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Chromogenic Reactions of Steroids with Strong Acids. IV.[1] Specificity of the Kober Reaction[2]

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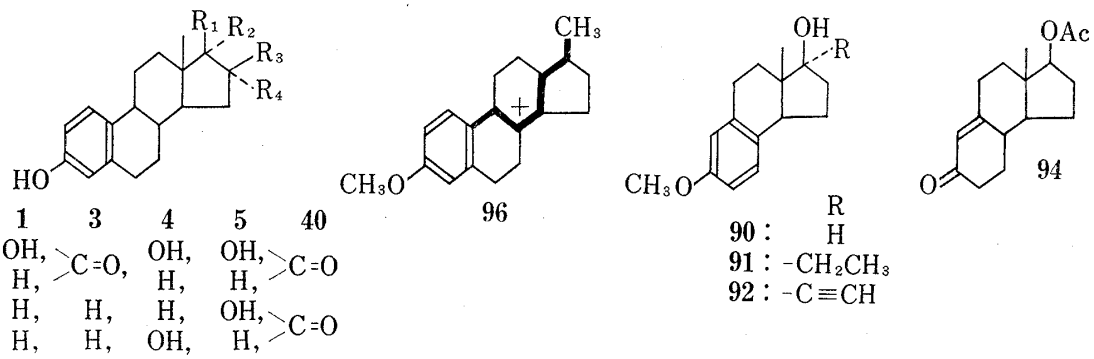
The structural requirements were investigated for the Kober reaction of the steroidal molecules. On the basis of the data given by the ninety-four specimens of phenolic steroid or related substance (Table I), a compound will give the positive Kober reaction when the following features are wholly present in its molecule: 1) steroidal ring system, 2) phenolic ring A, 3) double bond or oxygen function in ring D, 4) angular methyl group at C_{13}, and 5) angular hydrogen atom.

In 1930, Marrian[4] reported that estriol, estra-1,3,5(10)-triene-3,16α,17β-triol (**4**), gave an orange-yellow color with green fluorescence when it was heated with concentrated sulfuric acid. Kober[5] found that this color turned pink when the sulfuric acid solution was once diluted with water and heated again. He also found that the fluorescence was quenched in the presence of phenol or β-naphthol so that these procedures may be capable of applying for the colorimetric determination of **4**. Since then the Kober reaction and its modifications have extensively been studied and utilized for the analysis of various steroidal estrogens. Marrian, *et al.*,[6] guided by this reaction, were successful in isolating new estrogens such as 16-epiestriol, estra-1,3,5(10)-triene-3,16β,17β-triol (**5**), from urine. The modifications made by Bauld[7] and Brown[8] using hydroquinone-sulfuric acid mixture and by Ittrich[9] using solvent-extraction-method were probably most successful and have been widely used for the quantitation of steroidal estrogens in the biological materials.

While extensive studies on the colorimetric determination have thus been made, little work[2,10] has been reported on the detailed chemistry of the Kober reaction. The specificity and mechanism of this reaction have ever been studied by several workers.[11–13] Although the reagents and procedures employed are not unusual, the Kober reaction has been assumed to be surprisingly specific to estrone (3-hydroxyestra-1,3,5(10)-trien-17-one) (**3**), estradiol (estra-1,3,5(10)-triene-3,17β-diol) (**1**), estriol (**4**), and their derivatives and stereoisomers, as described by Fieser.[14] Marlow[11] studied on this reaction using β-naphtholsulfonic acid rea-

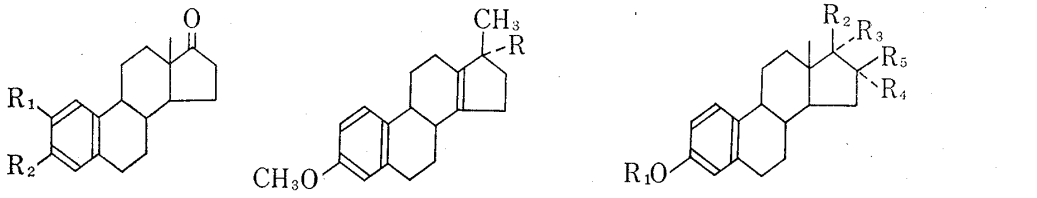
1) Part III: M. Kimura and K. Harita, *Chem. Pharm. Bull.* (Tokyo), **21**, 1261 (1973).
2) Preliminary accounts of a part of this work have been published: *a*) M. Kimura, K. Akiyama, K. Harita, T. Miura, and M. Kawata, *Tetrahedron Letters*, **1970**, 377; *b*) M. Kimura, K. Akiyama, and T. Miura, *Chem. Pharm. Bull.* (Tokyo), **20**, 2511 (1972).
3) Location: *Nishi-6-chome, Kita-12-jo, Kita-ku, Sapporo, 060, Japan.*
4) G.F. Marrian, *Biochem. J.*, **24**, 435 (1930).
5) S. Kober, *Biochem. Z.*, **239**, 209 (1931); *idem*, *Biochem. J.*, **32**, 357 (1938).
6) G.F. Marrian and W.S. Bauld, *Biochem. J.*, **59**, 136 (1955); G.F. Marrian, K.H. Loke, E.J.D. Watson, and M. Ponattoni, *ibid.*, **66**, 60 (1957); K.H. Loke, G.F. Marrian, and E.J.D. Watson, *ibid.*, **71**, 43 (1959).
7) W.S. Bauld, *Biochem. J.*, **56**, 425 (1954); *idem*, *ibid.*, **63**, 488 (1956).
8) J.B. Brown, *J. Endocrinol.*, **8**, 196 (1952); *idem*, *Biochem. J.*, **60**, 185 (1955).
9) G. Ittrich, *Z. Physiol. Chem.*, **312**, 1 (1958); *idem*, *Acta Endocrinol.*, **35**, 34 (1960).
10) H.A. Jones and R. Hähnel, *Nature*, **215**, 1381 (1967); *idem*, *Steroids*, **13**, 693 (1969).
11) H.W. Marlow, *Endocrinol.*, **42**, 479 (1948); *idem*, *J. Biol. Chem.*, **183**, 167 (1950).
12) R.J. Boscott, *Nature*, **164**, 140 (1949).
13) J.B. Brown, *CIBA Colloq. Endocrinol.*, **2**, 132 (1952).
14) L. Fieser and M. Fieser, "Steroids," Reinhold Publ. Co., New York, 1959, p. 469.

gent with seventeen phenolic steroids as well as three other steroids and found that 16-keto-estrone(3-hydroxyestra-1,3,5(10)-triene-16,17-dione) (**40**) was negative in the reaction. Although Marrian[15] assumed its oxonium salt to be the real chromophore, the diketone (**40**) cannot, thus, be the essential chromogen formed in the Kober reaction. Recently, Jones and Hähnel[10] suggested the molecular features for the positive (pink) reaction and proposed the mechanism for the formation of "delocalized resonating tertiary carbonium ion" (**96**), mainly on the basis of the spectroscopic observations. The present paper deals with the relation of the Kober chromogenesity to the steroidal structures and the functional groups involved.



	1	3	4	5	40
R_1=	OH,	>C=O,	OH,	OH,	>C=O
R_2=	H,		H,	H,	
R_3=	H,	H,	H,	OH,	>C=O
R_4=	H,	H,	OH,	H,	

Chart 1

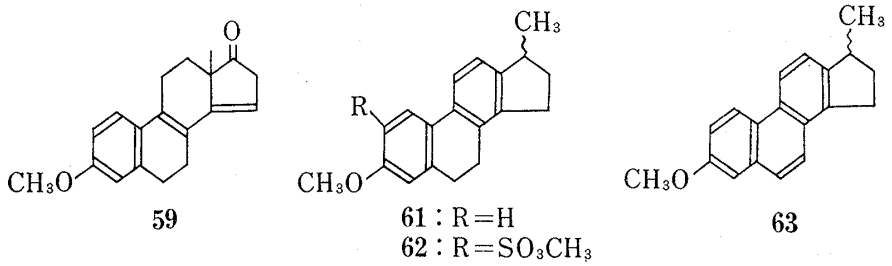


	R_1	R_2
13 :	H	H
16 :	H	Cl
17 :	H	-NHAc
19 :	OH	OH

33 : R=H
34 : R=CH_3

	31	32	7	35	36	37
R_1=	CH_3,	CH_3,	CH_3,	H,	H,	H
R_2=	H,	H,	OH,	H,	H,	OH
R_3=	H,	Δ^{16}	H,	H,	H,	H
R_4=	H,		H,	H,	>C=O,	>C=O
R_5=	H,	H,	H,	OH		

Chart 2



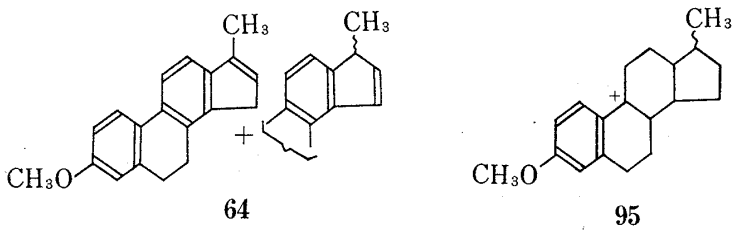


Chart 3

15) G.F. Marrian, *Harvey Lectures*, **34**, 37 (1938—1939); *Idem*, *Bull. New York Acad. Med.*, **15**, 27 (1939).

Result and Discussion

The Kober test is a two step reaction. The first is heating with sulfuric acid, which produces a yellow colored solution, and the second step is dilution with water or diluted sulfuric acid and reheating, which produces a pink colored solution. In this study, a steroid is estimated as giving a positive Kober reaction if it produces a pink chromophore giving light-absorption in the region of 500—540 nm under the conditions of the Kambegawa's modified method.[16] Among the modifications of Brown's method,[8] this procedure is the one which has been arranged to be as loosely specific as can be used commonly for all of the standard estrogens (**1**, **3**, and **4**) and is thus likely to be rather appropriate for such comparative examination of various substrates having different structures. On the basis of the data shown in Table I, it seems that a compound will be able to give the positive Kober reaction when the following features are wholly present in its molecule.

1) Steroidal Ring System

The tetracyclic system is necessary, which is characteristic of the steroidal structure. Thus, the bicyclic compounds (**88** and **89**) and tricyclic compounds (**84**—**86** and **90**—**94**) were negative in the reaction. It was interseting that the absorption maxima due to the tricyclic compounds (**90**—**92** and **94**) which were estimated to be negative from the definition mentioned above shifted towards more bathochromic region than those due to mono- (**87**) and bi-cyclic compounds (**88** and **89**).

2) Phenolic Ring A

It is essential for the positive reaction that the steroid molecule possesses an aromatic ring A having an oxygen function such as OH, OCH_3, or OAc at C_3 or C_2, contrary to that most of the ordinary steroids (**65**, **66**, **68**, **70**—**83**) carrying non-aromatic ring A were negative. Jones and Hähnel[10] found that androsta-1,4-diene-3,17-dione gave strongly positive Kober reaction, postulating the aromatization of ring A through the dienone-phenol rearrangement. The compounds (**67** and **69**) gave also positive reaction in this study, which are capable of being aromatized under the conditions examined. The steroids (**65** and **66**) carrying an aromatic system in ring B alone showed, on the other hand, maximum absorptions at the region of shorter wave-length and were estimated as negative. Phenolic steroids without any oxygen function at C_3 were positive when they have alternatively a hydroxyl group at C_2 (**18**) or C_1 (**28**); blue-shift and smaller ED-index (Table I) were observed in the latter case. Although the compound (**26**) having acetoxyl groups at both of C_1 and C_3 were also positive, the index was smaller than that due to the 3-acetate (**10**) of the ordinary phenolic steroid. These facts support the idea that the oxygen function at C_1 is inhibitory for producing the pink chromophore in the Kober reaction.[10] Contrary to that the compounds (**13**, **16**, **17**, and **29**) without oxygen function at C_3 were estimated to be negative on the basis of the definition mentioned above, introduction of 3-hydroxyl, -methoxyl, or -acetoxyl group and of 2,3-catechol function (**19**—**25**) to the 3-deoxy steroids (**13** and **29**) caused bathochromic shift by about 25 and 50 nm, respectively; this may be of fairly use in elucidating the chemical structure of the pink chromophore.

3) Double Bond or Oxygen Function in Ring D

It has been pointed out that the elimination reaction of the oxygen function in ring D plays an essential part in the early period of the Kober reaction.[12] In fact, the presence of a double bond or an oxygen function capable of forming it was necessary in ring D for the positive reaction, as shown below. Although deoxyestrone methyl ether (**31**) was slightly

16) A. Kambegawa, *Nippon Naibumpi Gakkai Zasshi*, 37, 807 (1961) [*C.A.*, 56, 9352*b* (1962)].

Table I. Kober Reaction of Phenolic Steroids and Related Compounds

Compound	Absorption maximum (nm)[a]					E.D. index[b]
Estra-1,3,5(10)-triene-3,17β-diol (**1**)	436′(4.02)	465 (4.26)	490′(4.27)	515 (4.32)		100
Estra-1,3,5(10)-triene-3,17α-diol (**2**)	436′(4.11)	462 (4.35)		517 (4.32)		100
3-Hydroxyestra-1,3,5(10)-trien-17-one (**3**)				514 (4.65)		214
Estra-1,3,5(10)-triene-3,16α,17β-triol (**4**)		462 (4.22)		512 (4.57)		178
Estra-1,3,5(10)-triene-3,16β,17β-triol (**5**)				513 (4.63)		204
3-Methoxyestra-1,3,5(10)-trien-17-one (**6**)		470′(4.44)		515 (4.57)		178
3-Methoxyestra-1,3,5(10)trien-17β-ol (**7**)	436′(4.08)	467 (4.36)		517 (4.34)		105
3-Methoxyestra-1,3,5(10)-trien-17α-ol (**8**)	438′(4.00)	468 (4.27)		524 (4.41)		123
3-Methoxyestra-1,3,5(10)-triene-16α,17β-diol (**9**)				515 (4.51)		155
3-Acetoxyestra-1,3,5(10)-trien-17-one (**10**)		465′(4.30)		514 (4.60)		191
Estra-1,3,5(10)-triene-3,17β-diol acetate (**11**)	436′(3.94)	465′(4.20)	490′(4.28)	514 (4.36)		110
Estra-1,3,5(10)-triene-3,16α,17β-triol triacetate (**12**)				513 (4.50)		152
Estra-1,3,5(10)-trien-17-one (**13**)	400′(3.51)		490 (4.06)			
3,17β-Dimethoxyestra-1,3,5(10)-triene (**14**)	440′(3.96)	468 (4.14)		513 (4.13)		64
3-*p*-Tolylsulfoxyestra-1,3,5(10)-trien-17-one (**15**)		466′(4.25)		516 (4.57)		178
3-Chloroestra-1,3,5(10)-trien-17-one (**16**)						
3-Acetamidoestra-1,3,5(10)-trien-17-one (**17**)			483 (4.01)			
Estra-1,3,5(10)-triene-2,17β-diol (**18**)				514 (4.50)		152
2,3-Dihydroxyestra-1,3,5(10)-trien-17-one (**19**)	414 (4.14)		490′(4.24)	510′(4.24)	536 (4.32)	100
2,3-Dimethoxyestra-1,3,5(10)-trien-17-one (**20**)	410 (4.07)		490 (4.20)	508′(4.32)	536 (4.37)	112
Estra-1,3,5(10)-triene-2,3,17β-triol (**21**)	412 (3.85)		490′(4.09)	508′(4.20)	540 (4.21)	78
2-Methoxyestra-1,3,5(10)-triene-3,17β-diol (**22**)	411 (3.91)		490′(4.18)	510′(4.23)	538 (4.25)	85
3-Methoxyestra-1,3,5(10)-triene-2,17β-diol (**23**)	412 (3.87)		490′(4.18)	510′(4.22)	536′(4.16)	69
2,3-Dimethoxyestra-1,3,5(10)-trien-17β-ol acetate (**24**)	410 (3.89)		490′(4.15)	508′(4.20)	540 (4.21)	78
Estra-1,3,5(10)-triene-2,3,16α,17β-tetrol (**25**)	415 (3.84)		490′(4.11)	510′(4.18)	537 (4.21)	78
1,3-Diacetoxyestra-1,3,5(10)-triene-17-one (**26**)					528 (3.47)	14
3-Hydroxy-1-methylestra-1,3,5(10)-trien-17-one (**27**)	430′(3.91)				520 (4.45)	135
1,11-Dihydroxy-4-methylestra-1,3,5(10)-trien-17-one (**28**)				505 (3.86)		35
Estra-1,3,5(10),16-tetraene (**29**)	470′(3.82)		490 (3.86)			
Estra-1,3,5(10)-triene (**30**)	430 (3.59)					
3-Methoxyestra-1,3,5(10)-triene (**31**)					523 (3.11)	6
3-Methoxyestra-1,3,5(10),16-tetraene (**32**)		466 (4.93)			521 (4.71)	246
3-Methoxy-17β-methyl-18-norestra-1,3,5(10),13-tetraene (**33**)		466 (4.61)			521 (4.47)	141
3-Methoxy-17ξ-methyl-18-nor-13ξ,14ξ-estra-1,3,5(10),8-tetraene (**34**)		466 (4.24)			520 (4.20)	76
Estra-1,3,5(10)-triene-3,16β-diol (**35**)		462 (4.40)			514 (4.24)	83
3-Hydroxyestra-1,3,5(10)-trien-16-one (**36**)	420 (3.76)			520′(3.33)		10
3,17β-Dihydroxyestra-1,3,5(10)-trien-16-one (**37**)				512 (4.61)		195
3-Acetoxy-16α-bromoestra-1,3,5(10)-trien-17-one (**38**)	428 (3.76)			506 (4.07)		56
16-Benzylidene-3-methoxyestra-1,3,5(10)-trien-17-one (**39**)					528 (3.54)	16
3-Hydroxyestra-1,3,5(10)-triene-16,17-dione (**40**)						
3-Methoxyestra-1,3,5(10)-triene-16,17-dione 16-oxime (**41**)						
3-Methoxyestra-1,3,5(10)-triene-16,17-dione 17-oxime (**42**)						
3-Hydroxyestra-1,3,5(10)-trien-17-oneoxime (**43**)				515 (2.32)		1
17ξ-Acetamidoestra-1,3,5(10)-trien-3-ol acetate (**44**)						
3-Hydroxy-17a-oxaestra-1,3,5(10)-trien-17-one (**45**)						
3-Hydroxy-17a-azaestra-1,3,5(10)-trien-17-one (**46**)			490 (2.99)			
3-Hydroxy-D-homoestra-1,3,5(10)-trien-17a-one (**47**)					523 (3.83)	22
17α-Ethinyl-3-methoxyestra-1,3,5(10)-trien-17β-ol (**48**)					537 (4.20)	74
17α-Cyanoestra-1,3,5(10)-triene-3,17β-diol diacetate (**49**)				515 (2.63)		2
3-Methoxy-17α-methylestra-1,3,5(10)-trien-17β-ol (**50**)	440′(4.09)	466 (4.25)			520 (3.42)	13

Compound	Absorption maximum (nm)[a]					E.D. index[b]
17,17-Dimethyl-3-methoxy-18-norestra-1,3,5(10),13-tetraene (**51**)	440′(4.10)	466 (4.27)			520 (3.42)	13
Estra-1,3,5(10),15-tetraene-3,17β-diol diacetate (**52**)	420 (3.76)				520′(3.33)	10
3-Benzyloxyestra-1,3,5(10),14-tetraen-17-one (**53**)		465′(4.02)		513 (4.16)		69
3-Acetoxyestra-1,3,5(10),15-tetraen-17-one (**54**)	417 (3.82)			514 (3.92)		40
3,15α-Dihydroxyestra-1,3,5(10)-trien-17-one (**55**)				512 (3.79)		30
3-Hydroxyestra-1,3,5(10),9(11)-tetraen-17-one (**56**)		470′(3.65)		510 (3.80)		30
Estra-1,3,5(10),6-tetraene-3,17β-diol (**57**)				512 (3.43)		13
3,11β-Dihydroxyestra-1,3,5(10),8-tetraen-17-one (**58**)	420′(3.95)	470 (4.22)	495′(4.11)			
3-Methoxyestra-1,3,5(10),8,14-pentaen-17-one (**59**)	444′(3.75)	474 (3.84)				
3-Hydroxyestra-1,3,5(10),6,8-pentaen-17-one (**60**)				510 (4.36)		101
9,10-Dihydro-7-methoxy-3′-methyl-1,2-cyclopentenophenanthrene (**61**)						
Methyl-9,10-dihydro-7-methoxy-3′-methyl-1,2-cyclopentenophenanthrene-6-sulfonate (**62**)						
7-Methoxy-3′-methyl-1,2-cyclopentenophenanthrene (**63**)						
9,10-Dihydro-7-methoxy-3′-methyl-1,2-cyclopenta-3′-dienophenanthrene and 9,10-Dihydro-7-methoxy-3′-methyl-1,2-cyclopenta-4′-dienophenanthrene (**64**)						
Estra-5(10),6,8-triene-3β,17β-diol (**65**)	416 (4.10)					
Estra-5(10),6,8-triene-3α,17β-diol (**66**)	416 (4.10)					
17β-Hydroxyestr-4-en-3-one (**67**)	398 (3.84)	465 (3.81)		515 (3.66)		22
17β-Hydroxyandrost-4-en-3-one (**68**)	416 (3.87)		483 (3.87)		593 (3.74)	
10β-Acetoxyestra-1,4-diene-3,17-dione (**69**)				515 (4.14)		66
17β-Hydroxy-17α-methylestr-4-en-3-one (**70**)	384 (3.71)		490 (3.33)			
Androst-5-ene-3β,17β-diol (**71**)	384 (4.02)	410 (3.97)	465 (3.48)			
17α-Methylandrost-5-ene-3β,17β-diol (**72**)	388 (3.54)	415 (3.44)	497 (3.51)			
3α-Hydroxy-5α-androstan-17-one (**73**)	390′(3.92)	410 (3.94)				
3β-Hydroxy-5α-androstan-17-one (**74**)	384′(4.06)	410 (4.14)				
5α-Androstane-3,17-dione (**75**)	380 (3.61)	412 (3.47)				
3α-Hydroxy-5β-androstan-17-one (**76**)	390′(4.00)	410 (4.04)				
17β-Hydroxy-17α-methyl-5α-androstan-3-one (**77**)	383 (3.78)					
Pregn-4-ene-3,20-dione (**78**)	388 (3.00)	480 (2.85)				
3β-Hydroxypregn-5-en-20-one (**79**)	400 (3.57)					
17α,21-Dihydroxypregn-4-ene-3,11,20-trione (**80**)	415 (3.48)					
11β,17α,21-Trihydroxypregn-4-ene-3,20-dione (**81**)	422 (3.93)					
11β,17α,21-Trihydroxypregna-1,4-diene-3,20-dione (**82**)	418 (3.75)					
Cholest-5-en-3β-ol (**83**)	415 (3.52)					
3-Methoxy-10-mtehyl-9,10-secoestra-1,3,5(10)-triene-11,17-dione (**84**)						
3-Methoxy-16,17-secoestra-1,3,5(10)-triene-16,17-dioic acid (**85**)						
3-Hydroxy-16,17-secoestra-1,3,5(10)-trien-17-oic acid (**86**)						
1-Methoxy-4-propenylbenzene (**87**)	418 (3.32)					
6-Methoxy-1,2,3,4-tetrahydronaphthalene (**88**)						
6-Methoxy-1-tetralone (**89**)						
2,3,3a,4,5,9bβ-Hexahydro-7-methoxy-3aα-methyl-1*H*-benz[e]inden-3α-ol (**90**)	430 (4.34)					
3β-Ethyl-2,3,3a,4,5,9bβ-hexahydro-7-methoxy-3aα-methyl-1H-benz[e]inden-3α-ol (**91**)	428 (3.48)					
3β-Ethinyl-2,3,3a,4,5,9bβ-hexahydro-7-methoxy-3aα-methyl-1*H*-benz[e]inden-3α-ol (**92**)	445 (4.00)					
1,2,3,4,4a,9,10,10aα-Octahydro-6-hydroxy-1α,4aβ-dimethyl-1-phenanthrenecarboxylic acid (**93**)						
3α-Acetoxy-1,2,3,3a,4,5,8,9,9a,-9bβ-decahydro-3aα-methyl-7*H*-benz[e]inden-7-one (**94**)	393 (4.18)	430′(3.48)		493 (2.48)		

a) Figures having apostrophe and those in parentheses represent the wave-lengths at the shoulder and the apparent extinction coefficients in logarithmic expression, respectively.

b) Estradiol indexes were calculated by the following equation: E.D. index$=\varepsilon'_S/\varepsilon'_E\times100$
where ε'_S: apparent extinction coefficient at the absorption maximum of the longest wave-length given by the sample and ε'_E: apparent extinction coefficient at 515 nm given by the standard estradiol.

positive, its dehydrogenated derivatives (**32** and **33**) thus produced intensive color more readily than estradiol methyl ether (**7**). When oxygen function is present at C_{16}, 16-hydroxy- (**35**) as well as 16-keto-17-hydroxysteroid (**37**) were both positive and 16-keto- (**36**) and 16,17-diketosteroid (**40**) were slightly positive and entirely negative, respectively; these are in agreement with the findings of Jones, *et al.*[10] and Marlow.[11] The estrane derivatives, in which ring D has nitrogen function, such as 17-acetamide derivative (**44**), 17-oxime (**43**), and heterocyclic compounds (**45** and **46**), were all negative. It was also of interest that D-homoestrone (**47**) gave slightly positive reaction.

4) Angular Methyl Group at C_{13}

With the fact that 18-norestradiol and 18-norestrone were nearly and entirely negative, respectively, Jones, *et al.*[10] pointed out the angular methyl group at C_{13} to be essential to the positive Kober reaction; the methyl migration from C_{13} to C_{17} occurred simultaneously with the dehydration.[12,17] Miesher, *et al.*[18] reported that the pseudo-androstene derivative (3β-formyloxy-17-methyl-18-nor-5α-androst-13(17)-ene) was an essential intermediate in the chromogenic reaction of 5α-androstane-3β,17α-diol with formic acid. Such intermediary olefins were also found in the reactions of testosterone (17β-hydroxyandrost-4-en-3-one) (**68**) with Brønsted acids.[19] The requirement that oxygen function and angular methyl group should be present in ring D for the positive Kober reaction seems to be explained by that they are both indispensable for the formation of such olefins. It is of interest that 17,17-dimethyl-18-norsteroid (**51**) and the compound **50** which is known to give **51** through acid catalysis were found to give fairly small E.D.-indexes. Formation of the olefinic 17-methyl-18-norsteroid having the tertiary 17-carbon atom, instead of those being secondary (18-norstradiol and 18-norestrone) or quartery (**50** and **51**), is thus essential to the positive reaction. This may be of great importance on elucidating the chemical structure of the pink Kober chromophore.

5) Angular Hydrogen Atom

E.D.-indexes were larger in the 17-carbonyl compounds (**3**, **6**, and **19**) than those in their corresponding 17-hydroxy compounds (**1**, **2**, **7**, **8**, and **21**). The steroids (**52**—**57**) having more double bond or oxygen function (s) in rings B, C, and D showed, with several exceptions, smaller indexes than those due to estradiol (1) and estrone (3). The more highly dehydrogenated compounds (**59**, **61**—**64**) were entirely negative in the Kober reaction. These facts are likely to indicate that the proper oxidation state of the substrate retaining angular hydrogen atom (s) is required for producing the pink chromophore.

In the chromogenic reactions of steroidal olefins or hydroxysteroids with acids, it may be reasonable to assume that the carbocations[20] are produced at the earlier period through protonation and/or dehydration, which are then transformed into the chromophoric species. The initial formation of the carbocation (**95**) and its subsequent change to the pink chromophore have been observed in the Kober reactions of several steroids (**7**, **8**, **32** and **33**).[2b] Thus, it may be concluded that a steroid which comes into reaction with acid to produce the cation (**95**) can be positive for the Kober reaction and that the above-mentioned requirements (1—5) for the positive reaction correspond to those for the formation of the steroidal cation (**95**) in acidic medium. However, the steroids giving the positive reaction do not always produce the pink chromophore merely through the cation (**95**) and some other processes may be probable correspondingly with the oxidation states of the substrates. Although the chemical structure of this characteristic chromophore remains unknown, valuable informations on dissolving it seem to be obtainable in the present study.

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Experimental

Material——Of the compounds listed in Table I, those (**1**—**4**, **13**, **48**, **60**, **67**, **68**, **70**—**83**, and **87**) were obtained from the commercially available sources and were purified by the ordinary methods. The compounds (**18**—**25**, **38**, **52**—**56**, and **57**), those (**27**, **28**, **36**, **37**, and **58**), those (**65** and **65**), and those (**59**, **84**, **90**—**93**, and **94**) were kindly supplied by Dr. I. Yoshizawa, Dr. H. Mori, Prof. D.K. Banerjee, and Dr. T. Miki, respectively. The compounds (**6**—**12**, **14**, **15**, **31**, **43**, **50**, **51**, **88**, and **89**) were derived from the respective material commercially available and were purified by the ordinary methods. The compounds **5**,[21] **16**,[22] **17**,[23] **26**,[24] **29**,[25] **30**,[26] **32**,[27] **33**,[28] **35**,[29] **39**,[30] **40**,[31] **41**,[32] **42**,[32] **44**,[22a] **45**,[33] **46**,[34] **47**,[35] **49**,[36] **69**,[24] **85**,[37] and **86**[37] were prepared and purified according to the methods ever reported or their modifications. The methylethers (**61**—**63**) were obtained from the reaction-mixture of estradiol methylether and sulfuric acid.[2a] Preparation of **34** and **64** will be described in the following paper.

Kambegawa's Modified Method[16]——A solution of the sample (15—35 μg) in EtOH–CH_2Cl_2 was evaporated on a water-bath. To the dried sample thus obtained was added 2 ml of the reagent (2% (v/v) hydroquinone in 65% (v/v) H_2SO_4) and the mixture was then heated at 100° for 20 min. After the solution was cooled with running water for 5 min, 0.25 ml of water was added and it was heated again at 100° for 5 min. The reaction mixture was cooled with running water for 5 min and was then mixed well with 2 ml of 30% (v/v) H_2SO_4 for the spectroscopic measurement. Absorption spectra were taken on a Hitachi EPS-3T spectrophotometer.

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