methionine and S-methyl-L-cysteine indicate that any influence the S-methyl-L-cysteine might have on the conformational behavior is not observable in the mixed $CHCl_3$:DCA solvents employed. It is thought that the necessity of using some DCA in the solvent mixtures probably is the cause of the seeming inconsistency of the solution and solid state results for the S-methyl-L-cysteine copolymer. As might have been expected, however, the influence of L-valine was unaltered by the change from solid to solution. One factor which is probably important in conformational stability in solution, as illustrated by the relative greater stability of the α-helix found in poly-L-methionine than in some other poly-α-amino acids, is the interaction of hydrophobic areas which contribute to the stabilization of any ordered conformation. Also significant was the finding that the helix→random transition of all the predominantly helical polymers studied was essentially independent of the non-helical regions.

We suggest that the conclusions derived from the conformational studies on the model polypeptides are applicable to proteins. Amino acid composition and sequence, therefore, should play an important role in determining the conformational states of proteins. Further the sharp change in optical rotation found on denaturation of some globular proteins[26] may be attributed to the breakdown of small helical regions, within the encompassing non-helical regions. Finally, we should like to emphasize that although the application of the concept of "α-forming" and "β-forming" α-amino acids will aid in understanding the conformational behavior of proteins, the importance of intra- and inter-molecular hydrogen bonds, disulfide bridges, salt links, etc., should not be neglected.[22,27]

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[CONTRIBUTION FROM THE DEPARTMENTS OF MICROBIOLOGY AND NEUROLOGY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY, NEW YORK, N. Y., AND THE NEUROLOGICAL INSTITUTE, PRESBYTERIAN HOSPITAL, NEW YORK 32, N. Y.]

Immunochemical Studies on Blood Groups.[1] XXVII. Periodate Oxidation of Blood Group A, B and O(H) Substances

BY GERALD SCHIFFMAN, ELVIN A. KABAT AND WILLIAM THOMPSON

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On exposure to periodate, blood group substances rapidly consume from 2.7 to 3.7 moles per 1000 g. The periodate consumption is essentially complete in one day at 0 to 4° and little or no further uptake is noted even up to 346 hours. The consumption of periodate can, for the most part, be accounted for by the destruction of fucose, galactose and hexosamine and the production of formic acid. Fucose is almost completely destroyed, galactose about 70 to 90% and hexosamine is the least affected. The glucosamine of two human cyst blood group substances is almost completely resistant to periodate. Galactosamine is affected to a varying extent in the five blood group substances and two P1 preparations used, but was only very slightly changed in the human B cyst and the hog O (H) substances. After periodate oxidation and borohydride reduction, the seven preparations were exposed to 1 N HCl and dialyzed. The non-dialyzable material contained appreciable amounts of galactose, hexosamine and amino acids. The N content of the non-dialyzable fraction was increased from the 5 to 6% originally present in blood group substances to 8 to 9%. The behavior of blood group substances on periodate oxidation is compatible with a highly-branched structure with a portion resistant to periodate made up either of carbohydrate residues or of amino acids in peptide linkage or both.

Periodate oxidation has been used extensively in investigations of the structures of many polysaccharides.[2–6] However, it has become increasingly evident that results based only on periodate consumption and the release of one-carbon fragments can be extremely misleading. Compounds with sterically hindered *trans*-hydroxyl groups consume less[7] and compounds with active hydrogen atoms consume more[8] periodate than expected. Other types of anomalous behaviour have been reported with hyaluronic acid[9a] and with glycoproteins[9b] in which periodate oxidizes the amino acids cysteine, cystine, methionine, tryptophan and tyrosine. Whenever possible studies should, therefore, include quantitative estimation of products as well as constituents destroyed by or resistant to periodate.[10]

With blood group substances, periodate oxidation data can be correlated with destruction or resistance of fucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and various amino acids.

In the past few years this Laboratory has modified standard periodate methods so that microgram quantities can be analyzed. For example, with a total of 50 to 100 μg. of a disaccharide, 8 points on a periodate uptake curve, a formaldehyde and a formic acid determination may be carried out, 5 to

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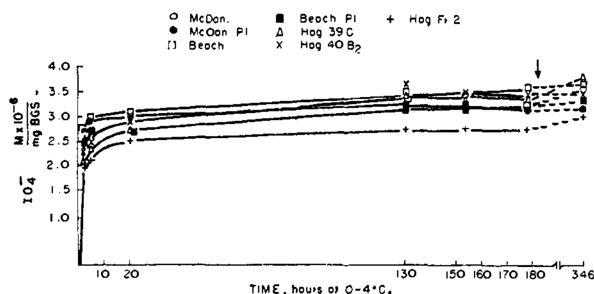

Fig. 1.—Periodate oxidation curves of the blood group substances and P1 fractions.

10 and 10 to 20 μg. of disaccharide being used for the latter two analyses, respectively. Various methods of analysis for microgram quantities of individual sugars are also available.[11,12]

In 1951, Aminoff and Morgan[13] oxidized with periodate a blood group A substance isolated from human ovarian cyst fluid. The results showed a remarkably rapid uptake of periodate complete in 3 hours at room temperature accompanied by loss of all methylpentose and galactose, but with much less destruction of hexosamine, as established by paper chromatography.

These studies have now been extended to other preparations including human A and B substances from pseudomucinous ovarian cyst fluid, hog A, O(H) and mucin preparations, and to the non-dialyzable residues obtained after mild acid hydrolysis of the human cyst substances (P1 fractions).[14] Effects of periodate on fucose, galactose, N-acetylglucosamine and N-acetylgalactosamine were followed. In addition, the oxidized polyaldehydes, after borohydride reduction,[15] were exposed to 1 *N* HCl[16]; on dialysis the bulk of the material remained non-dialyzable indicating non-random distribution of the periodate-sensitive carbohydrate residues.

Experimental

Materials and Methods. Blood Group Substances.—The following blood group substances and P1 fractions which have been previously described were used; human A substance from ovarian cyst fluid, McDon and its P1 fraction (non-dialyzable after hydrolysis at *p*H 1.6, McDon P1)[17]; human B substance from ovarian cyst fluid, Beach, and its P1 fraction, Beach P1[18,19]; A, O(H) and A + O(H) substances from hog stomach linings, hog 39C(A), hog $40B_2$-(O(H)) and mucin Fr. 2.[20,21]

Analytical Methods.—Reducing sugar was measured by a modification of the procedure of Park and Johnson[22,23] using glucose as a standard; methylpentose by the method of Dische and Shettles[24]; galactose by the method of Dische[11]; hexosamine by a modified[23] procedure of Elson and Morgan[25] using 5.0-ml. volumetric flasks; N-acetylhexosamine by the method of Reissig, *et al.*,[26] using a reduced volume.[23] N-Acetylation, after complete hydrolysis, and calculation of glucosamine and galactosamine ratios were performed by the method of Roseman and Daffner.[27] All of these methods are given in detail in ref. 12.

Periodate consumption and formic acid release were followed by the micro-titration procedure previously described.[17] Formaldehyde was determined by the chromotropic acid method described by Smith and Montgomery.[5,12]

Nitrogen was determined by the Markham modification of the micro-Kjeldahl procedure[12] for samples of 50 to 100 μg. N. When samples of 1 to 5 μg. N were determined, an adaptation of the procedure of Rosevear and Smith[28] was used as follows:

Samples were digested with 0.1 ml. of a digestion mixture containing 1.0 ml. concentrated sulfuric acid saturated with copper sulfate,[12] 0.5 g. of potassium sulfate and made up to 40 ml. Digestion is performed in 3-ml. conical tubes in a sand-bath. A slow stream of N_2 is passed over each tube during the heating until the water vapor is removed. This avoids refluxing and spattering. When the sample chars a small drop of superoxol is added and digestion is continued. More superoxol is added if needed and digestion is continued for 0.5 hour after the digest has become colorless. The samples are allowed to cool and the pellet dissolved in 0.3 ml. of water followed by 0.1 ml. of 4 *M* acetate buffer, *p*H 6.5. The solutions are mixed and 0.1 ml. of 5% ninhydrin in methyl Cellosolve is added. The 5% ninhydrin in methyl Cellosolve contains 0.050 ml. of 0.01 *M* KCN for each 2.0 ml. and is added just before use. The tubes are heated to 90 to 95° for 10 minutes, cooled and the contents transferred to 5.0- or 10.0-ml. volumetric flasks with 50% ethanol. Blanks and ammonium sulfate standards are included in each set of determinations. The colors are read at 5700 Å.

Periodate Oxidation.—Twenty to 50 mg. of the five blood group substances and the two P1 preparations were dried to constant weight over P_2O_5 and made up to 15.0 ml. with water. A 3.0-ml. portion was removed from each vessel and reserved for analysis. Exactly 3.0 ml. of 4.00 × 10^{-2} *M* sodium periodate was added and the solutions placed immediately in a refrigerator (4°). Portions of from 15 to 20 μl. were removed at 3, 5, 21, 130, 154, 178 and 346 hours and the amount of periodate consumed was measured. At 24 hours a 1.0-ml. portion was removed for further testing. The main preparations were dialyzed exhaustively, pervaporated to about 10 ml., and transferred to 15-ml. volumetric flasks and made up to volume with water. A 3-ml. portion of the polyaldehyde was reserved for analysis. To the remaining 12 ml., 240 mg. of sodium borohydride was added at 0° and the solutions were allowed to stand in the refrigerator overnight. A drop of caprylic alcohol was added to prevent foaming. The reaction mixtures were brought to room temperature for 3 hours and then kept at 37° for 1 hour. After exhaustive dialysis, pervaporation and transfer to 15-ml. volumetric flasks, the volume was adjusted to mark with water. A 3-ml. portion of the polyalcohol was reserved for analysis.

Results

The periodate uptake curves of the blood group preparations are shown in Fig. 1. Uptake is very rapid and is essentially complete in one day; the 130-hour point is only slightly higher than the 21-

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TABLE I

EFFECT OF PERIODATE OXIDATION AND BOROHYDRIDE REDUCTION ON THE COMPOSITION OF BLOOD GROUP SUBSTANCES AND THEIR P1 FRACTIONS

	Unhydrolyzed reducing sugar, %				Methylpentose, %			Galactose, %			Nitrogen, %		
	$NaBH_4$ reduced	Not treated	Polyaldehyde[a]	Polyalcohol[b]	Not treated	Polyaldehyde	Polyalcohol	Not treated	Polyaldehyde	Polyalcohol	Not treated	Polyaldehyde	Polyalcohol
McDon (A)	15	15	19	12	20	0.8	0.5	21	5.1	4.6	5.2	5.6	5.0
McDon P1	12	30	17	14	3	.3	.1	24	3.1	3.3	7.4	6.8	5.5
Beach (B)	14	15	16	13	22	.0	.5	26	7.3	6.5	5.2	5.2	4.9
Beach P1	17	23	16	15	5	.1	.4	30	6.6	7.1	6.6	6.3	5.5
Hog 39C (A)	13	15	20	9	7	.1	.5	21	6.3	5.9	5.9	6.3	5.8
Hog $40B_2$ (O(H))	12	10	18	7	9	.6	.9	21	3.2	2.3	6.2	6.8	5.7
Hog mucin Fr 2 (A + O(H))	9	13	22	9	9	.6	.8	20	3.8	3.9	6.4	6.4	6.1

[a] Periodate oxidized. [b] Periodate oxidized and reduced with sodium borohydride.

TABLE II

EFFECT OF PERIODATE OXIDATION AND BOROHYDRIDE REDUCTION ON THE AMINOSUGAR COMPOSITION OF BLOOD GROUP SUBSTANCES

	Hexosamine, %			Acetylhexosamine, % (glucosamine equivalents)			Glucosamine, %			Galactosamine, %		
	Not treated	Polyaldehyde	Polyalcohol	Not treated	Polyaldehyde	Polyalcohol	Not treated	Polyaldehyde	Polyalcohol	Not treated	Polyaldehyde	Polyalcohol
McDon (A)	33	22	21	20	16	16	14	13	14	19	9	7
McDon P1	36	18	17	19	11	11	11	8	8	25	10	9
Beach (B)	25	22	21	17	14	15	13	10	12	12	12	9
Beach P1	30	20	21	19	14	14	14	11	11	16	9	10
Hog 39C (A)	34	20	19	23	12	12	18	8	9	16	12	10
Hog $40B_2$ (O(H))	32	21	23	25	16	17	22	14	14	10	7	9
Hog mucin Fr 2 (A + O(H))	32	22	22	24	16	17	20	13	15	12	9	7

hour point with subsequent determinations to 346 hours not changing significantly and indicating little or no over-oxidation. Individual preparations consumed from 2.7 to 3.7 micromoles of periodate per milligram.

Analyses for reducing sugar, galactose, methylpentose, N, hexosamine and for N-acetylhexosamine after complete hydrolysis and N-acetylation were performed on the untreated substances, the polyaldehydes and the polyalcohols. The results are summarized in Tables I and II. Of the five blood group substances used, all showed from 10 to 15% reducing sugar without previous hydrolysis[14]; the two human P1 preparations had 23 and 30%, considerably higher than the hog P1 samples reported earlier.[14] These high initial values are not lowered by borohydride reduction of the blood group substances but reducing values of the P1 samples are significantly lowered by reduction with borohydride. After periodate oxidation the polyaldehydes have a higher reducing sugar value than the untreated substances, but the increase is less than twofold; however, the reducing sugar of the polyaldehydes of the P1 samples decreases to almost half their initial levels. On subsequent borohydride reduction the polyalcohols show reducing sugars of from 7 to 15%, which, except for the two P1 preparations, were only slightly lower than their initial values.

With the blood group substances and the P1 fractions, almost all the methylpentose was destroyed by periodate oxidation; all preparations contained less than 1% methylpentose after exposure to periodate. The same results were obtained before and after reduction, indicating absence of interference of the aldehyde groups with the methylpentose assay.

Most of the galactose of the seven preparations was destroyed, *i.e.*, 70 to 90%. As with methylpentose the findings obtained before and after reduction with borohydride were similar indicating no interference of the aldehyde groups in the galactose assay.

With the intact blood group substances there was no significant loss of N by any of the treatments. The losses in N with the P1 fractions were outside the limits of error and indicate some decomposition.

The amino sugar composition of the blood group substances and the P1 fractions before and after treatment is summarized in Table II. Destruction of hexosamine was less than that found for methylpentose and galactose, only McDon P1 showing as much as 50%. Hexosamine and N-acetylhexosamine values were unchanged after borohydride reduction. Calculation of glucosamine and galactosamine in the untreated and treated materials shows glucosamine in the two cyst materials to be more resistant to periodate than in the three hog preparations. Galactosamine is destroyed to varying extents in the seven substances; the greater the initial galactosamine content the greater the destruction. All the preparations had 7 to 10% galactosamine after treatment, while the untreated materials had from 10 to 25% galactosamine.

From the data in Table I and II the number of moles of each sugar destroyed by periodate can be calculated. Since a minimum of one mole of periodate is sufficient to destroy a mole of sugar as measured by the assay methods employed and uptake of

TABLE III

MOLES OF SUGAR DESTROYED, ACID PRODUCED AND PERIODATE CONSUMED PER 1000 GM. OF B.G.S.

	Galactose	Methyl-pentose	Hexosamine	Acid produced	Sum of sugar destroyed and acid produced	Periodate consumed, exptl.
McDon (A)	0.9	1.2	0.6	0.9	3.6	3.5
McDon P1	1.2	0.2	1.1	1.4	3.9	3.2
Beach (B)	1.1	1.3	0.2	1.1	3.7	3.6
Beach P1	1.3	0.3	.6	1.2	3.4	3.3
Hog 39C (A)	0.8	.4	.8	0.5	2.5	3.7
Hog $40B_2$ (O(H))	1.0	.6	.6	.4	2.6	3.5
Hog mucin Fr 2 (A + O(H))	0.9	.6	.6	.3	2.4	2.9

TABLE IV

COMPOSITION AND PERCENTAGE COMPOSITION OF NON-DIALYZABLE FRACTION OF ACID-TREATED POLYALCOHOLS

	Reducing sugar, mg./10 ml.	Galactose, mg./10 ml.	Hexosamine, mg./10 ml.	N-Acetylhexosamine, mg./10 ml.	Nitrogen, mg./10 ml.	Reducing sugar, %	Galactose, %	Hexosamine, %	Nitrogen, %
McDon (A)	2.1	1.1	2.0	2.8	0.66	30	16	19	9.4
McDon P1	1.5	0.3	1.7	1.2	.54	22	4	25	8.1
Beach (B)	2.0	1.5	2.7	2.4	.47	31	24	42	7.5
Beach P1	1.4	0.7	1.8	1.5	.42	26	13	34	7.9
Hog 39C (A)	0.4	.2	0.5	0.6	.27	13	7	17	9.0
Hog $40B_2$ (O(H))	0.9	.3	2.2	1.9	.52	16	5	39	9.3
Hog mucin Fr 2 (A + O(H))	1.8	.8	2.9	3.3	.79	21	9	33	9.1

two moles of periodate is needed to produce a mole of formic acid, the sum of the number of moles of sugar destroyed and moles of formic acid produced would equal the minimum number of moles of periodate consumed to produce the measured effect. Table III presents such a balance sheet. It can be seen that with the cyst substances the moles of periodate consumed can be accounted for by the sugars destroyed and the formic acid produced. The hog substances consume more periodate than can be accounted for in terms of the residues destroyed and formic acid formed. Whether this is because more than one mole of periodate is consumed per mole of sugar destroyed without liberation of formic acid or not must await further investigation. However, the possibility that blood group substances contain components other than amino acids and the four sugars studied cannot be disregarded. An attempt was made to determine whether there was any gross discrepancy between the sum of the components analyzed and the weight of substances used. The hexosamine N was subtracted from the total N to give non-hexosamine N. This value was multiplied by 7.15 to give the percentage of amino acid. The factor 7.15 is based on an amino acid residue weight of 100, calculated from data in ref. 20. From 86% of Hog 39C(A) to 98% for McDon Pl, with an average of 93%, of the weight of the seven preparations could be accounted for by the sum of the four sugars and the amino acids. It, therefore, seems unlikely that the excess periodate consumption can be attributed to unknown components but rather to more than one mole of periodate being consumed per residue destroyed.

Exposure of Polyalcohols to Acid.—The seven polyalcohols were acidified with HCl to a final concentration of 1 *N* and allowed to stand at room temperature for one hour. The solutions were exhaustively dialyzed, pervaporated and adjusted to 10.0 ml. Traces of precipitate tended to form at this stage. Before analysis these precipitates were centrifuged off and removed. Some accidental loss occurred in transfer of hog 39C(A). The results of the analyses of these materials are summarized in Table IV. Since these materials cannot be easily redissolved once they are lyophilized and since not sufficient material was available for taking an aliquot portion, weight was calculated by summation of the residue weights of galactose, acetylhexosamine and amino acids. The percentage composition calculated by this method is also given in Table IV. It can be seen that the N has risen from the 5 to 6% originally present to 8 to 9%.

Discussion

On exposure to periodate, blood group substances rapidly consume from 2.7 to 3.7 moles per 1000 g. This can, for the most part, be accounted for by the destruction of fucose, galactose and hexosamine and the liberation of acid, presumably formic. Fucose is almost completely destroyed, galactose is about 70 to 90% destroyed and hexosamine is the least affected. These results are in substantial agreement with those obtained by Aminoff and Morgan[13] in their study of a blood group A substance from human cyst fluid. The glucosamine in the two cyst blood group substances is almost completely resistant to periodate. Galactosamine is destroyed to a varying extent in all preparations, but least in the cyst B and hog O(H) substances. After periodate oxidation and borohydride reduction about 7 to 10% of all the polyalcohols is galactosamine. This rapid and specific consumption of periodate by blood group substances is in contrast to that observed with many polysaccharides. Several dextrans and pneumococcal type II polysaccharide exposed to periodate under similar conditions required several days to a week to reach a plateau, while pneumococcal type III polysaccharide needed even longer.[29] The rapid uptake by the blood

(29) R. Mage, personal communication.

group substances reflects a highly-branched structure with a portion made up either of carbohydrate residues resistant to periodate or of amino acids in peptide linkage or both. Such a highly-branched structure with a periodate-resistant region is rendered more likely by the behavior of the polyalcohols to acid. A linear polysaccharide, after about half of its sugar residues had been attacked by periodate, would be expected to become mostly dialyzable after reduction and exposure to acid if the periodate-sensitive residues were randomly dispersed. This does not happen. After exposure of the polyalcohols to acid, there are substantial amounts of non-dialyzable hexosamine and galactose, in addition to amino acids.

The high reducing sugar values of the five blood group substances before hydrolysis are apparently not a reflection of free reducing ends in the molecule. Borohydride reduction does not lower these values appreciably (Table I). They could be caused by the splitting of some alkali-labile bonds under the conditions of the reducing sugar assay. On the other hand, the lowering of the Pl values by borohydride and by periodate treatment could be explained by the presence of some free reducing ends.

It has been previously established[30] that on mild acid hydrolysis and dialysis the glucosamine: galactosamine ratio in the dialyzate was 2 to 4 for blood group A subtance, 13 to 14 for O(H) substance and only glucosamine was split off from human B substance. From these ratios it can be seen that relatively more galactosamine was split off from A, less from O(H) and no galactosamine from blood group B substance. With the blood group A substances from both human and hog sources a greater percentage of the galactosamine was destroyed by periodate than with the hog mucin preparation (A + O(H)) and very little of the galactosamine was destroyed in the hog O(H) and human B substances. Therefore, for galactosamine there appears to be a rough correlation between dialyzability on mild acid hydrolysis and periodate sensitivity on the one hand and non-dialyzability (acid stability) and periodate resistance on the other.

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Except for hog 39C(A), the amount of formic acid liberated on exposure to periodate is just slightly less than the amount of fucose destroyed. Since terminal non-reducing fucose is the only form of fucose which could liberate formic acid, this means that, if no acid is produced by oxidation of galactose, most of the fucose could be terminal and non-reducing. However some β-linked terminal galactose is known to be present since blood group substances cross react with type XIV anti-pneumococcal sera[31,32] before but not after treatment with *Cl. tertium* enzymes[33] which split off about 1 to 2% of the blood group substances as free galactose.

In addition, coffee bean α-galactosidase split off about 5% of Beach (B) as free galactose.[34] Thus an amount of fucose, equivalent to the terminal galactose, could then be non-terminal.

Although almost all of the fucose is periodate sensitive in all the preparations studied, fucosyl-(1→3)-fucose was isolated[19] by mild acid hydrolysis from blood group B substance (Beach). The amount isolated, 7 mg., represented only 0.4% of the dialyzable components and only 0.06% of the original blood group substance before hydrolysis. This amount of material is not determinable in the intact polymer by the analytical methods available.

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[CONTRIBUTION FROM THE STERLING-WINTHROP RESEARCH INSTITUTE, RENSSELAER, N. Y.]

Some Reactions of 2-Hydroxytestosterone and its Diacetate. II

BY ROBERT L. CLARKE

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The rearrangement of 2α-hydroxytestosterone (Ic) and its diacetate Ib to 2-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol (IIIa) is described. The structure of IIIa was proved by degradation to 4-methyl-1,3,5(10)-estratrien-17β-ol (IVb), by synthesis from 17β-hydroxy-2-methoxy-1,4-androstadien-3-one (V) and by n.m.r. characteristics. A mechanism for the rearrangement is suggested.

The rearrangement of 2β-hydroxytestosterone diacetate (Ia) by means of *p*-toluenesulfonic acid (tosyl acid) in boiling methanol to form 17β-hydroxyandrostane-3,6-dione (II) (51% yield) has been described recently.[1] The present paper describes the rearrangement of 2α-hydroxytestosterone (Ic) and its diacetate Ib under the same conditions to 2-methoxy-4-methyl-1,3,5(10)-estratrien-17β-ol (IIIa), m.p. 114.5–116°, in 24 and 11% yields, respectively. A 6% yield of the dione II was also isolated from the rearrangement of Ic.

(1) R. L. Clarke, *J. Am. Chem. Soc.*, **82**, 4629 (1960).

It was initially believed that the methoxyaromatic product (called III) from the rearrangement was the result of dehydration of Ic to produce a 1,4-dien-3-one which underwent a dieneone–phenol type of rearrangement[2] followed by etherification. The expected products, 1-methoxy-4-methyl-1,3,5-(10)-estratrien-17β-ol[3] and possibly 3-methoxy-1-methyl-1,3,5(10)-estratrien-17β-ol,[4] were synthe-

(2) *Cf.* C. Djerassi, G. Rosenkranz, J. Romo, J. Pataki and St. Kaufmann, *ibid.*, **72**, 4540 (1950).
(3) R. M. Dodson and R. D. Muir, *ibid.*, **80**, 5004 (1958).
(4) V. Petrow and I. A. Stuart-Webb, British Patent 807,225, Jan. 14, 1959.