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The Use of Polarography in the Examination of Ketosteroids Related to Cholesterol

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Wolfe, Hershberg & Fieser (1940) were pioneers in the polarography of ketosteroids in body fluids by introducing a method for determining 17-ketosteroids in urine. Morris and his co-workers have made valuable contributions in this field culminating in a neat method, combining partition chromatography and polarography, for the examination of corticosteroids in human blood (Morris & Williams 1953). In all these methods direct or classical polarography was used. Derivative technique was introduced by Heyrovsky (1946) with a circuit modified to produced a sharp peak in a polarogram instead of a stepped wave. A derived polarogram differs from the conventional type in that the rate of change (di/dE) of diffusion current (i) with applied potential (E) is measured instead of the diffusion current itself. The result is a peak, representing maximum di/dE occurring at or very near the half-wave potential of the conventional polarogram. This results in an improved definition of the value of the half-wave potential. In favourable cases, the height of the derived wave is proportional to the concentration of reacting material. The method used here embodied a more convenient circuit described by Leveque & Roth (1949).

The technique of derivative polarography has been applied to the examination of C_{27} -ketosteroids. The ketonic fraction from the neutral matter of the faecal extracts of animals fed large and small amounts of added cholesterol was investigated (see Riddell & Cook, 1955). In addition, a number of ketonic bile acids and other ketosteroids have been studied. The work of Prelog & Häfliger (1949) on the polarography of *cyclohexanone* formed a valuable source of reference.

METHODS AND MATERIALS

Apparatus. A Tinsley Three-Unit Ink Recording Polarograph was used in conjunction with a Tinsley Derivative Unit. Two Muirhead Decade Resistance Boxes were introduced into the potentiometer circuit, one at each end of the auto-dial wire. By this means recordings could be restricted to selected voltage ranges and the rate of voltage application could be decreased. A switch was used to eliminate the positive part of the potentiometer winding when it was desired to use the resistance boxes. The principle of these alterations is shown in Fig. 1. A dip type, saturated calomel electrode, with connexion through agar, was used. The dimensions are not critical.

Fig. 1. Alterations to the polarograph potentiometer circuit.

All measurements were made with the polarographic cell immersed in a thermostatically controlled bath at $25^\circ \pm 0.2^\circ$. A dropping mercury electrode was used having the characteristics of 1.54 sec. for the drop time (t) and

2.69 mg. for the weight of mercury/sec. (m) delivered in distilled water. The mercury head was 35 cm.

Buffers. The use of buffered solutions was considered necessary, since the half-wave potentials ($E_{1/2}$) of all ketosteroids, free or as the betainyl hydrazones, alter with pH. The addition of ethanol to the buffer solution was found to be essential with the C_{27} -ketosteroids and enhanced the diffusion currents of C_{19} - and C_{21} -ketosteroids. Determinations for the free ketosteroids and for their betainyl hydrazones were carried out in an ethanolic (90%, v/v) buffer with diethylbarbituric acid (0.05M), LiCl (0.1M) and LiOH to pH 8.5. All pH measurements were made with a glass electrode using a Doran Dual Titrimeter calibrated with Cambridge Standard Buffer Tablets (pH 4 and 9.27) in aqueous solution as pH controls. The pH value chosen gave optimum results with cholestan-3-one and coprostan-3-one and worked satisfactorily with all other ketosteroids tested.

Reagents. The salts used in the buffer solutions were of 'Polaritan' grade (Hopkin and Williams). The diethylbarbituric acid was from pharmaceutical sources and was found to be of sufficient purity. The reagent T (trimethylammoniumacetohydrazide chloride; Girard & Sandulesco, 1936) was crystallized three times from ethanol (Morris & Williams, 1953), and the methanol for the Girard reaction was distilled from CaO (5%, w/v) with a little Zn dust present. Anhydrous acetic acid was prepared by adding acetic anhydride (5%, v/v) and distilling the dried acid.

Preparation of 2:4-Dinitrophenylhydrazones (2:4-DNPH). The 2:4-DNPH derivatives of coprostan-3-one and of cholestan-3-one were prepared by allowing the ketonic material to stand overnight in ethanol saturated with 2:4-dinitrophenylhydrazine and containing HCl (1%, v/v).

Chromatography

Attempts to separate the C_{27} -ketosteroids by partition methods were unsuccessful, but adsorption-elution chromatography was found to be effective.

Alumina. Chromatography on alumina was used to separate the ketosteroids from other matter in extracts of biological origin. Some separation of coprostan-3-one, cholestan-3-one and cholest-4-en-3-one from cholesta-3:5-dien-7-one and cholesta-4:6-dien-3-one was achieved. The alumina used was that of Savory and Moore (Brockmann Grade II) and ten times the weight of alumina to the material to be chromatographed was used.

Silica gel-Celite. Following the suggestion of Bretschneider (1941), who gives no experimental details, silica gel was used for the chromatography of free ketosteroids. A partition system has been used by Katzenellenbogen, Dobriner & Kritchevsky (1954) for the separation of highly polar ketosteroids with silica gel as support for the stationary phase. In the method described here a good separation of the 2:4-DNPH of coprostan-3-one and cholestan-3-one was achieved upon silica gel. The silica gel was prepared from commercial water glass (sodium silicate) diluted with water (4 vol.) by the addition of HCl (10N) with stirring. The granules were washed, dried at room temperature and passed through a sieve. One-tenth of the total weight of this preparation in Celite was added and the mixture dried at 37° in an air oven for upwards of 3 hr. before use.

Solvents. All solvents used in the chromatography were of Analytical Reagent grade (British Drug Houses) and were not further purified.

Measurement of derived diffusion current and half-wave potential

The height of the derived diffusion current peak (the maximum value of di/dE) is given in tables and figures in mm., measured on the record: the sensitivity of the galvanometer settings used (mm./ μA) are given. The symbol D has been adopted for this peak-height. This procedure is analogous to the method of measuring 'wave-span' for a direct polarogram (Wolfe *et al.* 1940). Calibration of the indicated potential values on the potentiometer dial in terms of potential drop through the cell circuit was carried out for a resistance setting of 700 Ω on the negative side and one of 300 Ω on the positive side of the potentiometer wire, covering a range of -0.9 to -1.9 v. The values of $E_{1/2}$ for Zn^{2+} (-1.02 v) and Mn^{2+} (-1.51 v) in aqueous N-KCl, with reference to the saturated calomel electrode (Kolthoff & Lingane, 1952), were used as standards, 0.01% gelatine being used as maximum suppressor.

Preparation of polarographic samples

Free ketosteroids. Free ketosteroids were examined as such after solution in the ethanolic buffer described. Pure samples were weighed, dissolved in 2 ml. of buffer which had been deoxygenated separately with N_2 , and gelatine added as maximum suppressor. Ketonic fractions from biological material were treated similarly; no maximum suppressor was found to be necessary.

Betainyl hydrazones. These were prepared from the free ketones in the buffer after polarography had been performed to detect reducing ketosteroids. The contents of the polarographic cell (2 ml.) were diluted with water (2 vol.) and extracted with light petroleum (b.p. 40-60°) in two lots of 10 ml. The residue from this extract was heated for 8 min. over a steam bath with 0.5 ml. of a solution of Girard T reagent (0.02 g.) in methanol (9 ml.) and acetic acid (1 ml.). The material resulting from this was dried under water-pump vacuum at room temperature, buffer (2 ml.) was added and the pH of the solution of betainyl hydrazones in buffer was adjusted before polarography to 8.5 ± 0.1 .

RESULTS

Characteristics of the derived polarogram. Figs. 2 and 3 demonstrate the main features of the derived polarogram. A mixture of 3-keto- Δ^4 -steroids and 17-ketosteroids was polarographed in the form of the betainyl hydrazones. Resolution of the points of inflexion of the polarogram is much improved, but the sensitivity has decreased to about one-fifth of the former level. A low sensitivity was used in the production of the direct polarograms in order to get both waves (the Δ^4 -3-keto wave at -1.37 v and the 17-keto wave at -1.68 v) on the same trace. For the same sensitivity setting the derived wave peak (di/dE) will have one-fifth to one-quarter the magnitude of the current (i). The loss of sensitivity is largely offset by the sharp definition of the maximum, increasing the precision of measurement.

The improved resolution obtained has made it possible to assign different values of $E_{1/2}$ to cholesta-

3:5-dien-7-one (-1.24 v) and cholesta-4:6-dien-3-one (-1.18 v) as the free steroids; this difference corresponds to 10 mm. on the voltage scale of the polarogram, -0.9 to -1.9 v, covering 180 mm.

Effect of pH. Fig. 4 shows the effect of pH on the derived diffusion current peak (D) and the half-wave potential ($E_{\frac{1}{2}}$) of cortisone as its Girard complex.

Fig. 2. Three types of polarogram made from a mixture of 3-keto- Δ^4 -steroids and 17-ketosteroids ($80 \mu\text{g./ml.}$ of androst-4-en-3:17-dione and $150 \mu\text{g./ml.}$ of 3β -hydroxy-androst-5-en-17-one) as Girard T derivatives. Polarogram A was made using the technique of Werthessen & Baker (1945); C was made using the derivative unit. Sensitivity setting for A and B, $20 \mu\text{A.}$; for C, $1 \mu\text{A.}$

Fig. 3. Derivative polarogram (A) of a mixture of 3-keto- Δ^4 - and 17-keto-steroids (see Fig. 2) after alteration of the instrument as indicated in Fig. 1 and of a mixture (B) of cholesta-3:5-dien-7-one ($60 \mu\text{g./ml.}$) and of cholest-4-en-3-one ($60 \mu\text{g./ml.}$). Sensitivity setting; $1 \mu\text{A.}$

Linearity of derived current-concentration curves. A linear relationship between D and concentration of C_{21} -ketosteroids was found from about 10^{-4} M to 10^{-3} M (i.e. between 40 and $360 \mu\text{g./ml.}$) at pH 8.5 in the ethanolic buffer described, both for the free steroids and for the betainyl hydrazones (see Fig. 5). A similar relationship was found for coprostan-3-one and cholestan-3-one betainyl hydrazones, the change in the magnitude of D being 55 mm. between 10^{-4} and 10^{-3} M for a sensitivity setting of $2 \mu\text{A.}$

Reproducibility of polarograms. Using the ethanolic buffer in the making of serial polarograms from the same sample, a very slight increase in the maximum took place due to evaporation of ethanol from the cell. If precautions are taken to keep the volume of liquid in the cell constant, serial polarograms can be superimposed and seen to be identical except for a slight, insignificant variation caused

Fig. 4. Effect of pH on the derived diffusion current peak (D) and half-wave potential ($E_{\frac{1}{2}}$) of cortisone betainyl hydrazone ($180 \mu\text{g./ml.}$).

Fig. 5. Variation in derived diffusion current peak (D) and half-wave potential ($E_{\frac{1}{2}}$) with concentration. Cortisone betainyl hydrazone; pH 8.5.

by, for example, an occasional irregular mercury droplet from the dropping electrode. Polarograms made at different times of the same material are subject to a slight variation in the magnitude of D which seldom exceeds 2-3 mm. for a sensitivity setting of $1 \mu\text{A.}$ Change in concentration and pH produced slight changes of $E_{\frac{1}{2}}$, but these were negligibly small under standardized conditions of measurement. When a suitable pH value has been chosen for measuring D or for making calibration curves of D against concentration for quantitative estimations (e.g. pH 6.5 for cortisone at which the change in D is minimal for a slight change in pH), the slight change of $E_{\frac{1}{2}}$ with D presents no difficulty.

Sensitivity. About 30 $\mu\text{g./ml.}$ of a ketosteroid can be detected and estimated (error $\pm 10\%$) using the derivative technique described in this paper.

Free steroids and their betainyl hydrazones

In Table 1 are given the values for the half-wave potentials and derived diffusion currents of a number of ketosteroids in the free state and of their betainyl hydrazones.

range from -1.64 v (11-dehydrocorticosterone) to -1.77 v (5 α -pregnane-3 β -ol-20-one). These ranges do not overlap and it is thus possible to determine the position of the reactive group from the E_1 value.

For the betainyl hydrazones double waves were obtained with testosterone, cholest-4-en-3-one, progesterone, corticosterone and 11-dehydrocorticosterone. The first wave in the region of -1.34 v can be attributed to a reactive group in the 3-

Table 1. *Half-wave potentials and maximum derived diffusion currents of various free ketosteroids and of their betainyl hydrazones*

90% ethanolic barbitone buffer, pH 8.5.

Ketone	Concen- tration ($\mu\text{g./ml.}$)	Half-wave potentials E_1 (v)		Maximum derived diffusion current height D (mm.)		Reactive groups
		Free steroid	Betainyl hydrazone	Free steroid	Betainyl hydrazone	
C₁₉-ketosteroids						
3 β -Hydroxyandrost-5-en-17-one (Dehydroepiandrosterone)	116	—	-1.55	—	18	17-keto
Androst-4-en-17 β -ol-3-one (Testosterone)	116	-1.49	-1.34 -1.45	14 —	18 10	4-en-3-keto
C₂₁-ketosteroids						
Pregn-4-en-3:20-dione (Progesterone)	170	-1.45	-1.34 -1.64	18 —	25 25	4-en-4-keto 20-keto
3 β -Hydroxypregn-5-en-20-one (Pregnenolone)	170	—	-1.57	—	21	20-keto
3 β -Hydroxy-5 α -pregnan-20-one	175	-1.77	-1.51	25	36	20-keto
11 β :21-Dihydroxypregn-4-en-3:20- dione (Corticosterone)	180	-1.48 -1.68	-1.35 —	18 14	23 —	4-en-3-keto 20-keto
21-Hydroxypregn-4-en-3:11-20- trione (11-dehydrocorticosterone)	180	-1.49 -1.64	-1.37 -1.70	18 14	32 25	4-en-3-keto 20-keto
17:21-Dihydroxypregn-4-en- 3:11:20-trione (Cortisone)	180	-1.44 -1.65	-1.34 —	18 18	25 —	4-en-3-keto 20-keto
11 β :17 α :21-Trihydroxypregn-4- en-3:20-dione (Hydrocortisone)	180	-1.50	-1.42	20	25	4-en-4-keto
C₂₇-ketosteroids						
Coprostan-3-one	194	—	-1.34	—	34	3-keto
Cholestan-3-one	194	—	-1.44	—	25	3-keto
Cholest-4-en-3-one	192	-1.55	-1.34 -1.43	26 —	16 4	4-en-3-keto —
Cholesta-3:5-dien-7-one	190	-1.24	—	32	—	3:5-dien-7-keto
Cholesta-4:6-dien-3-one	190	-1.18	-1.12	27	13	4:6-dien-3-keto
3 β -Acetoxycholest-5-en-7-one	Approx. 200	—	-1.34	—	16	5-en-7-keto

With the free steroids the E_1 values occur within a narrow range for the particular reactive group irrespective of the type of steroid molecule. Thus the 4-en-3-keto group gives values between -1.44 v (cortisone) and -1.55 v (cholest-4-en-3-one). The values for the two dienones are -1.24 v (cholesta-3:5-dien-7-one) and -1.18 v (cholesta-4:6-dien-3-one). Values for the 20-keto groups

position. The second wave in testosterone at -1.45 v and in cholest-4-en-3-one at -1.43 v is probably due to the unsaturated bond in the 4:5-position, as this wave is absent in polarograms of betainyl hydrazones of saturated 3-ketosteroids and is much extended in the polarogram of cholesta-4:6-dien-3-one betainyl hydrazone. The only wave present in the polarogram of coprostan-3-one

betainyl hydrazone (-1.34 v) is due to a reaction in the hydrazine radical. The introduction of one double bond (4-5) into the molecule to give cholest-4-en-3-one betainyl hydrazone causes the appearance of a double-peaked wave (-1.34 and -1.43 v). The introduction of two double bonds (4-5, 6-7) to give cholesta-4:6-dien-3-one betainyl hydrazone causes the appearance of a double-peaked wave (-1.33 and -1.12 v). When derived polarograms of the free compounds are made, a single wave is obtained for each with the exception of coprostan-3-one which does not react within the region 0 to -1.80 v. The wave for cholest-4-en-3-one (-1.55 v) is 0.12 v more negative than the second peak from the betainyl hydrazone at -1.43 v, and that for cholesta-4:6-dien-3-one (-1.18 v) is 0.06 v more negative than the second peak from the betainyl hydrazone at -1.12 v. The above data suggest that the second peak in polarograms of the betainyl hydrazones and the single wave in polarograms of the free ketosteroids mentioned above (coprostan-3-one excepted) arises from a reaction at the unsaturated bond or bonds, the position of the half-wave potential being influenced by the group attached at position 3. The wave forms of different compounds are characteristic. The double-peaked wave from the 3-keto-4-ene grouping as in polarograms of the betainyl hydrazones of testosterone and cholest-4-en-3-one becomes a single-peaked wave in polarograms of the betainyl hydrazones of 3:20-diketo-4-ene compounds, the second peak being absorbed by the large 20-keto wave.

All the ketosteroids containing a 20-keto group yielded a discrete peak for this grouping either as the free steroid or as the betainyl hydrazone. These included the 17:21-dihydroxy-20-ketosteroids, cortisone and hydrocortisone (both as the betainyl hydrazones). The derived diffusion current from these two ketosteroids produced by the 20-keto group does not rise so steeply as for other 20-ketosteroids and will merge into the wave due to excess of Girard T reagent, if the concentration of this is more than about 0.05 mg./ml. The free ketosteroid

cortisone yielded a wave at -1.65 v for the 20-keto group, but that of hydrocortisone ran into the large supporting electrolyte wave and did not form a discrete peak. (See Wolfe *et al.* 1940; Morris & Williams, 1953).

Separation and estimation of coprostan-3-one and cholestan-3-one. These C_5 -epimeric saturated C_{27} -ketosteroids have different values of E_1 when estimated separately as the betainyl hydrazones. In a mixture of the two the waves overlap and one wave only appears. It was not found possible to resolve the waves of the two compounds by a change in pH or buffer composition or by reducing the rate of voltage application. Preliminary separation was therefore necessary.

To separate a mixture the ketones were converted into 2:4-DNPH derivatives, and pyruvic acid was added to take up the excess of the reagent. The solution of hydrazones was dried at reduced pressure at the water pump at room temperature and it was chromatographed on silica gel-Celite, using light petroleum (10):ethyl ether (1) as eluent. The pyruvic acid 2:4-DNPH remains at the top of the column but the ketosteroids separate well. Coprostan-3-one 2:4-DNPH emerges first. The amounts of the two C_{27} -ketosteroid 2:4-DNPH eluted from the column were estimated by weighing on a microbalance after drying off at room temperature at the water pump. Polarography of the C_{27} 2:4-DNPH derivatives was attempted. This was done in order to follow the elution of the hydrazones and was carried out in a mixture of water:ethanol:xylene (10:90:25 by vol.). This mixture contained LiCl (0.1 M) and 10N-HCl (1%, v/v). The xylene was necessary for complete solution of the hydrazones. E_1 values for the second peak in the derived polarogram were: coprostan-3-one 2:4-DNPH, -0.47 v; cholestan-3-one 2:4-DNPH, -0.62 v. The polarograms were well defined, and it was found possible to obtain separate waves, when both hydrazones were present in a mixture.

Ketonic bile acids and their esters. Pure samples of esters of ketonic bile acids yielded the results presented in Table 2. When the same experimental

Table 2. *Half-wave potentials and maximum derived diffusion current heights of some ketonic bile acids*

90% ethanol with LiCl 0.1 M, and the betainyl hydrazones of their methyl or ethyl esters in 90% ethanolic buffer, pH 8.5.

pH 8.5.

Ketonic bile acid or ester	Concentration (μ g./ml.)	Half-wave potentials E_1 (v)		Maximum derived diffusion current D (mm.)		Reactive groups
		Free acid	Betainyl hydrazone of ester	Free acid	Betainyl hydra- zone	
Ethyl 7:12-dihydroxy-3-oxocholanoate	20	—	-1.34	—	16	3-keto
12-Hydroxy-3:7-dioxocholanic acid	40	-1.33	No wave	36	—	Carboxyl
7-Hydroxy-3:12-dioxocholanic acid	40	-1.44	No wave	36	—	Carboxyl
Methyl 7:12-diacetoxy-3-oxocholanoate	1000	—	-1.50	—	25	3-keto
Methyl 12-hydroxy-7:11-diacetoxy-3-oxocholanoate	1000	—	-1.32	—	18	3-keto

conditions were used as for the betainyl hydrazones of the non-carboxylated ketosteroids, the 3-keto group only was manifest, but it failed to give a wave when other groups were present. Thus, the introduction of a 7- or 12-keto group instead of an hydroxy group or groups suppressed the 3-keto wave. Polarography of the free ketonic bile acids in 90 % ethanolic solution containing LiCl (0.1M) yielded E_1 values which varied with the nature of the attached groups.

Experiments using biological materials

Simple mixtures of ketosteroids may be determined polarographically, but in a complex lipid extract chromatography of the free steroids or of their 2:4-DNPH derivatives is necessary to separate and estimate the components. The method described has been used to determine the ketosteroid content of faecal unsaponifiable matter (UM) of rats and of humans fed diets low and high in cholesterol (see Riddell & Cook, 1955; Cook, Edwards & Riddell, 1956). Some experiments have been made on the ketones present in lipid extracts from atheromatous human arteries. The following illustrative examples are given.

Fig. 6. Polarograms of cholestan-3-one 2:4-DNPH using the derivative technique. (A, pure substance, 125 μ g./ml.; B, sample from rat faeces.)

Rat faecal ketones

A fraction (0.94 g.) giving a strong Zimmermann test, from the chromatography on alumina of the faecal UM of rats fed a diet rich in cholesterol, was treated with Girard's reagent and yielded ketones (0.08 g.) and non-ketones (0.82 g.).

Ketonic fraction. A sample of the ketones (3.3 mg.) was dissolved in the ethanolic buffer solution (2.0 ml.) and polarographed as the free ketosteroids. A wave characteristic of cholest-4-en-3-one was obtained, equivalent to 0.095 mg./ml. This gave a value of 0.5% cholest-4-en-3-one in the original fraction, or 4.75 mg. in 0.94 g. After treatment with Girard T reagent a large coprostan-3-one wave developed (equivalent to 5.5 mg./ml. or 67 mg. in 0.94 g. of ketonic material).

In order to make an estimation of cholestan-3-one and coprostan-3-one the remainder of the ketonic fraction (78.7 mg.) was treated with 2:4-dinitrophenylhydrazine and the hydrazones were chromatographed on alumina when five bands developed. The light petroleum-soluble fraction which formed the greater part was rechromatographed on silica gel-Celite with benzene when two fractions were obtained. The first fraction (37 mg., corresponding to 15 mg. of free ketosteroid) was examined by polarography (concentration approx. 200 μ g./ml. in the cell) and gave the polarogram marked B in Fig. 6. Pure cholestan-3-one 2:4-DNPH (125 μ g./ml.) gave the polarogram A in this figure.

The second fraction weighed 117 mg. ($\equiv$ 77 mg. of free ketosteroid) and a concentration of approximately 300 μ g./ml. in the polarographic cell gave the polarogram marked B in Fig. 7. Polarogram A in this figure was yielded by a concentration of 125 μ g./ml. of coprostan-3-one 2:4-DNPH.

Fig. 7. Polarograms of coprostan-3-one 2:4-DNPH using the derivative technique. (A, pure substance, 125 μ g./ml.; B, sample from rat faeces.)

When polarographed without pre-treatment with Girard T reagent, the non-ketonic fraction yielded the wave corresponding to cholesta-3:5-dien-7-one (1.4 mg. in 0.94 g. of ketonic material).

Ketosteroids in atheromatous arteries

An atheromatous human artery which had been freshly fixed in formalin as soon as possible after death, was kindly supplied by Professor A. C. Lendrum of the Pathology Department, Queen's College, Dundee.

A sample (50 g.) was washed with water, dried in a vacuum desiccator over CaCl_2 , extracted with ether and ethanol and yielded extract (1.46 g.). A sample of the extract (1.08 g.) was chromatographed on silica gel giving the following fractions: light petroleum (187 mg.), benzene (264 mg.), ether (279 mg.) and ethanol (137 mg.). The fractions were saponified and the unsaponifiable matter was recovered. The amounts were: light petroleum fraction (73 mg.), benzene (134 mg.), ether (195 mg.), ethanol (70 mg.). The benzene fraction gave a polarogram characteristic of cholesta-3:5-dien-7-one (approx. 200 μ g./ml.; equivalent to 3% of the UM). The ether fraction gave a polarogram of cholesta-4:6-dien-3-one ($\sim$ 30 μ g./ml.).

The presence of the dienone cholesta-3:5-dien-7-one in atheromatous arteries has been reported by Hardegger, Ruzicka & Tagmann (1943) and by Kantiengar & Morton (1955).

DISCUSSION

The value of polarography in the examination of ketosteroid material from biological sources has been well established by Morris & Williams (1953). In their work on the corticosteroid constituents of peripheral human blood these two workers used direct polarography in conjunction with partition chromatography. It was not found possible, in the course of the work described in this paper, to find an effective partition system for the separation of the C_{27} -ketosteroids. The resolving power of the polarograph was increased as far as possible by the use of a derivative unit and reduction of the rate of applied voltage.

When derivative polarography was used in place of the classical or direct method, it was found that a clearer indication was given of the position and number of the carbonyl groups in ketosteroids. The position of the half-wave potential was clearly defined and the magnitude of the derived diffusion current maximum was much easier to determine. It was found that two or more waves of large magnitude could be recorded on the same paper at a high sensitivity setting without loss of continuity. This is not possible with the conventional stepped wave, as two or more successive waves will mount up out of the upward limit of the record and the polarogram must be divided. Mixtures giving badly defined direct polarograms appearing as an undulating line will often yield clear peaks and derived diffusion currents on derivative polarography.

Compared with conventional methods there is some loss in sensitivity when the derivative method employing a large capacity electrolytic condenser is used. A loss of sensitivity is inherent in the method since a derived wave has a magnitude less than half that of its underived equivalent. With the Tinsley instrument this loss of sensitivity was 80% but it was never found necessary to use the highest-sensitivity setting ($0.2\mu A$) for ketosteroid estimations.

The saturated ketones cholestan-3-one and coprostan-3-one yield satisfactory waves only as the betainyl hydrazones within narrow limits of pH and require an organic solvent in the buffer solution. The choice of a buffer for general use was determined by these considerations.

A method for the examination of ketosteroids in biological material could be based on the chromatography of their 2:4-DNPH derivatives and subsequent polarography of the fractions. 2:4-Dinitrophenylhydrazine is a more sensitive reagent

than Girard T and the hydrazones are more easily prepared and separated. The application of heat is unnecessary. 2:4-DNPH derivatives are sometimes obtained from lipid extracts which have yielded no polarographic wave for betainyl hydrazones. The labile betainyl hydrazones of coprostan-3-one and cholestan-3-one cannot be chromatographed without decomposition, but the 2:4-DNPH derivatives form visible, sharp bands on alumina and particularly on silica gel. Paper partition chromatography can also be applied.

Disadvantages of polarography of 2:4-DNPH lie in the decreased sensitivity of the estimations possible as compared with the betainyl hydrazones. Using a derivative method as described, the threshold concentration is in the region of $100\mu g./ml.$ in terms of free ketosteroid. The wave form is complicated; three peaks appear on the polarogram and only one is useful for examination.

When 90% aqueous dioxan with LiCl ($0.1M$) is used for the polarography of coprostan-3-one and cholestan-3-one 2:4-DNPH, the wave useful for estimations is increased in magnitude, while the others decrease. A complexing reagent is required for the chromatographic separation of ketosteroids of low polarity which would form compounds more stable than the betainyl hydrazones, while still possessing their excellent polarographic characteristics.

It was found that the 20-keto group in C_{21} -ketosteroids showed up well under the working conditions chosen, though a lower pH value than 8.5 is recommended.

SUMMARY

1. A standard type of recording polarograph has been modified and improved polarograms were obtained.

2. A buffer with an ethanol content of 90% (v/v) has been found to facilitate polarography of C_{19} -, C_{21} - and C_{27} -ketosteroids in the free state and as the betainyl hydrazones (Table 1). Some ketonic bile acids were also investigated (Table 2).

3. A method for the examination of C_{27} -ketosteroids in biological material has been developed.

4. The separation of cholestan-3-one and coprostan-3-one as 2:4-dinitrophenylhydrazones is described.

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The Nature of the Protein in the Hyaluronic Complex of Bovine Synovial Fluid

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Ogston & Stanier (1950, 1951, 1952) found that the hyaluronic acid of ox synovial fluid is always combined with 25–30% of protein when it is isolated by ultrafiltration under such conditions that its physicochemical properties are unchanged. Approximately the same amount of protein accompanies hyaluronic acid migrating by electrophoresis, sedimentation or diffusion. The protein complex is considered to be the form in which the hyaluronic acid occurs in the native fluid, since removal of the protein leads to changes in physicochemical properties of the material. Relatively little is known of the origin or composition of the protein, although Humphrey & Stanier (unpublished) found that the hyaluronic acid complex gave antisera in the rabbit which cross-reacted with some of the components of ox blood serum. Since, apart from its hyaluronic acid content, synovial fluid has the composition of a plasma exudate, similar to lymph and ascitic fluid (Hesslevik, 1940), it appears that at least some of the proteins in the hyaluronic acid complex could be derived from the free proteins of the synovial fluid. It was considered desirable to confirm this conclusion by isolation and characterization of the protein and this is described in this paper.

MATERIALS AND METHODS

Ox synovial fluid. This was collected and stored according to the methods of Ogston & Stanier (1950).

Hyaluronic acid complex. This was prepared as an ultrafiltrate residue using a sintered-glass '5 on 3' filter, average

pore diameter 1 μ . (Ogston & Stanier, 1952). The residue was taken up in phosphate buffer (pH 7.0, $I=0.1$) and the small amount of insoluble material was removed by centrifuging at 15 000 g for 20 min. Where studies were made on the ultrafiltrate this included the initial filtrate and the first five washings, concentrated by freeze-drying, if necessary.

Buffers. The following buffers were used, diluted to the desired concentrations:

pH 5.8	0.53M-KH ₂ PO ₄ , 0.056M-KOH
pH 7.0	0.50M-KH ₂ PO ₄ , 0.177M-KOH
pH 8.0	0.50M-KH ₂ PO ₄ , 0.47M-KOH
pH 10.0	0.62M glycine, 0.62M-KCl, 0.38M-KOH.

Physiological saline. This was 0.155M-NaCl. All solutions were saturated with chloroform as an antiseptic precaution.

Hyaluronidase. Benger hyaluronidase (Hyalase) was used. This preparation has been found to be free from proteolytic activity (Ogston & Stanier, 1952). Immunological analysis showed that it contained 0.8 mg. of substances reacting with antialbumin per mg. of N.

Ox serum fractions. These were prepared by an extension of the low-temperature ethanol precipitation method used by Hess & Deutsch (1948) to isolate γ -globulin from ox serum. The techniques of fractionation followed those established by Cohn (Edsall, 1947). Details of the fractionation are given in Fig. 1. Where no exact temperature is marked the value is in the range 0–1°. pH values were adjusted with 0.05N acetic acid or 0.05M-Na₂HPO₄. Ethanol-containing solutions were diluted 5 times with distilled water before their pH values were measured with the glass electrode. Each precipitate was washed twice with 500 ml. of fluid having the same pH and salt and alcohol concentration as its supernatant. All the fractions were dissolved in and dialysed against physiological saline and were stored as frozen 3% stock solutions. After one cycle of freezing and thawing it was found that some lipid material separated from F3. This was discarded. The fractions, with the exception of F5, gave single peaks on

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