Experimental Evidence for the Existence of an Adrenochrome–Adrenolutin Complex

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Horizontal linear paper chromatography was utilized to achieve the semiquantitative separation of adrenochrome–adrenolutin mixtures. Data obtained by the spectrophotofluorometric assay of bands eluted from these chromatograms show evidence of the formation of a 1:1 complex of the two components.

As a facet of a continuing study of the photodegradative mechanisms of epinephrine, chromatographic separations of its degradation products were undertaken with a view toward quantitative estimation of the various components present. Heacock and his group used an adsorption method to separate the oxidative degradation products of epinephrine (1). Heacock and Mahon reported the separation of adrenochrome, adrenolutin, and 5,6-dihydroxy-*N*-methylindole on acetylated paper (2).

The accepted mechanism of oxidation and subsequent degradation of epinephrine was first elucidated about 1950 (3–6). An expanded scheme has been published by Harrison (7). Adrenolutin, one of the degradation products, is characterized by an intense apple-green fluorescence. This fluoresence has been utilized as the basis for the assay of epinephrine, adrenochrome, or adrenolutin (8–11).

EXPERIMENTAL

Adrenochrome Preparation—Adrenochrome was prepared by the silver oxide oxidation of epinephrine, using a modification of the method of Somkaite (12), which was derived from that of Sobotka and Austin (13). Oxygen was displaced from the adrenochrome solution during crystallization by placing a small piece of dry ice in the solution. In order to minimize the tendency of moisture from the air to condense in the −40° solution, a cover filled with dry ice was placed over the container holding the crystallizing solution, m.p. 125° dec.[1]

Adrenolutin Preparation—All operations were performed under a carbon dioxide atmosphere using solutions prepared with doubly distilled carbon dioxide saturated water unless otherwise stated. These solutions ranged in concentration from 0.05 to 1.0 mg./ml.

An amount of adrenochrome equivalent to the concentration of adrenolutin desired was placed in a carbon dioxide filled 10-ml. flask. Nitrogen saturated 0.05 *N* tertiary sodium phosphate solution (4.0 ml.) containing ascorbic acid (0.1 mg./ml.) was added. After 2 min., 0.1 *N* phosphoric acid (4.0 ml.) was introduced and the solution brought to volume with buffer (0.1 *M* sodium dihydrogen phosphate adjusted to pH 5.5 with 0.1 *N* sodium hydroxide). The resultant solution was always used immediately. It exhibited the characteristic fluorescence peak of adrenolutin at 518 mμ (corrected) when activated with 412 mμ (corrected) light.

Chromatographic Method—The chromatographic paper[2] (25-cm. strips) was suspended from a glass rod and washed with 2% acetic acid for 18 hr. The strips were next washed with two portions of distilled water and soaked in distilled water for 1 hr. The paper was then dried over silica gel for 6 hr. and stored in a 100% humidity chamber for a minimum of 48 hr. before use.

A modification of the chromatographic apparatus of Hauton (14) was employed. A continuous posterboard strip was placed between the edges of the glass plates to effect a slight separation and to serve as a vapor barrier. The saturation strips described by Hauton were omitted and his procedure of clamping the plates together during runs was found unnecessary.

All chromatographic procedures and elutions were performed in a dry box[3] under a positive carbon dioxide pressure. The running solvent used was normal saline solution saturated with carbon dioxide. Fifty-microliter samples were used in all cases.

Adrenochrome Assay—The sample was applied to the prepared paper as a band 5 cm. from one end. Chromatographic development was allowed to proceed in total darkness until the solvent front was a few centimeters from the terminal end. The section of R_f 0.6–0.9 including the red adrenochrome band at R_f 0.75 was cut from the strip, rolled into a cylinder, and inserted into a micro-filter tube-type funnel and this assembly placed in a 10-ml. flask. The adrenochrome band was eluted by dropwise addition (0.5 ml./min.) of bicarbonate buffer (8.0 ml.) (0.05 *M* sodium bicarbonate adjusted to pH 6.0 with 0.2 *N* hydrochloric acid.) Four per cent ascorbic acid (100 μl.) was added to the eluate and the flask filled to the mark with 10.0 *N* sodium hydroxide, stoppered, and inverted five times. An aliquot of the now pale yellow solution was placed in the spectrophotofluorometer[4] and its fluorescent intensity read at 518 mμ (corrected) exactly 5 min. after the time of addition of the sodium hydroxide. Light at 412 mμ (corrected) was used to activate the solution. The results were given the designa-

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[1] Lit. 125° dec. (12).

[2] Whatman No. 1, 1.5 in. wide strip.

[3] Manostat Dry Box, model 41-905.

[4] Aminco-Bowman spectrophotofluorometer. The No. 3 slit arrangement and the 0.3 M. multiplier setting were used for all fluorescence determinations.

tion (A). (All assays throughout this investigation utilized the same activation and emission wavelengths.)

To obtain a time zero fluorescent intensity reading, a 50-μl. sample of each original adrenochrome solution was placed in bicarbonate buffer (8.0 ml.). This solution was treated and read using the same procedure as that for the eluate from the adrenochrome band of the chromatogram. The time zero adrenochrome reading was designated (A_0).

Adrenolutin Assay—Pure adrenolutin solution was analyzed in a manner similar to that for adrenochrome at time zero. A 50-μl. sample was placed in a 10-ml. flask containing bicarbonate buffer (8.0 ml.) and the fluorescent intensity obtained as above; (L_0) was used to designate these results.

Preparation and Analysis of Adrenochrome–Adrenolutin Mixtures—Adrenolutin was prepared as previously described by the addition of 0.05 N tertiary sodium phosphate (4.0 ml.) to an amount of adrenochrome equivalent to the desired quantity of adrenolutin in a 10-ml. flask. The resulting adrenolutin solution was adjusted to a pH below 7 by the addition of 0.1 N phosphoric acid (4.0 ml.). The adrenochrome portion of the mixture was then added, adrenochrome being stable at pH's slightly below 7. The flask was then filled to the mark with pH 5.5 phosphate buffer and the contents mixed by inverting the flask for 1 min. A 50-μl. sample was streaked on the chromatogram and development begun. Upon completion of the chromatographic run, the segment of R_f 0.6–0.9 was cut from the strip and treated according to the procedure outlined for adrenochrome. The value of the fluorescent intensity found was given the designation (F'). (The adrenochrome band was located between R_f 0.70 and 0.80 and the adrenolutin band between R_f 0.40 and 0.50.)

While the chromatogram was being developed, a second 50-μl. sample of the same adrenochrome–adrenolutin mixture was taken and placed in a 10-ml. flask containing bicarbonate buffer (8.0 ml.). Subsequent treatment was identical to that used in the adrenochrome assay. Since this procedure should rearrange all adrenochrome present to adrenolutin and should merely stabilize the adrenolutin already present, the fluorescent intensity found should equal the sum of the values expected from each. This experimentally found total fluorescent intensity at time zero was designated (F_t).

The mixtures of adrenochrome and adrenolutin chromatographed varied in ratio but always totaled 1.5 mg./ml. in solute concentration.

RESULTS AND DISCUSSION

Calibration data are shown in Table I and in graphical form in Fig. 1. The three sets of calibration data gave straight line plots on log-log paper between 0.05 mg./ml. and 1.0 mg./ml. concentration (2.6×10^{-4} to 5.3×10^{-3} M) and were utilized to calculate the amount of each compound present in the mixtures studied.

Adrenochrome concentration in the mixture was found by extending a line from the value of its fluorescent intensity (F') to line (A). Its concentration was then taken as that point on the concentration axis. Adrenolutin concentration was determined by subtracting the fluorescent intensity value on line (A_0) at the above experimental concentration of adrenochrome from the total fluorescent intensity value at time zero (F_t) and extending a line from the fluorescent intensity calculated to line (L_0). The mg./ml. of adrenolutin was then read on the concentration axis directly below the point of intersection.

The results obtained for the adrenochrome–adrenolutin mixtures are represented graphically by Fig. 2. As seen from this figure, when mixtures of adrenochrome and adrenolutin are separated chromatographically, although a qualitative separation is achieved, theoretical recovery of either component was not obtained. The adrenochrome recovery was always above the theoretical line of slope unity, indicating greater than theoretical recovery. Adrenolutin, on the other hand, showed indicated recoveries well below theoretical for all mixtures. Considering the method of analysis used, these experimental results necessitate the conclusion that some adrenolutin or substance giving rise to it was being carried in the adrenochrome band.

The above data can be used to show the existence

TABLE I—FLUORESCENCE-CONCENTRATION CALIBRATION DATA[a]

Concn., mg./ml.	Fluorescence: Adrenochrome-Eluted from Chromatogram (A)	Fluorescence: Adrenochrome at Time Zero (A_0)	Fluorescence: Adrenolutin (L_0)
0.01	1.16	1.16	...
0.05	5.2	6.3	3.58
0.1	9.6	10.5	7.17
0.2	17.9	18.6	14.0
0.5	37.5	42.3	32.6
1.0	64.8	71.5	63.6
2.0	99.8	103.0	...

[a] All fluorescent values were obtained on an Aminco-Bowman spectrophotofluorometer with a No. 3 slit arrangement, a 412 mμ (corrected) activation wavelength, and a 518 mμ (corrected) emission wavelength. All fluorescent values are adjusted to the equivalent of a 0.3 M. multiplier setting. Each value is the average of 4–7 runs.

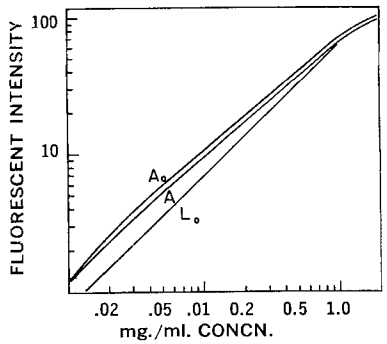


Fig. 1—Standard curves for the analysis of adrenochrome–adrenolutin mixtures. Key: A_0, *the fluorescent intensity obtained from adrenochrome assayed at time zero;* A, *the fluorescent intensity obtained from alkaline ascorbate treated adrenochrome after chromatography and elution;* L_0, *the fluorescent intensity obtained from adrenolutin assayed at time zero.*

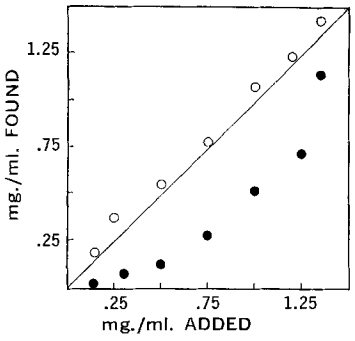


Fig. 2—Analysis results for adrenochrome–adrenolutin mixtures.

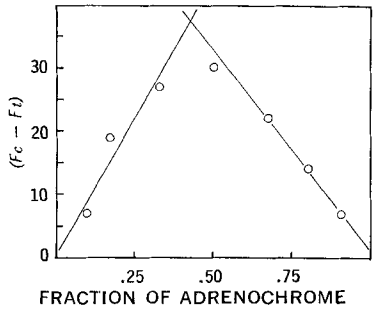
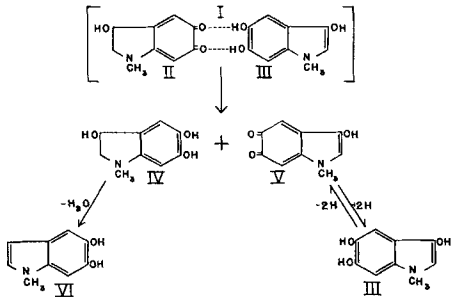


Fig. 3—Modified plot of continuous variation.

of an adrenochrome–adrenolutin complex in the mixture. By use of a modified method of continuous variation, the difference between the theoretical total fluorescent intensities (F_c) and the actual total fluorescent intensities (F_t) for the various solute ratios studied are plotted against the mole fraction of adrenochrome in the mixtures. Two lines are generated which intersect at approximately 45% adrenochrome (Fig. 3), indicating the greatest decrease in fluorescence near the 1:1 *M* ratio.

It was also observed that when a mixture of adrenochrome and adrenolutin was being separated chromatographically, a nonfluorescent yellow band appeared at the trailing edge of the adrenochrome band. When the chromatogram was sprayed with Ehrlich's reagent [*p*-dimethylaminobenzaldehyde (1 Gm.) dissolved in a mixture of concentrated hydrochloric acid (30 ml.) and ethanol (30 ml.) and the solution diluted to 100 ml. with water], a blue-violet band indicating 5,6-dihydroxy-*N*-methylindole formed at the location of the zone; adrenochrome and adrenolutin did not give the color with this reagent. The nonfluorescent yellow band did not appear when either adrenochrome or adrenolutin were chromatographed separately.

These observations can be explained by assuming a hydrogen bonded 1:1 complex (I) of adrenochrome (II) and adrenolutin (III). As a result of the complexation, the adrenolutin can transfer two hydrogens to the adrenochrome, giving an equimolar mixture of leucoadrenochrome (IV) and adrenolutin-quinone (V) (Scheme I). The latter substance should be stable but the former will dehydrate to 5,6-dihydroxy-*N*-methylindole (VI), in line with the



Adrenochrome–Adrenolutin Complex Degradation Scheme

Scheme I

indicated presence of this substance on the chromatogram. For every molecule of adrenochrome which gains two hydrogens and is transformed to leucoadrenochrome, one molecule of adrenolutin-quinone should also be obtained. When the adrenolutin-quinone is reduced to adrenolutin by the addition of the alkaline ascorbate solution, an amount of fluorescence equivalent to that obtainable from the same number of adrenochrome molecules should result. In addition, the fluorescence due to the now rearranged adrenochrome which originally did not complex is also read. Thus the developed fluorescence of the adrenochrome band should be the sum of the rearranged uncomplexed adrenochrome, adrenolutin carried as the complex, and reduced adrenolutin-quinone formed by degradation of the complex. This sum should be greater than the amount of fluorescence expected from the adrenochrome concentration originally present.

Additional conclusions may be drawn. Since part of the adrenolutin is traveling as a complex in the adrenochrome band, the fluorescent intensity of adrenolutin should be decreased, as observed. Experimental results also show that as expected from these interpretations, the sum (F_t) of the fluorescent intensity related to adrenochrome plus that of adrenolutin is always less than theoretical since part of the original adrenochrome has been converted to the nonfluorescent 5,6-dihydroxy-*N*-methylindole.

REFERENCES

(1) Heacock, R. A., Nerenberg, C., and Payza, A. N., *Can. J. Chem.*, **36**, 853(1958).
(2) Heacock, R. A., and Mahon, M. E., *J. Chromatog.*, **6**, 91(1961).
(3) Bu'Lock, J. D., and Harley-Mason, J., *J. Chem. Soc.*, **1951**, 712.
(4) Falk, J. E., *Biochem. J.*, **44**, 369(1949).
(5) Lund, A., *Acta Pharmacol. Toxicol.*, **5**, 75(1949); through *Chem. Abstr.*, **43**, 5816*b*(1949).
(6) Ruiz-Gijon, J., *Nature*, **166**, 831(1950).
(7) Harrison, W. H., *Arch. Biochem. Biophys.*, **101**, 116 (1963).
(8) Anton, A. N., and Sayre, D. F., *J. Pharmacol. Exptl. Therap.*, **138**, 360(1962).
(9) Atkinson, R., and Wynne, N., *J. Pharm. Pharmacol.*, **14**, 794(1962).
(10) Bertler, A., Carlsson, A., and Rosengren, E., *Acta Physiol. Scand.*, **44**, 273(1958); through *Chem. Abstr.*, **53**, 9344*e*(1959).
(11) Cohen, G., and Goldenberg, M., *J. Neurochem.*, **2**, 58(1957).
(12) Somkaite, R., Ph.D. Thesis, Rutgers—The State University, New Brunswick, N. J., 1962.
(13) Sobotka, H., and Austin, J., *J. Am. Chem. Soc.*, **73**, 3077(1951).
(14) Hauton, J. C., *J. Chromatog.*, **7**, 341(1962).