The Separation of Steroid Alcohols by Chromatography of their Benzoates on Alumina

By R. V. BROOKS, W. KLYNE AND E. MILLER

Postgraduate Medical School, London, W. 12

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The separation of steroids by chromatography on alumina has been practised by nearly all workers in this field during the last 15 years (for a review, see Reichstein & Shoppee, 1949). Steroids containing hydroxyl groups have commonly been subjected to chromatography as free alcohols or as acetates. Recently, in work on the steroids of pregnant mares' urine, we were faced with the problem of separating epimeric 20-hydroxysteroids. These are almost impossible to separate by chromatography as alcohols, and difficult to separate as acetates (for references, see p. 215). We therefore attempted the chromatography of the benzoates and found that pairs of 20-epimeric benzoates could be separated almost quantitatively in one chromatogram. Other examples of the chromatography of steroid benzoates in the literature are the separation of 17 α - and 17 β -hydroxy derivatives of *D*-homosteroids (Goldberg & Wylder, 1943 and subsequent papers by the same school), of the 5 α :6 α -epoxy- and 5 β :6 β -epoxy-cholestan-3 β -ols (Barton & Miller, 1950) and of some steroid ketones lacking ring *D* (Cardwell, Cornforth, Duff, Holtermann & Robinson, 1951).

Since no systematic study of the chromatography of steroid benzoates had been made, we examined the behaviour of synthetic mixtures of a number of pairs of isomeric steroid benzoates of types likely to occur in work on natural products, especially urine. The results have shown that the chromatography of benzoates always offers at least as good a chance of separating isomers as does the chromatography of acetates, and sometimes a much better one. Turner & Voile (1951) have independently discovered the value of benzoates as derivatives for the separation of 20-epimeric hydroxysteroids.

A preliminary account of this work has been given to the Biochemical Society (Brooks, Klyne & Miller, 1951). The application of the method to the separation of the non-ketonic β -hydroxy steroids of mares' urine has been described by Brooks, Klyne, Miller & Paterson (1952).

Nomenclature. This follows the proposals of the Ciba Foundation Conference (1951). Since this work has been concerned with the separation of 5-epimeric steroids, among

others, it has seemed desirable to specify the configurations at C₍₅₎ explicitly in the names of all compounds—thus '5 β -pregnane-3 β :20 β -diol'—to distinguish this isomer from 5 α -pregnane-3 β :20 β -diol, although the name 'pregnane', according to the rules, implies the β -configuration at C₍₅₎.

EXPERIMENTAL

Melting points and optical rotations. Melting points and optical rotations were determined as described by Brooks *et al.* (1952). Melting points are all corrected. Optical rotations are all determined in CHCl₃ (c, 1–2 %) at 20–25°; experimental error for $[\alpha]_D$ approx. $\pm 1^\circ$. Samples for optical rotations and analyses were dried for 2 hr. *in vacuo* at 110° (except for compounds whose melting points were less than 140°, which were dried at 80°).

Analyses. These were by Weiler and Strauss, Oxford.

Starting materials. The hydroxysteroids used as starting materials for the preparation of benzoates and acetates were mostly gifts from colleagues to whom acknowledgements are made on p. 217.

Androstane-3 β :17 β -diol was prepared by reduction of the corresponding 17-ketone by LiAlH₄ (cf. preparation of pregn-5-ene-3 β :20 β -diol by Klyne & Miller, 1950). Pregn-5-ene-3 β :20 α -diol was obtained by reduction of the corresponding 20-ketone by Na and ethanol (cf. Wieland & Miescher, 1949) and separated from its 20 β -epimer by chromatography of the benzoates.

Preparation of benzoates. These were prepared by the use of benzoyl chloride and pyridine on the alcohols (cf. Brooks *et al.*, 1952). They were usually freed from traces of coloured impurities by passage in light petroleum-benzene through a short column of Al₂O₃. The physical properties of all the benzoates used and the analytical data for new compounds are summarized in Table 1. Most of the benzoates were recrystallized from CHCl₃:methanol (1:4, v/v). Exceptions are noted in Table 1. For comparison this table includes some data obtained by other workers on related compounds.

Chromatograms. These were carried out using Al₂O₃ (Savory & Moore Ltd.) of activity II–III (Brockmann & Schodder, 1941) and mixtures of light petroleum (b.p. 60–80°) and benzene of gradually increasing benzene content. All proportions of solvent mixtures are given as % (v/v). Standard proportions were steroid (1 g.), Al₂O₃ (60 g.), eluent (100 ml. fractions).

Most pairs of epimeric steroids were subjected to chromatography as acetates and as benzoates, using mixtures of about 15 mg. of each isomer. In a few cases the products of preparations were examined on a larger scale.

Table 1. *Properties of steroid benzoates*

(Values for $[\alpha]_D$ were determined in CHCl_3 and have been rounded off to the nearest 1° ; error $\pm 1^\circ$. Analyses are given for new compounds only. Data determined by other workers are in italics.)

Compound	Molecular formula	M.p. (corr.)	Crystalline form	[α] _D	[M] _D	Found		Required		Notes
						C (%)	H (%)	C (%)	H (%)	
<i>C₁₉ steroids: monobenzoates</i>										
3α-Hydroxy-5α-androstan-17-one 3β-Hydroxy-5α-androstan-17-one 3α-Hydroxy-5β-androstan-17-one 3β-Hydroxy-5β-androstan-17-one	C ₂₈ H ₄₈ O ₃	178°	Needles	+75°	+295°	—	—	—	—	(a)
		216°	Prisms	+69°	+272°	—	—	—	—	(b)
		164°	Needles	+98°	+387°	—	—	—	—	(c)
		243°	Prisms	+79°	+314°	78.8	9.0	79.2	8.7	—
3β-Hydroxyandrost-5-en-17-one 17α-Hydroxyandrost-4-en-3-one 17β-Hydroxyandrost-4-en-3-one	C ₂₈ H ₃₈ O ₃	257°	Prisms	+24°	+94°	—	—	—	—	(b)
		136–138°	Prisms	—	—	—	—	—	—	(d)
		193°	Prisms	+156°	+615°	—	—	—	—	(b)
<i>C₁₉ steroids: dibenzoates</i>										
5α-Androstane-3β:17β-diol Androst-5-ene-3β:17α-diol Androst-5-ene-3β:17β-diol	C ₃₃ H ₄₀ O ₄ C ₃₃ H ₃₈ O ₄	198–200°	Needles	+36°	+188°	79.1	7.9	79.2	8.1	—
		152°	Needles	–68°	–339°	—	—	—	—	(b)
		217°	Prisms	+13°	+65°	—	—	—	—	(b)
<i>C₃₁ steroids: monobenzoates</i>										
3β-Hydroxy-5α-pregnan-20-one 3β-Hydroxy-5β-pregnan-20-one 3β-Hydroxypregn-5-en-20-one 3β-Hydroxypregna-5:16-dien-20-one 3β-Hydroxy-16α-methoxypregn-5-en-20-one	C ₃₈ H ₅₆ O ₃ C ₃₈ H ₅₄ O ₃ C ₃₈ H ₅₄ O ₃ C ₃₉ H ₅₈ O ₄	197°	Prisms	+76°	+319°	—	—	—	—	(b)
		154–155°	Prisms	+80°	+338°	79.3	9.2	79.6	9.0	(e)
		197°	Leaflets	+40°	+168°	—	—	—	—	(b)
		171–172°	Needles	–9°	–38°	80.3	7.9	80.3	8.2	(f)
		156–157°	Prisms	–1°	–3°	77.6	8.6	77.3	8.5	(f, g)
<i>C₃₁ steroids: dibenzoates</i>										
5α-Pregnane-3β:20α-diol 5α-Pregnane-3β:20β-diol 5β-Pregnane-3α:20α-diol 5β-Pregnane-3β:20α-diol	C ₃₈ H ₅₄ O ₄	171–172°	Prisms	+27°	+139°	—	—	—	—	(h)
		237–239°	Needles	–10°	–53°	—	—	—	—	(h, i)
		131–133°	Prisms	+31°	+165°	79.3	8.2	79.5	8.4	(j)
		169–170°	Prisms	+23°	+123°	—	—	—	—	(k)
17α-Methyl-D-homoandrostane-3β:17αβ-diol (uranediol) Pregn-5-ene-3β:20α-diol Pregn-5-ene-3β:20β-diol	C ₃₈ H ₅₄ O ₄ C ₃₈ H ₅₄ O ₅	211–213°	Leaflets	+22°	+118°	—	—	—	—	(h)
		194–196°	Needles	+5°	+26°	80.1	8.2	79.8	8.0	—
		228–230°	Needles	–48°	–252°	80.0	7.8	—	—	—
5α-Pregnane-3β:17α:20α-triol	C ₃₈ H ₅₄ O ₅	216–217°	Prisms	–3°	–14°	77.5	8.1	77.2	8.1	—
<i>C₃₁ steroids: tribenzoates</i>										
5α-Pregnane-3β:16α:20β-triol Uranetriol	C ₄₃ H ₆₆ O ₆	211–213°	Needles	–14°	–91°	—	—	—	—	(h)
		234–235°	Needles	+81°	+524°	—	—	—	—	(h)

(a) Lieberman *et al.* (1948) give [α]_D + 3° in ethanol, which must be a misprint.
(c) Lieberman *et al.* (1948) give [α]_D + 111° in ethanol, [M]_D + 437°.
(f) These compounds were prepared from 3β-acetoxypregna-5:16-dien-20-one by hydrolysis with KOH in aqueous methanol, benzylation and chromatography (cf. Fukushima & Gallagher (1950, 1951), who prepared the corresponding acetates and discovered the nature of the reaction between steroid 16-en-20-ones and alcoholic alkali).
(g) Found: OCH₃, 7.9, 8.9. C₃₉H₅₈O₄ (1 OCH₃) requires 6.9%.
(h) Brook's *et al.* (1952).
(i) Pearlman (1946).
(j) Barton & Cox (1948).
(k) Recrystallized from methanol.
(l) Recrystallized from methanol, benzylation and chromatography (cf. Pearlman (1944)).

(a) Barton & Cox (1948).

(b) Recrystallized from methanol.

(c) Lieberman *et al.* (1948) give $[\alpha]_D + 3^\circ$ in ethanol, which must be a misprint. (d) Ruzicka & Kagi (1936).
 (e) Lieberman *et al.* (1948) give $[\alpha]_D + 111^\circ$ in ethanol, $[M]_D + 437^\circ$. (f) These compounds were prepared from 3 β -acetoxypregna-5:16-dien-20-one by hydrolysis with KOH in aqueous methanol, benzoylation and chromatography (cf. Fukushima & Gallagher (1950, 1951), who prepared the corresponding acetates and discovered the nature of the reaction between steroid 16-en-20-ones and alcoholic alkali).
 (g) Found: OCH_3 , 7.9, 8.9. $\text{C}_{39}\text{H}_{58}\text{O}_4$ (1 OCH_3) requires 6.9%.
 (h) Brooks *et al.* (1952).
 (i) Pearlman (1946).
 (j) Recrystallized from acetone.

Table 2. *Chromatography of 20-epimers*

Run	Eluent, light petroleum: benzene (% v/v)	Fraction no.	Eluate	
			Weight (mg.)	M.p.
<i>Chromatogram I. As benzoates.</i> (15 mg. each of 5 α -pregnane-3 β :20 α -diol dibenzoate and its 20 β -epimer. 1.8 g. Al ₂ O ₃ ; each run 3 ml. eluent.)				
1-7	90:10	IA	12.1	236-237°
8 and 9	90:10	IB	3.5	156-227°
10	85:15			
11-16	85:15	IC	13.0	166-169°
17-23	80:20			
24-26	70:30			
<i>Chromatogram II. As acetates.</i> (15 mg. each of 5 α -pregnane-3 β :20 α -diol diacetate and its 20 β -epimer. 1.8 g. Al ₂ O ₃ ; each run 3 ml. eluent.)				
1-6	90:10	IIA	7.4	140-142°
7-9	90:10	IIB	5.5	124-137°
10-12	85:15			
13-16	85:15	IIC	2.5	132-157°
17-21	80:20	IID	3.1	149-159°
22-25	70:30	IIE	8.5	160-163°
26-28	50:50			

Fraction IIE recrystallized from methanol gave 4.9 mg. (m.p. 166-168°). The melting points of the pure diacetates are as follows: 3 β :20 α -diol, 166-168°; 3 β :20 β -diol, 142-143°.

Table 3. *Chromatography of epimeric and related pairs of hydroxysteroids*

(Mixtures used for elution are all light petroleum:benzene and are described in terms of % benzene. 'Recovery x% pure' means that x% of each component was separated from the chromatogram in a state of purity such that the m.p. was only a few degrees below the m.p. of an analytically pure sample.)

Types <i>Epimers at</i>	Compounds	Result of chromatography	
		As acetates	As benzoates
C ₍₃₎	3 α - and 3 β -Hydroxy-5 α -androstan-17-ones	Good separation: 3 α -epimer eluted by 10-20% benzene; 3 β - by 17-100% benzene. Recovery 90% pure	Good separation: 3 α -epimer eluted by 20-30% benzene, 3 β - by 40-100% benzene. Recovery nearly 100% pure
C ₍₃₎	3 α - and 3 β -Hydroxy-5 β -androstan-17-ones	—	Good separation: 3 α -epimer eluted by 20-40% benzene, 3 β - by 40-100% benzene
C ₍₅₎	3 β -Hydroxy-5 α - (and 5 β -) androstan-17-ones	No apparent separation. Mixtures eluted continuously from 15-100% benzene	Partial separation: mixtures eluted by 25-100% benzene; 5 α -compound enriched in early fractions and 5 β -compound in later fractions
C ₍₅₎	3 β -Hydroxy-5 α - (and 5 β -) pregnan-20-ones	—	Partial separation: mixtures eluted by 5-100% benzene; enrichment of 5 α -compound in early fractions
C ₍₁₇₎	Androst-5-ene-3 β :17 α - (and 3 β :17 β -) diols (3-acetates)	Good separation: 17 α -epimer eluted by 10-20% benzene, 17 β - by 15-100% benzene. Recovery 80% pure	Good separation: 17 α -epimer eluted by 15% benzene, 17 β - by 20% benzene. Recovery 90% pure
C ₍₁₇₎	17 α - and 17 β -Hydroxyandrost-4-en-3-ones	—	Good separation: 17 α -epimer eluted by 35-50% benzene; 17 β -epimer eluted by 50-100% benzene. Recovery 85% pure
C ₍₂₀₎	Pregn-5-ene-3 β :20 α - (and 3 β :20 β -) diols	Partial separation: mixtures eluted by 15-40% benzene; enrichment of 20 β -compound in early fractions	Good separation: 20 β -epimer eluted by 10% benzene; 20 α - by 15-20% benzene. Recovery 90% pure
C ₍₂₀₎	5 α -Pregnane-3 β :20 α - (and 3 β :20 β -) diols	Fair separation. For details see Table 2	Good separation. For details see Table 2
C ₍₂₀₎	5 β -Pregnane-3 β :20 α - (and 3 β :20 β -) diols	—	Good separation: 20 β -epimer eluted by 15-20% benzene; 20 α -epimer eluted by 20-30% benzene

Table 3 (cont.)

Types	Compounds	Result of chromatography	
		As acetates	As benzoates
<i>Other pairs</i>			
5 α and Δ^5	3 β -Hydroxy-5 α -androstan-17-one and 3 β -hydroxy-androst-5-en-17-one	Poor separation: mixtures eluted by 20–100% benzene. Slight concentration of unsaturated compound in later fractions*	Poor separation: mixtures eluted by 40–100% benzene. Slight concentration of unsaturated compound in later fractions†
5 α and Δ^5	3 β -Hydroxy-5 α -pregnan-20-one and 3 β -hydroxypregn-5-en-20-one	—	Poor separation: mixtures eluted by 7–40% benzene. Slight concentration of unsaturated compound in later fractions‡
Δ^5 and $\Delta^{5:16}$	3 β -Hydroxypregn-5-en-20-one and 3 β -hydroxypregna-5:16-dien-20-one	—	Poor separation: mixtures eluted by 20–100% benzene. Slight concentration of 5:16-diene compound in late fractions§
17- and 20-Ketones	3 β -Hydroxy-5 α -androstan-17-one and 3 β -hydroxy-5 α -pregnan-20-one	Good separation: 20-ketone eluted by 10–30% benzene, 17-ketone by 20–100% benzene. Recovery 90% pure	Good separation: 20-ketone eluted by 15–30% benzene; 17-ketone by 20–100% benzene. Recovery 90% pure

* Fractions eluted by the same solvent mixture were combined and 1 mg. samples were treated with four drops of conc. H_2SO_4 . All the fractions developed an immediate yellow-brown colour; after 48 hr. the first fraction was brown and all the others purple-brown. Pure 3 β -acetoxyandrost-5-en-17-one gave an immediate orange colour with conc. H_2SO_4 (after 1 hr., orange-brown; 48 hr., violet). 3 β -Acetoxy-5 α -androstan-17-one under these conditions gave no immediate colour (after 1 hr., orange; 48 hr., reddish brown) (cf. Dirscherl & Zilliken, 1943; Paterson, 1947).

† Each solid fraction was treated with one drop of conc. H_2SO_4 ; all turned red in a few minutes. After 48 hr. early fractions were brown and later fractions blue. The pure benzoates gave the same H_2SO_4 colour reactions as the corresponding acetates.

‡ Conc. H_2SO_4 (four drops) was added to every fourth fraction. After 5 min. early fractions were yellow and late fractions red. Pure 3 β -benzoyloxy-5 α -pregnan-20-one gave a pale-yellow colour under these conditions and 3 β -benzoyloxy-5-en-20-one a dark-orange colour.

§ Selected fractions were dissolved in a mixture of two drops of $CHCl_3$ and three drops of ethanol. On addition of two drops of dinitrophenylhydrazine (1% in ethanol), early fractions gave a light-orange precipitate and late fractions gave a dark-orange precipitate. Pure 3 β -benzoyloxy-5-en-20-one gave a yellow precipitate in this test while the pure 5:16-diene gave a dark-orange precipitate.

RESULTS

Details of typical chromatograms of 20-epimeric diols as acetates and as benzoates are given in Table 2. The behaviour of the other pairs of compounds examined is described briefly in Table 3.

DISCUSSION

20-Epimeric hydroxysteroids. In all cases, the benzoates separated well, whilst the acetates separated only partially. Previous workers have found that 20-epimeric acetates can be separated only with difficulty by chromatography or crystallization (cf., for example, Reichstein & Gätzi, 1938; Hirschmann & Hirschmann, 1950a, b; Julian, Meyer, Karpel & Cole, 1951).

The separation of 20-epimers is the only example where chromatography of benzoates gives appreciably better results than chromatography of acetates; but since we have yet found no examples where the use of benzoates is disadvantageous it may be profitable to chromatograph the benzoates first in trying to separate mixtures of natural steroids of unknown composition.

3-Epimeric hydroxysteroids. These can be separated sharply by chromatography, either as acetates or benzoates. Although 3 α - and 3 β -hydroxysteroids are commonly separated by digitonin, this method is often attended with losses. Furthermore, recent work in this Department (Haslam & Klyne, 1952) extending some observations by Haslewood (1947) has shown that many 3 β -hydroxy-5 β -steroids are precipitated by digitonin only when present in high concentrations. The separation of such steroids from their 3 α -epimers could best be carried out by chromatography of their acetates or benzoates.

Some pairs of 3-epimeric hydroxysteroids have been separated by chromatography as alcohols (Shoppee (1946), 5 α -cholestan-3-ols; Ruzicka, Meister & Prelog (1947), 3-methyl-5 α -androstan-3-ols; Bridgwater & Shoppee (1952), 5 β -cholestan-3-ols (coprostanols)). In each case the 3 α -hydroxy epimer was eluted first.

5-Epimeric steroids. 5 α - and 5 β -Epimeric steroids are difficult to separate by chromatography as alcohols or as acetates (cf. for example, Reichstein & Lardon, 1941; Dobriner, Lieberman & Rhoads, 1948; Wenner & Reichstein, 1944; Lieberman, Dobriner, Hill, Fieser & Rhoads, 1948). The

Table 4. *Conformation of acyloxyl groups and order of elution of epimers from alumina*

(The symbols (e) and (p) indicate the conformation (equatorial or polar) of the acyloxyl groups concerned.)

Position of epimerism	Type of compound	Order of elution of acetates and benzoates
3	5 α -Androstan-3-ols	3 α (p) before 3 β (e)*
3	5 β -Androstan-3-ols	3 α (e) before 3 β (p)*
5	5 α - and 5 β -Androstan-3 β -ols	3 β (e): 5 α before 3 β (p): 5 β
17	Androstan-17-ols	17 α (p) before 17 β (e)*

* The same order of elution has been found for the free alcohols (Shoppee, 1946; Bridgwater & Shoppee, 1952; Elks & Shoppee, 1953).

benzoates give results which are a little better, but still disappointing. The chromatographic method used by Dingemans, Huis in't Veld & de Laat (1946) for the fractionation of urinary 17-ketosteroids permits sharp separation of 5-epimers (3 α -hydroxy-5 α (and 5 β)-androstan-17-ones) as the alcohols, but it could hardly be used for quantities larger than a few milligrams.

5 α -Saturated and Δ^5 -unsaturated steroids. The problem of trying to separate related steroids of these types often occurs in dealing with natural steroid mixtures, especially urinary extracts. Lieberman *et al.* (1948) have pointed out the difficulties of this separation (cf. also Seebeck & Reichstein, 1943).

Ruzicka & Prelog (1943) surmounted the difficulty by treating a mixture of saturated and unsaturated compounds (3 β -hydroxy-5 α -pregnan-20-one and 3 β -hydroxy-pregn-5-en-20-one from swine testis) with osmium tetroxide; the unsaturated compound gave a 5 α :6 α -diol, which could be readily separated from the unchanged saturated steroid, but which could not be reconverted into the Δ^5 -unsaturated compound.

The use of benzoates for chromatography offers little advantage over the use of the acetates.

17-Epimeric hydroxysteroids. These can be separated sharply by chromatography either as acetates or as benzoates. Elks & Shoppee (1953) have found that the epimeric androstan-17-ols can be separated as alcohols, the 17 α -epimer being eluted before the 17 β -epimer.

17-Ketosteroids and 20-ketosteroids. Related pairs of 3-hydroxy-17 (and 20)-ketosteroids can be separated sharply either as acetates or as benzoates. Lieberman *et al.* (1948) showed that such pairs of compounds in urinary extracts could be separated as alcohols, the 20-ketone being eluted first in each case.

Δ^{16} -20-Ketosteroids and the related 16:17-saturated compounds. One attempt at the separation of a pair of related compounds (3 β -hydroxypregn-5-en-20-one and its Δ^{16} analogue) by chromatography of the benzoates gave disappointing results. The conjugated 16-en-20-one was concentrated slightly in the later fractions of the chromatogram.

Conformation of acyloxyl groups and order of elution of epimers. The results of this work were considered in an attempt to correlate the conformation of acyloxyl groups in the steroid skeleton (cf. Barton, 1950; Barton & Rosenfelder, 1951) with the order in which the epimeric compounds were eluted from alumina (as acetates or benzoates). The results, summarized in Table 4, together with a few results from the free alcohols, show no regularity. Dr D. H. R. Barton has suggested to the authors that the lack of regularity here may be due to the fact that when compounds are adsorbed on to a solid surface, they may well take up conformations different from those which are preferred in solution. In contrast, the behaviour of steroid alcohols in partition chromatograms seems to show a consistent relationship to the equatorial or polar nature of the hydroxyl groups. Savard (1953) has shown that on paper partition chromatograms (using propylene glycol as stationary phase and light petroleum (b.p. 60–110°) or other saturated hydrocarbon as moving phase) the alcohols carrying polar hydroxyl groups move faster than their epimers carrying equatorial hydroxyl groups; examples tested include 3-epimers (both in the 5 α - and 5 β -series) 6-, 7-, 11- and 17-epimers. Presumably the more hindered polar hydroxyl groups in the steroids associate with the hydroxyl groups of the propylene glycol less than do the equatorial hydroxyl groups in the corresponding epimeric steroids.

SUMMARY

1. Different types of epimeric and related hydroxysteroids have been subjected to chromatography as acetates and benzoates.

2. Hydroxysteroids which are epimeric at C₍₂₀₎ can be separated readily by chromatography as benzoates, but not as acetates. Compounds epimeric at C₍₃₎, or at C₍₁₇₎ can be separated readily either as acetates or as benzoates.

3. Steroids epimeric at C₍₅₎ and some related pairs of steroids (a 5 α -saturated compound and the corresponding Δ^5 -unsaturated compound) cannot be separated easily either as acetates or benzoates.

4. Nine new steroid benzoates are described.

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Protein-bound Iodine in Extrathyroidal Tissues

BY E. A. CARR, JUN. AND D. S. RIGGS

Department of Pharmacology, Harvard Medical School, Boston, Massachusetts

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It has been shown that the concentration of protein-bound iodine in serum usually reflects the concentration of the thyroid hormone, increasing in hyperthyroidism and decreasing in hypothyroidism. Several reasonably satisfactory analytical methods have been developed in the past decade and give results which are in essential agreement. There is evidence that normally most of the protein-bound

iodine which is separable from inorganic iodide in serum by zinc precipitation is present as thyroxine (Taurog & Chaikoff, 1948; Man, Kydd & Peters, 1951).

The desirability of determining the concentration of a hormone not merely in the manufacturing gland or in the blood stream, but also in the peripheral tissues, has led to many attempts, beginning with