chromatography (see refs 3,4 for reviews) that, while the separation of many such pairs of epimers readily is effected, some pairs separate adequately† only after extensive over-running and a few, notably the cortols and cortolones,[5] have not been separated by this means.

Following a suggestion by Dr. Ian Bush that such epimeric pairs might best be separated as their boric acid complexes, we investigated the paper chromatographic separation of a number of 5β-pregnane or pregnene-20α,21-glycols, 17α,20α-glycols and 17α,20α,21-glycerols from the corresponding C-20 epimers using paper pretreated with boric acid or borate buffers in the pH range 7·6 to 10. The purpose of this paper is to detail the method used and to illustrate the separations obtained with 13 such pairs of epimers. As an extension of this work, we studied also the characteristics of several pairs or groups of epimeric nuclear *vic*-glycols when chromatographed in

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† We maintain that substances are separated only if the R_f difference is 0·1 or greater.

[1] L. L. Engel and L. J. Langer, *Ann. Rev. Biochem.* **30**, 499 (1961).
[2] L. F. Fieser and M. Fieser, *Steroids* Reinhold (1959).
[3] I. E. Bush, *The Chromatography of Steroids* Pergamon (1961).
[4] R. Neher, *Chrom. Rev.* **1**, 99 (1959).
[5] D. K. Fukushima, H. L. Bradlow, L. Hellman, B. Zumoff and T. F. Gallagher, *J. Biol. Chem.* **235**, 2246 (1960).

this fashion. Here the aim was less one of improving separations and more one of determining, within the limits of sample availability, the scope of cycloborate formation.

MATERIALS AND METHODS

Steroids. The steroids used in this study either were prepared in this laboratory* or supplied by other investigators as indicated. We are most grateful to the many people who assisted us in this way.

Paper chromatography. A descending technique was used, employing paper-lined glass cylindrical jars measuring 12 in. in diameter and 24 in. in length. At the initiation of equilibration the lining paper was wet with both phases of the system, a center strip was wet with the stationary phase only and an extra trough, containing mobile phase only, was placed in the upper part of the jar. The equilibration period varied from 8 to 12 hr and it was usually possible to maintain the temperature during each run at 25 ± 1°.

Buffers used were a boric acid: sodium borate buffer, 0·02 M, pH 7·6,[6] boric acid:sodium hydroxide buffers, 0·02 M, pH 8·0, 8·4, 8·8, 9·2, 9·6, and 10·0,[7] Tris:hydrochloric acid buffers, pH 7·6 and 8·4 and glycine:sodium hydroxide buffers, pH 9·2 and 10·0.[8] All were checked using a Beckman model H-2 meter equipped with fiber junction calomel and glass electrodes and adjusted as necessary.

Prior to spotting, 19 × 60 cm sheets of Whatman No. 1 paper were dipped in distilled water, 5% aqueous boric acid,[9] or the appropriate borate or non-borate buffer and allowed to air-dry in a hanging position. The steroids were then applied to the starting line in amounts just sufficient for subsequent detection and chromatography was carried out as indicated. In those cases where a Zaffaroni-type[10] system was used, the dried, buffer-impregnated paper was dipped in 30% formamide in acetone just prior to spotting.

The systems were of the 3 to 4-component, 2-phase type. Their composition was determined less by a need for superior resolving power than by our aim of obtaining round, discrete spots (as evidence of adequate capacity) together with R_f values in the range 0·2 to 0·6. It was found unnecessary to incorporate buffers in the systems.

Techniques used to detect the steroids on the developed chromatograms included scanning under a UV lamp emitting maximally at about 250 mμ where applicable, *in-situ* periodate oxidation followed by treatment with the Zimmermann reagent in the case of the 17α,20,21-glycerols,[11] and dipping in 10% alcoholic phosphomolybdic acid followed by heating at 60 to 70° for the remaining steroids. No difficulty in detecting the steroids was encountered.

In the course of these experiments we noted a property of boric acid which has an important practical bearing on its use in paper chromatography. We observed, initially in the case of the epimeric 17α,20-glycols VI-α and VI-β† that, if a control sheet (water-dipped, air-dried and spotted with VI-α and VI-β) is placed in a separate trough but within the same jar as a sheet pretreated with 5% aqueous boric acid and both chromatographed with system 8, the specific borate-induced acceleration of the 20-β epimer occurred, and to the same extent, on both sheets. The same effect was observed, but to a lesser extent, if a borate buffer pH 7·6 was substituted for boric acid. If borate buffers above pH 8·4 were used, or if boric acid was employed together with a methanol-lacking system such as isopropyl ether, 60; n-heptane, 140; t-butanol, 10; water, 190 ml (system 9), the effect was not noted. It has been our practice, therefore, to place the control sheet and those adjusted to pH 8·4 or lower in separate tanks. This phenomenon, for which the term "transbortation"

* Details of the preparation and characterization, including assignments of configuration, of the 20,21-glycols and certain of the 17α,20-glycols will be presented in another paper.

† Steroids and systems concerned in this point are given in the experimental section under 17α,20-glycols.

[6] W. Holmes, *Anat. Record* **86,** 163 (1943).
[7] W. M. Clark and H. A. Lubs, *J. Bact.* **2,** 1 (1917).
[8] G. Gomeri, *Methods in Enzymology* **1,** 138 Academic Press (1955).
[9] S. G. Brooks, J. S. Hunt, A. G. Long and B. Mooney, *J. Chem. Soc.* 1175 (1957).
[10] A. Zaffaroni, *Recent Prog. in Hormone Res.* **8,** 51 (1953).
[11] C. de Courcy and J. J. Schneider, *J. Biol. Chem.* **223,** 865 (1956).

seems suitable, is understandable in terms of a mechanism suggested by Dr. G. W. Willcockson. During the equilibrium period trimethyl borate is readily formed on the boric acid-impregnated sheet and is distributed to the remaining sheets as the volatile methyl alcohol-trimethyl borate azeotrope, $(CH_3O)_3B{\cdot}CH_3OH$, b.p. 54·6°. There it undergoes hydrolysis to yield boric acid. We have not tested this hypothesis experimentally.

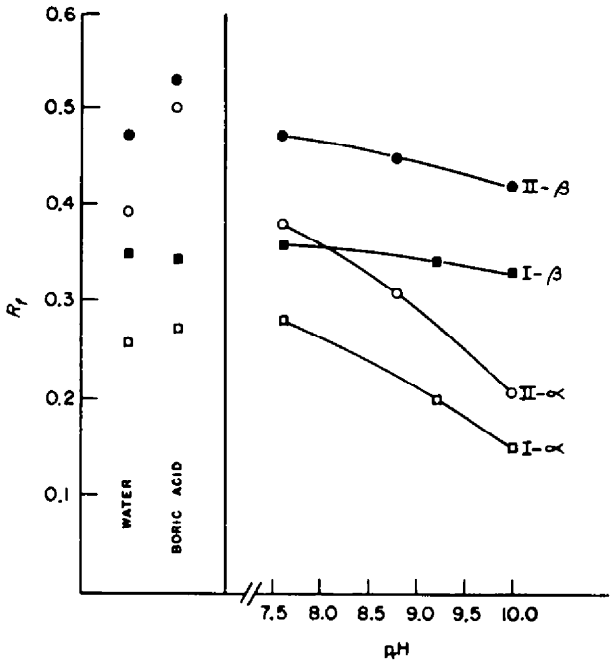

FIG. 1. R_f:pH curves for the pair I-α, I-β (system 1) and the pair II-α, II-β (system 3).

RESULTS

Since it was soon apparent that the single most important controllable factor governing the R_f values of steroid glycols chromatographed on borate-impregnated paper is the pH imposed, each pair of epimers was chromatographed with one or more suitable systems over the pH range 7·6 to 10. The shapes of the R_f:pH curves thus obtained were found, with but one exception, to be characteristically dependent upon whether the steroid employed was a 17α,20-glycol, a 20,21-glycol or a 17α,20,21-glycerol. Curves from representatives of these three classes will serve to illustrate this relationship.

20,21-*Glycols*

Five pairs of epimers were examined. All gave similar R_f:pH curves.

5β-Pregnane-3α,20α,21-triol (1-α) *and its C*-20 *epimer* (1-β). Chromatography of the pure epimers using the system isopropyl ether, 120; n-heptane, 80; methanol, 150; water, 50 ml (system 1) and paper impregnated with borate buffers in the pH range 7·6 to 10 gave the R_f:pH curves shown in Fig. 1. The R_f values obtained using paper dipped in distilled water are included as points of reference. The extent of what we judge to be complexing with boric acid increases with the increase in pH and, since its effect is to reduce the mobility of the 20-α epimer (1-α) chiefly, it serves considerably to improve the separation of 1-α from 1-β.* This result was not obtained when non-borate buffers

* The improvement in separating individual pairs of epimers is given in each case as a value, B, defined as the ratio of the R_f difference at optimum separation to that obtained using paper pretreated with water alone. The condition under which maximal separation is obtained appears in parentheses following the value. In the case of this pair of epimers, B = 1·7 (pH 10).

were employed. Repetition of this experiment using the system toluene, 200; methanol, 150; water, 50 ml (system 2) gave curves very similar to those illustrated in Fig. 1 thus showing that the observed effects are not due to any particular properties of system 1.

The use of this technique in column partition chromatography is illustrated by the separation of I-α from I-β on a preparative scale. System 1 was modified by replacing the water with 0·1 M sodium borate:sodium hydroxide buffer, pH 9·4. Two hundred grams of grade 545 Celite, stirred in the mobile phase, were treated with 100 ml of the stationary phase and packed with proper care[12] into

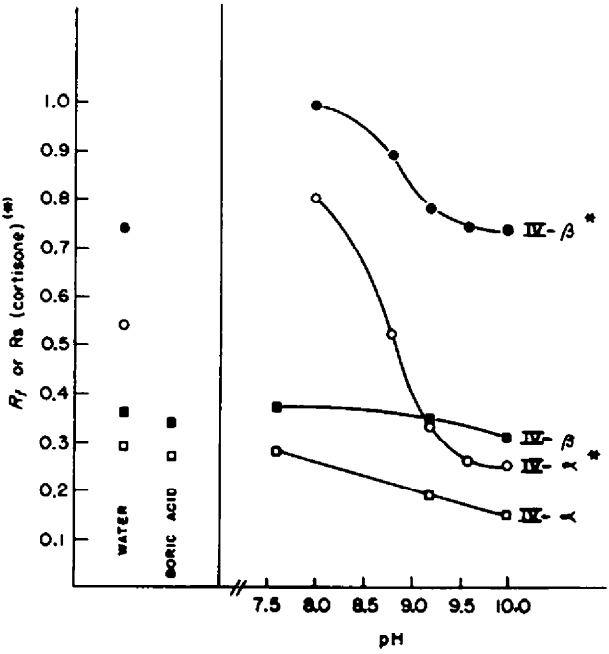

FIG. 2. R_f:pH curves for the pair IV-α, IV-β using systems 5 and 6. Curves obtained using the latter system are indicated by stars.

a 32 × 720 mm column. After applying the crude product obtained by reducing 170 mg of 5β-pregnane-3α,21-diol-20-one in dimethylformamide with sodium borohydride, the column was developed with the mobile phase. The individual epimers emerged in the expected order, were well separated and were readily recovered in pure form.*

4-Pregnene-20α,21-diol-3-one (II-α) *and its* C-20 *epimer* (II-β). This pair was chromatographed using the system toluene, 150; isooctane, 50; methanol, 150; water, 50 ml. (system 3) and gave R_f:pH curves (Fig. 1) very similar to those illustrated for the pair I-α, I-β, B = 2·4 (pH 10).

5β-Pregnane-3α,11β,20α,21-tetrol (III-α) *and its* C-20 *epimer* (III-β). Chromatography of this pair of epimers under the conditions indicated above and employing the system toluene, 150; ethyl acetate, 50; methanol, 120; water, 80 ml. (system 4) furnished R_f:pH curves (not illustrated) similar in all respects to those shown in Fig. 1. B = 2·5 (pH 10).

4-Pregnene-11β,20α,21-triol-3-one (IV-α) *and its* C-20 *epimer* (IV-β). This pair of epimers was chromatographed both on borate-buffered paper over the pH range 7·6 to 10 using a conventional system [toluene, 175; ethyl acetate, 25; methanol, 120; water, 80 ml. (system 5)] and on similarly

* Column partition chromatography, with Celite as the support, has since been used to separate the pairs III-α and III-β, V-α and V-β, IX-α and IX-β and XIII-α and XIII-β (*vide infra*). Our present procedure is to prepare the column using a suitable paper chromatography system in which the water is replaced with the appropriate 0·02 M borate buffer, and to develop it with the unmodified system.

[12] M. L. Lewbart and V. R. Mattox, *J. Org. Chem.* **28**, 1779 (1963).

buffered paper impregnated also with formamide, using chloroform saturated with formamide as the mobile phase (system 6). The results (Fig. 2) are given in terms of R_f:pH curves where system 5 was used and in the form of $R_{s(cortisone)}$:pH curves where system 6 was employed. The latter expedient was used in order to overcome the difficulty in visualizing the solvent front, and hence accurately in determining R_f values, utilizing system 6. The general characteristics of the R_f:pH curves for the epimeric pair IV-α, IV-β (Fig. 2) closely resemble those obtained for the pairs I-α, I-β and II-α, II-β (Fig. 1) when compared in terms of conventional systems. It is evident from Fig. 2 that the specific effects of the borate buffers are manifest on paper treated also with formamide. For the pair IV-α, IV-β B = 2·1 (system 5, pH 10) and 2·4 [system 6, pH 10, using $R_{s(cortisone)}$ differences].

The use of this technique in preparative paper chromatography is illustrated by the recovery, in pure form, of milligram amounts of IV-α and IV-β from the crude product obtained by reducing 100 mg of corticosterone in dimethylformamide with sodium borohydride. Chromatography of an aliquot of the neutral fraction with system 6 on paper pretreated with borate buffer pH 9·0 and formamide showed that, while IV-α was well separated from IV-β, the former had a mobility close to that of various UV-negative, phosphomolybdic acid-positive products. Since these substances were well separated from IV-α on paper pretreated with formamide only, the separation of IV-α from IV-β and the former from the noted contaminants was effected on a preparative scale by dipping the lower two-thirds only of sixteen 19 × 60 cm sheets with the borate buffer, air-drying, impregnating each sheet over its entire length with 30% formamide in acetone, applying the reaction product and chromatographing for 6 hr using system 6. Detection by UV light scanning and recovery by a previously described method[13] gave IV-α and IV-β in pure form.

5β-Pregnane-3α,6α,20α,21-tetrol (V-α) *and its* C-20 *epimer* (V-β). This pair of epimers, prepared from 5β-pregnane-3α,6α-diol-20-one (Canada Packers Limited, Toronto) by lead tetraacetate acetoxylation[14] followed by lithium aluminum hydride reduction, was chromatographed over the pH range 7·6 to 10 using the system toluene, 110; ethyl acetate, 90; methanol, 130; water, 70 ml (system 7). The R_f:pH curves (not illustrated) were very similar to those shown in Figs. 1 and 2. This epimeric pair well serves to illustrate the utility of the method since they remained unseparated on the control (water-impregnated) sheet but had an R_f difference of 0·15 at pH 10. Repetition of this experiment, using two wholly different systems, confirmed this result.

The effect of impregnating paper with boric acid alone on the mobilities of the 5 epimeric pairs of glycols in this section also was examined. Alterations in R_f values, consisting of a significant increase in mobility (as determined by comparison with water-control and pH 7·6 values) of the 20-α epimer and a slight increase in mobility of the 20-β epimer, were noted only in the case of the epimeric pair II-α, II-β (Fig. 1).

17α,20-*Glycols*

Five pairs of epimers were examined. Four gave similar results while one pair furnished wholly different R_f:pH curves.

5β-Pregnane-3α,17α,20α-triol (VI-α) *and its* C-20 *epimer* (VI-β). These epimers, which were prepared from 5β-pregnane-3α-ol-20-one (Canada Packers Limited, Toronto) by 17-hydroxylation[15] followed by lithium aluminum hydride reduction of the diacetate,[16] were chromatographed on paper dipped in 5% aqueous boric acid and on paper pretreated with borate buffers using the system isopropyl ether, 90; n-heptane, 110; methanol, 160; water, 40 ml. (system 8). Inspection of the R_f:pH curves (Fig. 3) shows that the effect is limited to the 20-β epimer and consists of a marked increase in its mobility at low pH values and particularly in the presence of boric acid itself (pH approximately 5·1), B = 4·0 (boric acid).

5β-Pregnane-3α,17α,20α-triol-11-one (VII-α) *and its* C-20 *epimer* (VII-β); *5β-Pregnane-3α,11β,17α,20α-tetrol* (VIII-α) *and its* C-20 *epimer* (VIII-β). These two pairs of epimers were prepared by the procedure of Fukushima and Meyer.[17] Chromatography of both pairs in system 2, using the

[13] J. J. Schneider, *Arch. Biochem. and Biophys.* **98**, 249 (1960).
[14] F. Sondheimer, G. Rosenkranz, O. Mancera and C. Djerassi, *J. Amer. Chem. Soc.* **75**, 2601 (1953).
[15] T. H. Kritchevsky and T. F. Gallagher, *J. Amer. Chem. Soc.* **73**, 184 (1951).
[16] R. B. Turner, *J. Amer. Chem. Soc.* **75**, 3489 (1953).
[17] D. K. Fukushima and E. D. Meyer, *J. Org. Chem.* **23**, 174 (1958).

conditions indicated above, gave R_f:pH curves closely resembling those obtained with the epimeric triols VI-α and VI-β. The curves for the pair VII-α, VII-β are indicated in Fig. 3. For the pair VII-α, VII-β B = 1·9 (boric acid) and for the pair VIII-α, VIII-β B = 2·6 (boric acid).

5β-Pregnane-3α,6α,17α,20α-tetrol (IX-α) *and its* C-20 *epimer* (IX-β). These epimers, prepared from 5β-pregnane-3α,6α-diol-20-one by the method indicated for the pair VI-α and VI-β, were chromatographed on paper pretreated with boric acid and the usual borate buffers using the system toluene, 170; t-butanol, 30; methanol, 50; water, 100 ml (system 10). The R_f:pH curves (not illustrated) again showed a specific increase in the mobility of the 20-β epimer at low pH values, particularly in the presence of boric acid itself, B = 3·7 (boric acid).

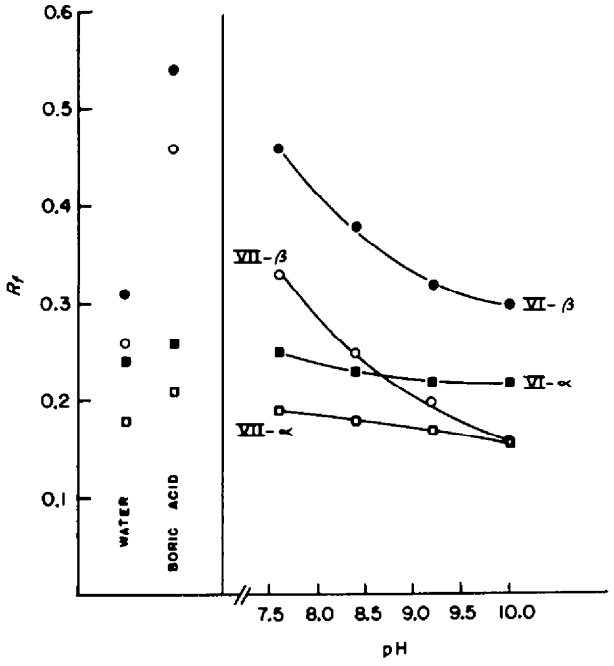

FIG. 3. R_f:pH curves for the pair VI-α, VI-β (system 8) and the pair VII-α, VII-β (system 2).

4-Pregnene-17α,20α-diol-3,11-dione-21-oic acid (X-α) *and its* C-20 *epimer* (X-β). This previously prepared[18] pair of epimeric 17α,20-glycols was chromatographed as the methyl and ethyl esters on borate-buffered paper using the system isopropyl ether, 200; methanol, 130; water, 70 ml (system 11). The R_f:pH curves (Fig. 4) differ from those obtained with the other 17α,20-glycols (Fig. 3) in that, as in the case of the 20,21-glycols (Figs. 1 and 2), separations are best effected at a high rather than at a low pH. While this pair of epimers remains unseparated when chromatographed in the usual fashion using untreated paper together with system 11, it was established by means of overrunning that the usual order of mobility, namely 20-β more mobile than 20-α, was observed. It is to be noted (Fig. 4) that this order of movement is reversed in the presence of borate buffers. Inspection of Figs. 1, 2 and 3 and those in subsequent sections shows that the pair X-α, X-β constitute the sole exception to this general order-of-mobility rule. As an approximation for this pair, B = 12 [as the methyl esters (pH 9·2)] and 13 [as the ethyl esters (pH 9·2)].

17α,20,21-Glycerols

Three pairs of epimers were examined. All gave similar R_f:pH curves.

4-Pregnene-11β,17α,20α,21-tetrol-3-one (XI-α) *and its* C-20 *epimer* (XI-β). This pair, previously prepared,[18] was chromatographed on paper pretreated with 5% boric acid and with borate buffers

[18] M. L. Lewbart and V. R. Mattox, *J. Org. Chem.* **28**, 1773 (1963).

using the system toluene, 175; t-butanol, 35; methanol, 30; water, 100 ml (system 12). The reversed sigmoid curves thus obtained (Fig. 5) are characteristic for glycerols of this class and show that, in the case of this particular pair, the best separations are obtained at pH 7·6 (B = 2·3) or on paper pretreated with boric acid (B = 2·5).

5β-*Pregnane*-3α,17α,20α,21-*tetrol*-11-*one* (XII-α) and its C-20 *epimer* (XII-β); 5β-*Pregnane*-3α,11β,17α,20α,21-*pentol* (XIII-α) *and its* C-20 *epimer* (XIII-β). These two pairs of epimers, supplied by Dr. D. K. Fukushima and Dr. T. F. Gallagher, were chromatographed on borate-buffered paper

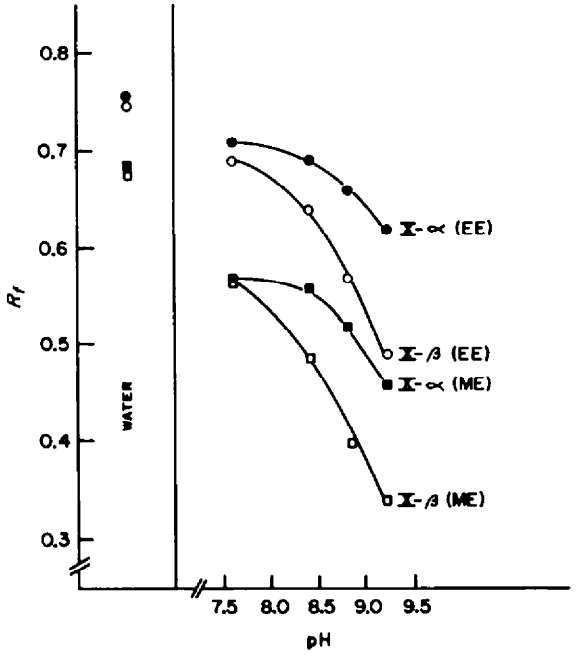

FIG. 4. R_f:pH curves for the pair X-α, X-β using system 11. ME = methyl ester, EE = ethyl ester. Separations suggested on water-control sheet actually were not obtained.

over the pH range 7·6 to 10 using a wide variety of systems. Of these, the most satisfactory was the system toluene, 170; t-butanol, 40; methanol, 30; water, 100 ml (system 13). The R_f:pH curves differed from those illustrated in Fig. 5 only to the extent of showing that separations are best effected in the pH range 8·4 to 8·8 for the pair XII-α, XII-β [B = 4·3 (pH 8·8)] and in the pH range 7·6 to 8·4 for the pair XIII-α, XIII-β [B = 2·0 (pH 7·6)].

In order better to show the characteristics of these important pairs of epimers when so chromatographed, results obtained using system 13 are shown (Fig. 6) as representations of 6 chromatograms simultaneously developed at pH 8·8. It is clear that the separation of XII-α from XII-β and of XIII-α from XIII-β is improved in the presence of borate buffers (sheet 3 compared with sheets 1 and 2) and that added improvement results on over-running (sheet 6 compared with sheets 3, 4 and 5). But it is also to be noted that the gain in separating the individual pairs of epimers (such as XII-α from XII-β, the more difficult separation) is, under circumstances where both pairs of epimers are present, largely negated by a failure to obtain the usual group separations based on 11-ketone: 11β-ol mobility differences. Fig. 6 illustrates: the pair XII-α, XII-β are well separated from the pair XIII-α, XIII-β in the absence of borate buffers (sheets 1, 2, 4 and 5) but not in their presence (sheets 3 and 6). This difficulty partially can be overcome by employing system 12 and a running time of from 12 to 15 hr under which conditions XII-α moves between XIII-α and XIII-β and XIII-β between XII-α and XII-β as suggested on sheet 6. But it is perhaps better, as Dr. Fukushima has suggested, first to effect the group separation using untreated paper and then to separate the epimers as above.

Nuclear vic-glycols

The first group in this division consists of various 16,17-glycols wherein true *cis*:*trans* relationships are apparent and which complexed or failed to complex in accord with their established configurations. A second group is made up of various saturated ring A, B or C *vic*-glycols in which true *cis*:*trans* assignments generally cannot be made and which complexed only in certain cases.

1,3,5-*Estratriene*-3,16α,17β-*triol* (XIV), 1,3,5-*Estratriene*-3,16β,17β-*triol* (XIV-a) and 1,3,5-*Estratriene*-3,16α,17α-*triol* (XIV-b). These 3 estrogens, supplied by Dr. Jack Fishman, were converted to the 3-methyl ethers and chromatographed on borate-buffered paper over the pH range

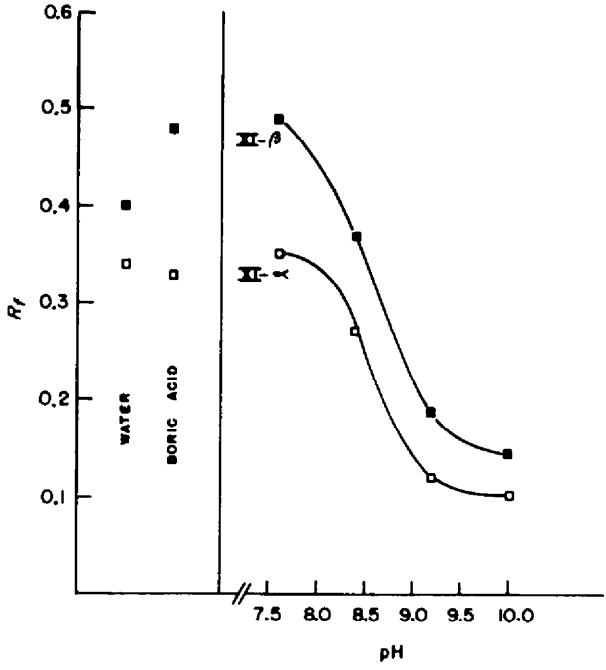

FIG. 5. R_f:pH curves for the pair XI-α, XI-β using system 12.

7·6 to 10 using the system toluene, 70; isooctane, 130; methanol, 150; water, 50 ml (system 14). As the curves in Fig. 7 indicate, there is a progressive reduction in the mobilities of the *cis*-glycols XIV-a and XIV-b as the imposed pH is increased and a lack of effect on the mobility of the *trans*-glycol XIV. It is of interest that conversion to the 3-methyl ether did not wholly eliminate the influence of pH on mobility in the case of the *cis*-glycol XIV-b when the point was checked using non-borate buffers. It seems likely, as Dr. Fishman has suggested, that this can be ascribed to the greater hydrogen bonding of XIV-b as compared with XIV-a.

Several attempts were made to develop systems suitable for the chromatography of the three A-ring *vic*-glycols 2-hydroxyestrone, 2-hydroxyestradiol and 2-hydroxyestriol, also supplied by Dr. Fishman, on borate-buffered paper. Since the fractionation of these estrogens results in unaccountable losses,[19] the development of such systems seemed desirable on the assumption that these substances may be more stable in their complexed forms. Two difficulties have to date frustrated these attempts. All three compounds are complexed as the imposed pH is increased. In the pH range 9·2 to 10, 2-hydroxyestriol remained nearly immobile even when polar systems were employed. At pH values under 8·8, in which range adequate mobilities were assured, excessive streaking of all three substances was noted.

5β-*Androstane*-3α,16α,17β-*triol* (XV) *and its* C-16 *epimer* (XV-a). This pair of epimers was

[19] J. Fishman, *J. Clin. End. and Met.* **23**, 207 (1963).

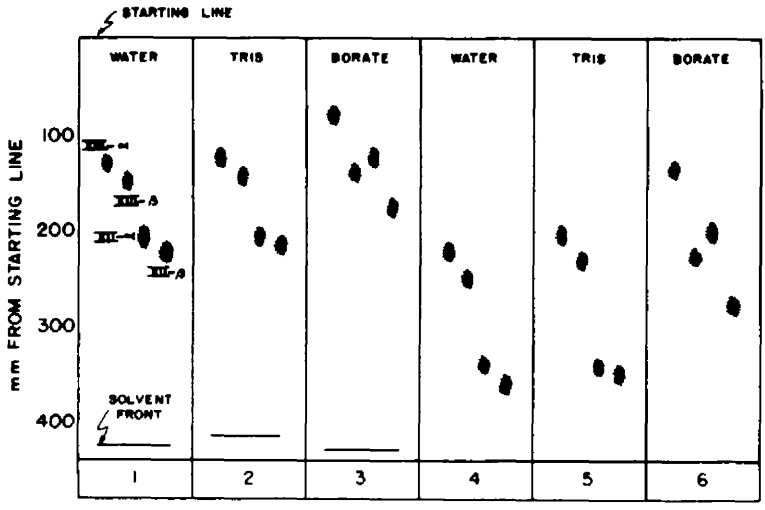

FIG. 6. Paper chromatogram representations for the epimeric pairs XII-α, XII-β and XIII-α, XIII-β using system 13.

prepared from 5β-androstane-3α-ol-17-one by the method of Lieberman *et al.*[10] modified to the extent that the separation of the triols from each other and from extraneous material was effected by partition column chromatography using the system toluene, 190; ethyl acetate, 10; methanol, 140; water, 60 ml. (system 15) with grade 545 Celite as the support. Chromatography of the pure triols on borate-impregnated paper using the system toluene, 180; ethyl acetate, 20; methanol, 150; water, 50 ml. (system 16) gave R_f:pH curves (Fig. 7) in accord with the established *trans* and *cis*-glycol configurations of XV and XV-a respectively.

4-*Pregnene*-11β,16α,17α,21-*tetrol*-9α-*fluoro*-3,20-*dione* (XVI) *and its* 16,17-*cycloborate* (XVI-a),

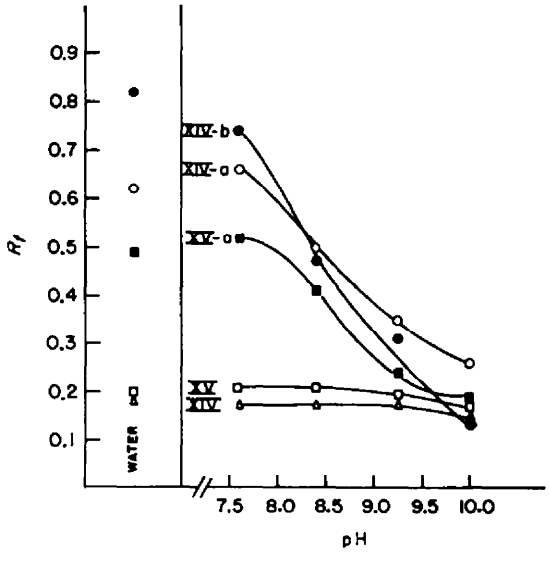

FIG. 7. R_f:pH curves for epimers XIV, XIV-a and XIV-b (system 14) and the pair XV, XV-a (system 16).

[10] S. Lieberman, B. Praetz, P. Humphries and K. Dobriner, *J. Biol. Chem.* **204**, 491 (1953).

1,4-*Pregnadiene*-11β,16α,17α,21-*tetrol*-9α-*fluoro*-3,20-*dione* (XVI-b). Glycols XVI and XVI-a, supplied by Dr. Josef Fried, were chromatographed over the pH range 7·6 to 10 using the system toluene; 100; t-butanol, 100; water, 150 ml (system 17). The R_f:pH curve for XVI was identical with that given by XVI-a and closely resembled the curves illustrated in Fig. 7.

The related glycol XVI-b, furnished by Dr. Seymour Bernstein, was chromatographed in system 17 and gave a R_f:pH curve similar to that obtained from XVI. Unfortunately, the C-16 epimers of neither XVI nor XVI-b were available for comparison.

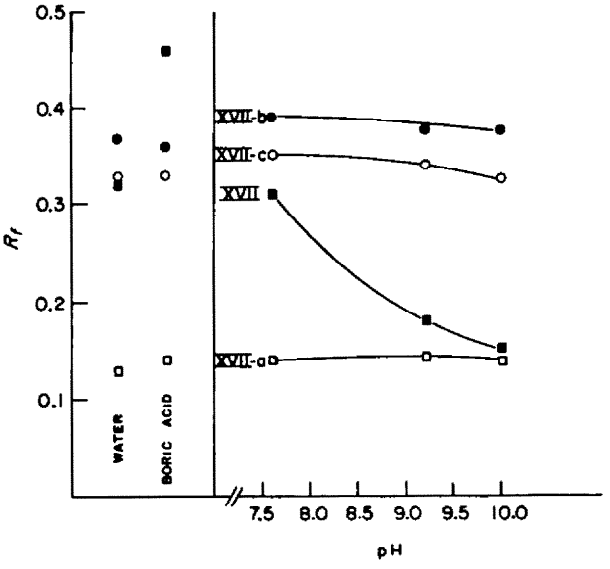

FIG. 8. R_f:pH curves for the four diastereoisomers XVII, XVII-a, XVII-b and XVII-c using system 18.

3α,6β,7β-*Trihydroxycholanic acid* (XVII), 3α,6β,7α-*Trihydroxycholanic acid* (XVII-a), 3α,6α,7α-*Trihydroxycholanic acid* (XVII-b) *and* 3α,6α,7β-*Trihydroxycholanic acid* (XVII-c). These 4 isomeric acids, furnished by Dr. S. L. Hsia, were chromatographed as the methyl esters on paper impregnated with boric acid and the usual borate buffers using the system toluene, 120; isooctane, 80; methanol, 160; water, 40 ml (system 18). The R_f:pH curves (Fig. 8) show that the mobilities of the esters derived from acids XVII-a, XVII-b and XVII-c were unaffected. The mobility of the ester obtained from acid XVII, using the R_f value obtained from the water-treated sheet as point of reference, was increased greatly in the presence of boric acid but decreased, significantly below the control value, as the imposed pH was increased.

11α,12α-*Dihydroxytigogenin* (XVIII), 11β,12α-*Dihydroxytigogenin* (XVIII-a), 11β,12β-*Dihydroxytigogenin* (XVIII-b) *and* 11α,12β-*Dihydroxytigogenin* (XVIII-c). These ring C *vic*-glycols, supplied by Dr. A. G. Long, were chromatographed as above using system 18. The R_f:pH curves (not illustrated) showed that the mobilities of the 11β,12α (XVIII-a) and the 11α,12β (XVIII-c) glycols were unaffected. The mobility of the 11β,12β (XVIII-b) glycol was increased slightly, but consistently, in the presence of boric acid itself. Of chief interest were the mobility changes noted in the case of the 11α,12α glycol XVIII. Its mobility, as compared to the R_f value obtained from the water-treated sheet, was increased greatly in the presence of boric acid (and to some extent at pH 7·6) but decreased significantly as the imposed pH was increased, a result closely paralleling that given by the bile acid XVII. These results confirm and slightly extend those of Brooks *et al.*[9] who studied the mobility changes induced when the 4 diastereoisomers were chromatographed on paper pretreated with 5% boric acid, using a system similar to system 16 to which 8·5% of boric acid had been added. They noted the mobility increases described above.

Androstane-2α,3α,17β-*triol* (XIX), *Androstane*-2β,3α,17β-*triol* (XIX-a), *Androstane*-2β,3β,17β *triol* (XIX-b) *and Androstane*-2α,3β,17β-*triol* (XIX-c). These triols, supplied by Dr. Leon Bradlow, were chromatographed on paper pretreated with boric acid or borate buffers using the system toluene, 200; methanol, 130; water, 70 ml (system 19). An effect on mobility, consisting of a slight but reproducible retardation as the imposed pH was increased, was noted only in the case of the 2β,3β,17β triol XIX-b.

Samples of androstane-2α,3α-diol-17-one (XX) and its C-2 epimer (XX-a), also supplied by Dr. Bradlow, were chromatographed as above using the system toluene, 130; isooctane, 70; methanol, 130; water, 70 ml. (system 20). No effects on mobility were noted.

1β,3β,5,11α,14,19-*hexahydroxy*-20:22-*cardenolide* (*ouabagenin*) (XXI). This genin, supplied by Dr. Maximilian Ehrenstein, was chromatographed on paper pretreated with water, boric acid and the usual borate buffers using the system toluene, 80; t-butanol, 120; water, 150 ml (system 21). R_f values obtained for the indicated conditions were: water, 0·29; 5% boric acid, 0·10 with marked tailing; pH 7·6, 0·01 with tailing; pH 8·4, 8·8 and 10, genin did not leave the starting line. This substance is therefore strongly complexed with boric acid, particularly at high pH values.

DISCUSSION

As illustrated in the recent paper of Krauss *et al.*,[21] differential complexing with boric acid as a means of improving the paper chromatographic separation of carbohydrates is of proved utility. Detailed studies of this sort in the steroid field have not been reported. Balaschenko *et al.*[22] observed anodic migration in borate buffer at pH 8·6 or 9·6 of several unidentified steroid glycols. Leeson *et al.* studied the preparation and composition of the cycloborates of XVI and XVI-b[23] and the use of these complexes in fractionation[24] and preparative procedures.[25]

As a means of improving the paper chromatographic separation of epimeric pairs of steroid glycols, esterification with boric acid is clearly useful where applicable. Complexing occurs *in situ* under mild conditions, does not interfere with the detection of the steroid and is freely reversible. In addition to its use in paper chromatography, the technique can be extended to partition column chromatography and to countercurrent distribution. It may prove possible to adapt the technique of Leeson *et al.*[24] to the selective removal of various strongly complexed steroids from crude extracts of biological origin.

Discussion of the paper chromatographic characteristics of the steroid cycloborates involves a consideration of several aspects. Our results are consistent with the view that the observed changes in mobility are a consequence of complex formation and that the complex chromatographs as an acid. Thus in those cases where mobility changes were observed at low pH values or in the presence of boric acid itself, the effect uniformly was to increase R_f values indicating an increase in the partition coefficient, K, of the anionic species in a given system. Conversely, the effect at high pH values was consistently to reduce R_f values indicating that, as the cation, the complex was preferentially soluble in the stationary phase.

The reason why the 17α,20β-glycols are complexed only at low pH values and the 20α,21-glycols, the three 2-hydroxylated estrogens and all the C-16,17 *cis*-glycols only at an elevated pH must center on the nature of the complex itself. It is known from

[21] M. T. Krauss, H. Jager, O. Schindler and T. Reichstein, *J. Chrom.* **3**, 63 (1960).
[22] H. Bulaschenko, E. M. Richardson and F. C. Dohan, *Arch. Biochem. and Biophys.* **87**, 81 (1960).
[23] L. J. Leeson, J. A. Lowery, G. M. Sieger and S. J. Muller, *J. Pharm. Sci.* **50**, 193 (1960).
[24] L. J. Leeson, J. A. Lowery, G. M. Sieger and S. Muller, *J. Pharm. Sci.* **50**, 606 (1960).
[25] L. J. Leeson, J. A. Lowery, G. M. Sieger and C. Krieger, *J. Pharm. Sci.* **50**, 856 (1960).

the notable researches of Boeseken[26] and of Isbell *et al.*[27] that, under conditions where excess boric acid is present or a low pH prevails, formation of the faintly acidic monodiol boric acid (*A*, Fig. 9) is favored. The strongly acidic *bis*-diol boric acid (*B*, Fig. 9) is believed to be the sole product under circumstances where an elevated pH is maintained. It is held, generally, that the latter type of complex is formed by those diols which complex most readily and that it is with this type of complexing that the greatest changes in optical rotation are associated. We believe, wholly on analogy and without supporting evidence, that the 17α,20β-glycols form

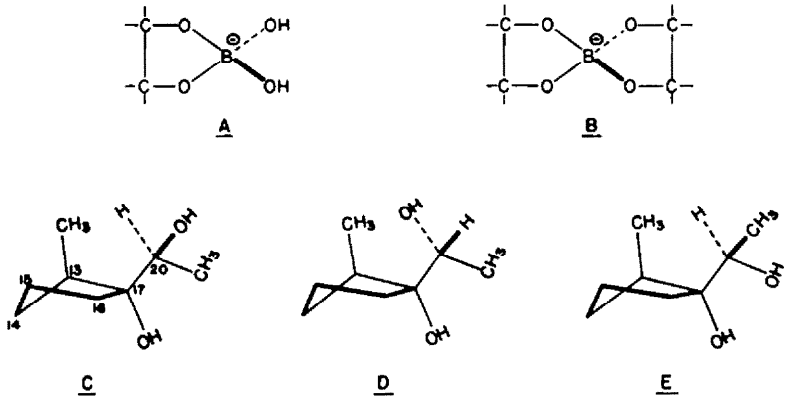

FIG. 9. Representations of a monodiol boric acid (*A*), a *bis* diol boric acid (*B*), a steroid 17α,20α-glycol (*C*) and a 17α,20β-glycol (*D* and *E*).

predominantly the monodiol type of complex and that the 20α,21-glycols, the 2-hydroxylated estrogens and the *cis* 16,17-glycols give the *bis*-diol diesters. This view is supported by the work of Leeson *et al.*[23] who prepared the cycloborate of XVI-b and concluded, on the basis of various analytic results, that the structure is of the *bis* type.

It seemed of interest to interpret the results in terms of conformational analysis, and here we wish to indicate our gratitude to Dr. D. H. R. Barton whose interpretation of our data has been transposed, with little change, into the section which follows. Assuming a *bis* type structure, complexing with boric acid requires ideally a coplanarity of the two separate halves of the borate anion (*B*, Fig. 9). It is understandable, therefore, why *cis* cyclopentane-1,2-diols complex and *trans* do not, and it follows that steroid ring D *vic*-glycols should complex, or fail to complex, in a predictable fashion. Our results with seven 16,17-glycols are in accord with this view. Compounds XIV-a, XIV-b, XV-a, XVI and XVI-b, all *cis*, were strongly complexed under conditions where compounds XIV and XV, both *trans*, were not.

The complexing behavior of cyclohexane-1,2-diols depends on whether the basic conformation can be easily deformed or not and whether the conformation imposed by angle strain tends to give the required coplanarity. Thus of the 14 saturated ring A, B or C *vic*-glycols examined, ten (XVII-a, XVII-b, XVII-c, XVIII-a, XVIII-c, XIX, XIX-a, XIX-c, XX and XX-a) failed to complex under the conditions imposed

[26] J. Boeseken, *Adv. in Carb. Chem.* **4**, 189 (1949).
[27] H. S. Isbell, J. F. Brewster, N. B. Holt and H. L. Frush, *J. Res. Nat. Bur. Stand.* **40**, 129 (1948).

because their conformations are not sufficiently flexible. Complexing did occur with the remaining four (XVII, XVIII, XVIII-b and XIX-b) because in these cases the angle strain in the molecule happens to give the required coplanarity.

The results with the eight 17α,20-glycols VI-α, VI-β, VII-α, VII-β, VIII-α, VIII-β, IX-α and IX-β also can be understood in terms of this analysis. The preferred conformation for the 17α,20α-diols is *C* (Fig. 9) and the corresponding conformation for the 17α,20β-diols would be *D*. *C* is more stable than *D* because of the C-13-methyl-C-20-hydroxyl interaction in *D*. Complexing of a 17α,20β-diol seems reasonable since the more stable conformation for the 20β-hydroxyl group should be that in *E* in which the 17α,20β-diol system is perfectly coplanar. With the 17α,20α-diols the conformation has no reason, of its own choice, to become coplanar. Our results admirably fit this analysis. Of the eight 17α,20-glycols studied, only the four 20β epimers (VI-β, VII-β, VIII-β and IX-β) were complexed.

The pregnenoic acids X-α and X-β, not included in this discussion, constitute an exception of interest. Their wholly different complexing properties clearly demonstrate the influence of the large substituent at C-21. It is possible that this structural feature accounts also for the observation that acetylation at C-20 of the 17α,20-dihydroxy-pregnenoates does not result in shifts of molecular rotation characteristic of other 20α and 20β-ols.[18]

Complexing in the case of the ten 20,21-glycols uniformly was characterized by a moderate to marked reduction in the mobilities of the 20α,21-glycols and a small decrease in the R_f values of the 20β,21-glycols as illustrated in Figs 1 and 2. This less than complete differentiation is understandable, at least in part, in conformational terms. When the 20α hydroxyl group is in the preferred orientation, or in any of a number of adjacent positions, the required coplanarity with the freely rotating hydroxyl group at C-21 easily is achieved. When the orientation of the 20β hydroxyl group approximates the stable arrangement, its approach to the hydroxyl group at C-21 appears similarly unhindered. The point of difference rests on the observation that the various approaches to the coplanar system are less obstructed by neighboring groups in the case of the 20α,21-glycols. It seems likely, however, that the differences observed experimentally are due largly to factors not readily discerned by inspecting models.

The 17α,20,21-glycerols comprise a special and interesting group. All three pairs in this class gave similar, reversed-sigmoid, curves as illustrated in Fig. 5. It seems reasonable to regard such a curve as a rough composite in which the portion below pH 8·5 resembles the curves given by the 17α,20β-glycols (Fig. 3) and the part above that point similar to the curves obtained from the 20,21-glycols (Figs 1 and 2). We believe that these similarities are sufficiently real to permit the conclusion that complexing of the 17α,20,21-glycerols involves primarily the 17α,20-glycol system at low pH values (giving the monodiol type of complex) and the C-20,21 hydroxyl groups at an elevated pH under which conditions the *bis*-diol type of complex predominates.

If this reasoning is correct, it follows that the 3α,6β,7β-trihydroxycholanic acid XVII and the 11α,12α-dihydroxytigogenin XVIII, which are complexed at both low and high pH values, are capable of forming both types of complex at the same site. It is of interest also to speculate on the site of complexing in the case of ouabagenin.

Since acetonide formation involves the hydroxyl groups at C-1 and C-19,[28] it seems likely that these positions serve also as the chief site for cycloborate formation. But the common axial orientation of the hydroxyl groups at C-1,3 and 5 suggests that complexing of a 1,3 type is a possibility. The complexing of 1,3 diols is known in the carbohydrate field.[29]

Our results with the four diastereoisomeric bile acid esters, namely that the 6β,7β isomer XVII alone is complexed, may be compared with those of Angyal and Young[30] who studied the rates of reaction of the four cholestane-3β,6,7-triols with lead tetraacetate. They noted that the 6β,7β (a, e) isomer reacted most rapidly and attributed this to the influence of C-6-hydroxyl-C-10-methyl repulsion in promoting an approach to the coplanar state. An extension of this reasoning to our results with the four dihydroxytigogenins furnishes only a partial explanation. Thus the complexing of the 11β,12β isomer would appear to be favored by C-11-hydroxyl-C-10-methyl interaction, but this factor clearly cannot explain the even greater complexing tendency of the 11α,12α system. It seems possible that the unencumbered rearside approach to the 11α,12α hydroxyl groups promotes complex formation in this case.

Although the number of epimeric pairs examined to date is small and the structural variations within each class very limited, the borate-induced mobility changes in the simple 17α,20β-glycols, the 20α,21-glycols and the *cis* 16,17-glycols are so uniformly reproducible as to suggest that the method has utility in configuration assignment.

[28] G. Volpp and Ch. Tamm, *Helv. Chim. Acta* **40**, 1860 (1957).
[29] R. E. Reeves, *Adv. in Carb. Chem.* **6**, 107 (1951).
[30] S. J. Angyal and R. J. Young, *J. Amer. Chem. Soc.* **81**, 5251 (1959).