ing 100 Gm. of diethyl-(3-cyclohexenal)-malonate. The mixture was refluxed for 6 hr. The color of the solution turned red. Distillation of the solvents under reduced pressure followed using a flash evaporator. The residue was dissolved in cold distilled water. The solution was extracted twice with ether. The aqueous layer was adjusted to pH 7 and then it was extracted with ether once more. The water layer was acidified with 3 *N* hydrochloric acid. White crystals resulted which were filtered, washed with distilled water, filtered, and then washed with a 50:50 mixture of ether–petroleum ether, m.p. 111°–113°. Recrystallized 4 times from absolute alcohol, m.p. 133°–137°.

Anal.—Calcd. for $C_{18}H_{24}O_6$: C, 64.26; H, 7.19. Found: C, 64.45; H, 7.32; C, 64.45; H, 7.19.

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Comparative Study of the Alternating and Direct Current Polarography of Several Δ^4-3-Ketosteroids

By JAMES L. SPAHR and ADELBERT M. KNEVEL

A study was undertaken to compare alternating current polarography with direct current polarography as a method of analysis of testosterone, methyltestosterone, and progesterone. A solution consisting of 50 per cent ethanol, buffer (pH 1.3), and tetrabutylammonium iodide was used as the sample medium. Results showed that the lowest practical concentrations of detection for both a.c. and d.c. polarography was $3.3 \times 10^{-5}M$. However, a.c. polarography gave greater precision than did the d.c. method.

A STUDY OF the analysis of Δ^4-3-ketosteroids by direct current (d.c.) polarography has been reported by several groups of workers (1–3). In the study conducted by Kabasakalian and McGlotten (3), it was reported that the diffusion current of testosterone and other related Δ^4-3-ketosteroids was directly proportional to the concentration in the range of 2×10^{-4} to $1 \times 10^{-2}M$. At low concentrations, however, deviations from linearity were observed in some cases. These workers pointed out that the deviations may have been due to the method of measuring the diffusion current rather than a change in diffusion properties, because the diffusion current plateau at low concentration was too steep. This explanation seems reasonable since ill-defined polarographic waves are not uncommon with ketones. One factor contributing to this poor definition may be that ketone half-wave potentials occur very close to the discharge potentials of the buffer components. This effect often makes it difficult to separate ketone diffusion current from buffer discharge current. Alternating current (a.c.) polarography offers the advantage of producing polarograms in which the reduction waves of the ketone and buffer components are often sufficiently separated so that diffusion currents can be measured more accurately. Furthermore, this technique is often more sensitive to organic compounds than is d.c. polarography. The objective of this study was to compare a.c. polarography with d.c. polarography as a method of analysis for several different Δ^4-3-ketosteroids.

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EXPERIMENTAL

Apparatus.—The dropping mercury electrode capillary used in this study had a length of 9.3 cm. Under a pressure of 26.5 cm. of mercury and with an open circuit, the drop time was 4.86 sec. and *m* was 1.13 mg. $sec.^{-1}$. These characteristics were determined at 25° with the mercury dropping into 50% ethanol which was 0.1 *M* in tetrabutylammonium iodide.

The electrolysis cell was a tube 7 cm. in length with an inside diameter of 2.1 cm. The saturated calomel reference used throughout this work was contained in a Hildebrand half-cell. Junction be-

tween the sample solution and the reference was made with a bridge of 4% agar in saturated KCl.

The instrument used in this study was a Sargent polarograph model XXI (E. H. Sargent & Co., Chicago, Ill.) modified according to Miller (4) for a.c. polarography. During the course of this study it was found necessary to modify the Miller circuit by adding a downscale compensator. The reason for the modification was that the base current of a.c. polarography was somewhat larger than the corresponding residual current of d.c. polarography. Many times at high sensitivity settings the supporting electrolyte alone caused a pen deflection so large that the a.c. peak went off scale. The downscale compensator on the model XXI polarograph could not be used to bring the peak on scale, because it is inoperative during a.c. operation using the Miller modification. In this study it was found necessary to introduce a downscale compensator in the a.c. circuit since low concentrations of steroid required high sensitivity values. Although this accessory was introduced into the circuit specifically for this study, it should increase the value of the a.c. modification of the model XXI in cases where high sensitivity is required. The details of this circuit have been reported elsewhere (5).

Reagents.—The buffers used were those reported by Kabasakalian and McGlotten (3). The components were observed to be polarographically inert in the voltage range employed. The need for a medium of 50% ethanol was dictated by the limited solubility of the steroids. The ethanol was distilled from commercial absolute ethanol in the presence of sodium ethoxide and diethyl phthalate (6). This preparation was necessary because the commercial material contained some reducible impurities. Alkyl phenoxy polyethoxy ethanol[1] was used as the maximum suppressor. A polarographic grade of tetrabutylammonium iodide was used as the supporting electrolyte. All other reagents were of A.R. grade. The steroids studied were testosterone,[2] methyltestosterone,[3] and progesterone.[4]

Sample Preparation and Handling.—In most of this work 50% ethanol containing the buffer and supporting electrolyte was used as the sample medium. Although potassium chloride is the most commonly used supporting electrolyte in d.c. polarography, it was not satisfactory for this study. Preliminary a.c. polarograms using methyltestosterone in 50% ethanol with 0.5 *M* KCl revealed that ethanol produced a tensametric peak. This phenomenon has been described by Breyer and Hacobian (7). The peak appeared at a potential very close to the reduction potential of the steroid and completely obscured the steroid wave. Other common inorganic supporting electrolytes such as KNO_3 gave the same results. Organic quaternary ammonium compounds were then tested and found to eliminate the ethanol tensametric peak in the potential region of interest. The supporting electrolyte finally chosen for this study was tetrabutylammonium iodide.

Electrolysis solutions were prepared by pipeting the appropriate aliquot of a standard ethanolic steroid solution into a 10-ml. volumetric flask, adding 0.1 ml. of 0.2% alkyl phenoxy polyethoxy ethanol, sufficient ethanol to measure exactly 5 ml., a calculated amount of supporting electrolyte, and finally diluting to the mark with aqueous buffer. Since the volume of the aliquot varied with the final concentration of the steroid desired, it was necessary to add enough ethanol to measure exactly 5 ml. before diluting with buffer. Tetrabutylammonium iodide was added to the flask before diluting with the aqueous buffer because it is only slightly soluble in water. The concentrations of the aqueous buffers were twice the values desired so that after dilution by the ethanol the final buffer concentrations were those listed in Table I. Sample solutions were transferred to the electrolysis cell and deaerated with nitrogen for 15 min. During the deaeration period and during the polarographic run the sample container was partially immersed in a water bath held at 25° ± 0.1°. The d.c. run was made immediately after deaeration. The instrument was then switched to a.c. operation and the a.c. run was made. pH determinations of each solution were made after electrolysis. The pH of individual samples varied slightly (0.1–0.2 units) from the values listed in Table I.

TABLE I.—COMPOSITION OF BUFFERS IN 50% ETHANOL

pH		Buffer Components
1.3	0.100 *M* HCl	
2.9	0.100 *M* malonic acid	0.025 *M* KOH
3.8	0.100 *M* malonic acid	0.075 *M* KOH
5.3	0.075 *M* acetic acid	0.025 *M* NaOAc
5.6	0.050 *M* acetic acid	0.050 *M* NaOAc
6.1	0.025 *M* acetic acid	0.075 *M* NaOAc
6.9	0.100 *M* malonic acid	0.170 *M* KOH
8.7	0.100 *M* trimethylamine	0.075 *M* HCl
9.1	0.100 *M* trimethylamine	0.050 *M* HCl
9.4	0.100 *M* triethylamine	0.075 *M* HCl
9.9	0.100 *M* triethylamine	0.050 *M* HCl
10.3	0.100 *M* triethylamine	0.025 *M* HCl

pH Effect on Wave Form.—The object of this portion of the research was to determine the pH at which the polarographic wave had the form best suited for quantitative analysis. The judgment was made on the basis of peak height and form.

Alternating current and d.c. polarograms were recorded for all three steroids at all pH values. In order to assure that observed effects were due to pH it was necessary to keep the ionic strength constant. The amount of tetrabutylammonium iodide required to bring the ionic strength to 0.2 was calculated and added to the sample at the time of preparation.

The solutions for the pH study were prepared by pipeting 5 ml. of an alcoholic steroid solution containing 1 mg./ml. of steroid into a 10-ml. volumetric flask, adding maximum suppressor and supporting electrolyte, and diluting to the mark with the appropriate aqueous buffer.

The a.c. calibration point was determined as follows: using a 2800 ohm resistance[5] substituted for the cell; a sensitivity setting of 0.300; and an applied a.c. voltage of 70 mv., the slidewire calibration potentiometer was adjusted until the re-

[1] Marketed as Triton X-100 by Rohm & Haas Co., Philadelphia, Pa.
[2] Courtesy of Eli Lilly and Co., Indianapolis, Ind.
[3] Courtesy of Wyeth Laboratories, Inc., Philadelphia, Pa.
[4] Courtesy of The Upjohn Co., Kalamazoo, Mich.

[5] Leeds & Northrup AC–DC Decade Resistor.

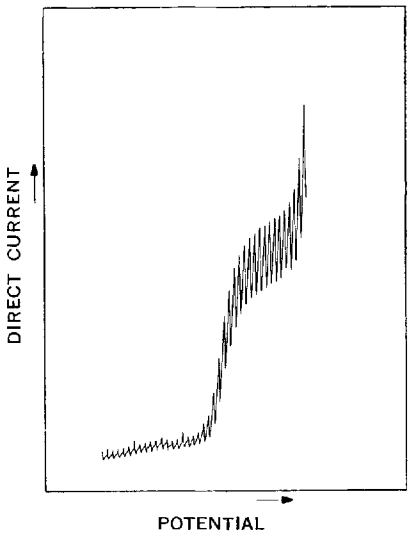

Fig. 1.—Direct current polarogram of methyltestosterone in 50% ethanol and tetrabutylammonium iodide at pH 1.3.

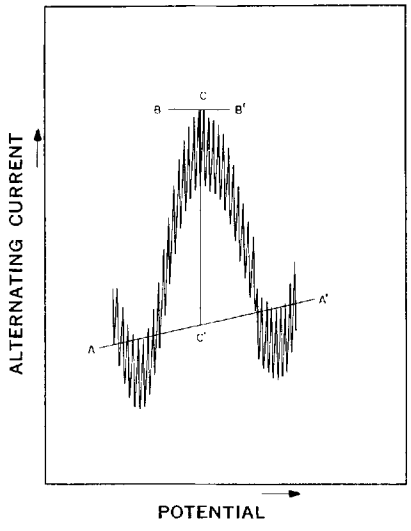

Fig. 2.—Alternating current polarogram of methyltestosterone in 50% ethanol and tetrabutylammonium iodide at pH 1.3. CC′ is the peak current.

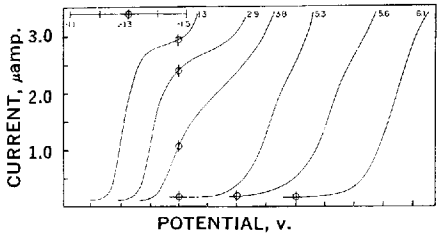

Fig. 3.—Effect of pH on the half-wave potentials and wave form of testosterone in 50% ethanol with tetrabutylammonium iodide as supporting electrolyte.

corder read 250 mm. The samples were then run at a sensitivity setting of 0.600.

Quantitative Studies.—All the quantitative work was carried out at pH 1.3 using methyltestosterone. For the steroid concentration range from $3.3 \times 10^{-4}M$ (100 mcg./ml.) to $6.6 \times 10^{-3}M$ (2 mg./ml.) a 50% ethanol solution was used. For the lower concentration range, from $3.3 \times 10^{-5}M$ (10 mcg./ml.) to $3.3 \times 10^{-4}M$, 25% ethanol was used. It was observed that the a.c. peaks were slightly higher in the lesser alcohol concentration. This was to be expected due to the series resistance effect.

For all the d.c. studies a direct potential scanning rate of 2.34 mv./sec. was used. For the a.c. studies a scanning rate of 2.34 mv./sec. was used in the high concentration range and 1.85 mv./sec. in the low concentration range.

The following a.c. calibration point was used for the high concentration range. The resistance substituted for the cell was 2800 ohms, the sensitivity setting was 0.300, the applied alternating potential was 70 mv., and the slidewire calibration potentiometer was adjusted until the recorder pen read 250 mm. The sensitivity was adjusted to the setting which produced a peak height of 50–125 mm.

For the low concentration range the instrument was standardized as follows. The resistance substituted for the cell was 2800 ohms, the sensitivity setting was 0.300, the applied alternating potential was 70 mv., the slidewire calibration potentiometer was adjusted until the pen read 250 mm., the downscale compensator was adjusted until the pen read 100 mm., and the slidewire potentiometer was readjusted until the pen again read 250 mm. The sensitivity was then set to 0.020 when the samples were polarographed.

In this low concentration range the downscale compensator was of the most value because it permitted the use of high sensitivity settings not possible without it. It enabled zero to be shifted downscale and thus the a.c. curve could be positioned on the chart paper. In other words, at high sensitivity settings the a.c. curve appeared high on the electrical scale due to high base current, and the downscale compensator enabled the electrical scale to be shifted with respect to the chart paper.

RESULTS

Results of the pH Study.—The current values of the d.c. polarographic waves which are shown are the average of the oscillations due to the dropping mercury. All the polarograms were recorded with the damping switch off. The amount of oscillation which resulted is shown by a typical polarogram of methyltestosterone in 50% ethanol at pH 1.3 in Fig. 1.

The current values of the a.c. curves are the values at the maximum age of the mercury drop, *i.e.*, the highest point in the recorder pen oscillation. All alternating current scales are arbitrary since the recorder scale was not calibrated in absolute alternating current. The values on this scale were obtained by multiplying the peak height in mm. by the polarograph sensitivity setting. Figure 2 is an a.c. polarogram of methyltestosterone which shows: (*a*) the oscillations due to the dropping mercury, (*b*) how the a.c. peak height was measured.

Figure 3 shows the d.c. waves for testosterone at

pH values 1.3 to 6.1. Figure 4 shows the corresponding a.c. waves. Methyltestosterone and progesterone gave essentially the same a.c. and d.c. results as those obtained for testosterone. Above the pH range shown, the waves were very similar to that obtained at pH 6.1.

In general, the polarographic waves of ketones are not ideal. They occur at very negative potentials and the diffusion current plateau and residual current have distinctly different slopes. In order to obtain reliable analytical results with the d.c. procedure, the method of evaluating the wave must be rigidly standardized. The a.c. wave tends to facilitate quantitative interpretation. The reduction of the steroid and buffer discharge are separated and the chances for error are lessened.

From Fig. 4 it was decided that pH 1.3 was optimum for quantitative work.

Results of Quantitative Studies.—The method used for determining the diffusion current from recorded d.c. polarographic waves was that described by Willard, Merritt, and Dean (8). Figure 2 illustrates how the a.c. waves were evaluated.

The d.c. results of the high concentration range are shown in Fig. 5 and the a.c. results are shown in Fig. 6. The relationship between a.c. current and concentration is not linear at high concentration and, therefore, is not as analytically useful as the d.c. curve.

The d.c. results of the low concentration range are shown in Fig. 7 and the a.c. results are shown in Fig. 8. Each concentration shown in Figs. 7 and 8 was run in triplicate. Where only two points appear, it was found that two results were identical. The concentration-current curves are calculated regression lines and the experimental points are plotted. R for the d.c. relationship is 0.994 and for the a.c. it is 0.997.

The value of the downscale compensator is illustrated by comparing Fig. 8 and Fig. 9. These are plotted on the same scale. The slope for the line in Fig. 8 is 0.034 and the slope in Fig. 9 is 0.019. Using the downscale compensator, the peak was about twice as high as without it.

DISCUSSION

While d.c. diffusion current depends on concentration gradients near the electrode surface, the a.c. current depends on the concentration of electroactive species on the drop surface. When adsorption of either the oxidized or reduced form occurs on the mercury drop, as is usually the case with organic compounds, the concentration-peak current curve is nonlinear. According to the Langmuir adsorption theory, molecules will be adsorbed on a surface until the surface is covered with a monolayer of adsorbed molecules (9). This means that no matter what the bulk concentration of a solution of electroactive species, the a.c. current reaches a limiting value when the surface of the mercury drop becomes saturated. The a.c. current is, therefore, not a linear function of bulk concentration, but approximates an adsorption isotherm. However, at low bulk concentration, when the mercury drop surface is nearly bare, the peak current-concentration relationship is essentially linear, and can be used for quantitative analysis.

In the quantitative study of methyltestosterone,

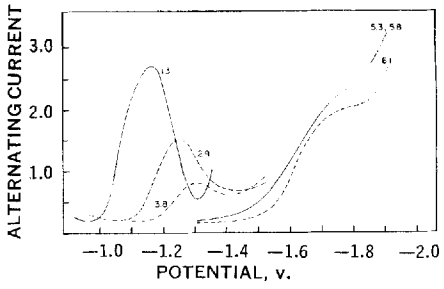

Fig. 4.—Effect of pH on the a.c. wave form of testosterone in 50% ethanol with tetrabutylammonium iodide as supporting electrolyte.

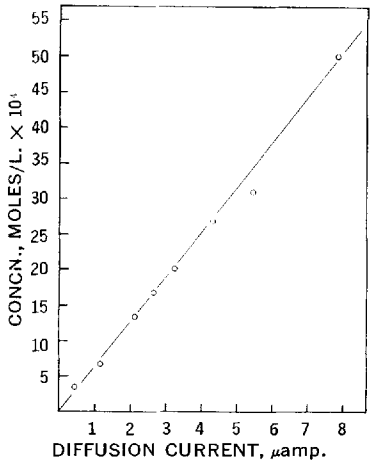

Fig. 5.—Diffusion current-concentration curve for methyltestosterone: concentration range, 6.6 × 10^{-3} *M* to 3.3 × 10^{-4} *M*; medium, 50% ethanol and tetrabutylammonium iodide at pH 1.3.

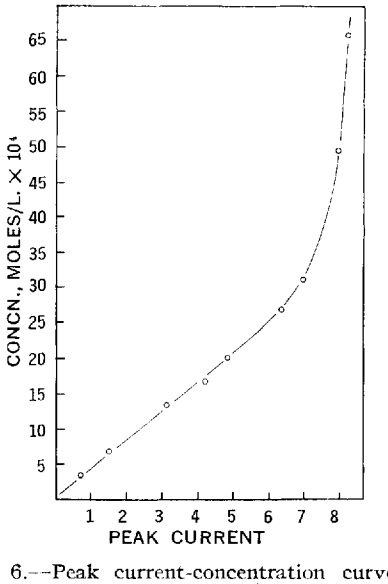

Fig. 6.—Peak current-concentration curve for methyltestosterone: concentration range, 6.6 × 10^{-3} *M* to 3.3 × 10^{-4} *M*; medium, 50% ethanol and tetrabutylammonium iodide at pH 1.3.

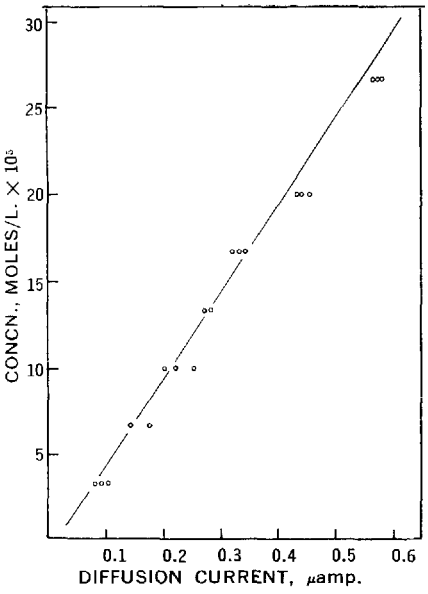

Fig. 7.—Diffusion current-concentration curve for methyltestosterone: concentration range, 3.3 × 10^{-4} *M* to 3.3 × 10^{-5} *M*; medium, 25% ethanol and tetrabutylammonium iodide at pH 1.3.

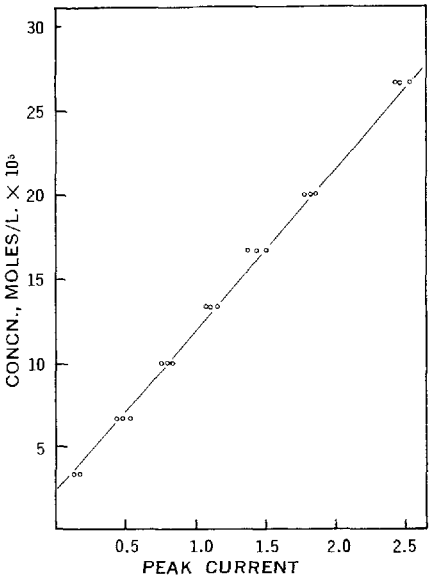

Fig. 8.—Peak current-concentration curve for methyltestosterone using the downscale compensator: concentration range, 3.3 × 10^{-4} *M* to 3.3 × 10^{-5} *M*; medium, 25% ethanol and tetrabutylammonium iodide at pH 1.3.

it was determined that the lowest practical limit of detection for both the a.c. and the d.c. methods was 3.3 × 10^{-5}*M*. Below this concentration the instrument did not give a reliable response with either method.

Although the lower limits of detection are essentially the same for a.c. and d.c., the a.c. procedure was found to be more precise. The presence of trace amounts of oxygen at low concentrations

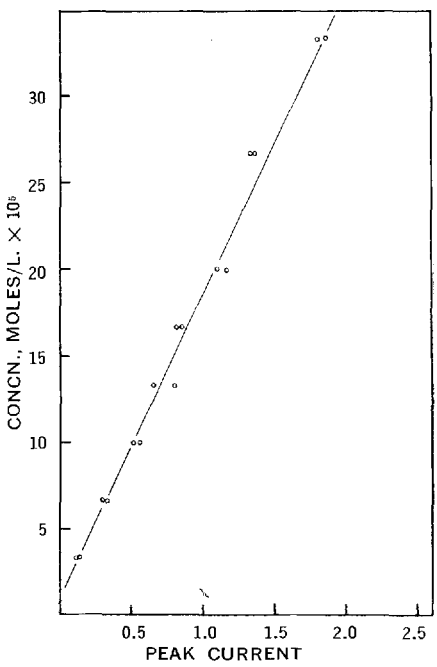

Fig. 9.—Peak current-concentration curve for methyltestosterone without using the downscale compensator: concentration range, 3.3 × 10^{-4} *M* to 3.3 × 10^{-5} *M*; medium, 25% ethanol and tetrabutylammonium iodide at pH 1.3.

of steroid can result in large errors in d.c. polarography, whereas the presence of oxygen does not affect a.c. results. It was observed during this study that occasionally 20 min. of deaeration did not completely remove all of the oxygen present. It was further observed that adequate grounding of the chassis of the a.c. accessory was absolutely essential. Without grounding a stable reference point could not be maintained. Stray a.c. voltages from the line cause undesirable electrical noise which can be minimized by shielding leads to the polarograph.

SUMMARY

An a.c. polarographic method has been developed for the analysis of several Δ^4-3-ketosteroids and compared to the d.c. method. The results show that lower limits of detection for both methods were essentially the same. However, the a.c. procedure was found to be more precise.

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