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SIMULTANEOUS DETERMINATION OF 5-HYDROXYTRYPTAMINE AND CATECHOLA--ETC(U)
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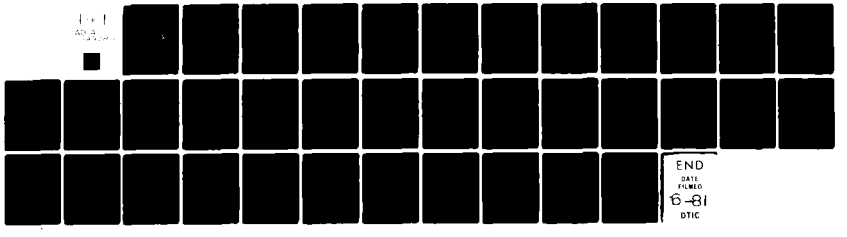

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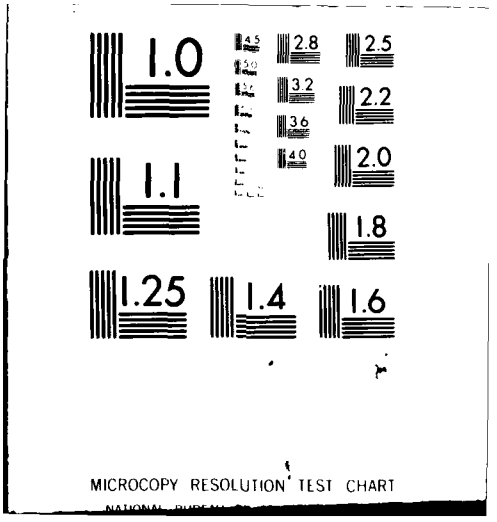
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LEVEL II

SIMULTANEOUS DETERMINATION OF 5-HYDROXYTRYPTAMINE AND CATECHOLAMINES IN TISSUES USING A WEAK CATION EXCHANGE RESIN[1,2]

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Running Title: 5-HT, NE, E, DA Determination

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During the past decade, a number of procedures have been used for routine assay of catecholamines and the indoleamine, serotonin, in various tissues. Many of these procedures do not allow simultaneous assay of the amines, or, if they do, as in the case of some solvent extraction procedures, do not allow for separation of amines from their precursor amino acids (1-6). The successful application of the weak cation exchange resins by some authors (7-13) for one or two amines made it possible for us to adapt and improve those methods and, at the same time, to study various parameters of theprocedure which have not been previously reported in order to increase the versatility of the assay method. The procedure can be used for simultaneous determination of norepinephrine, epinephrine, dopamine and serotonin. Basically, the methods involve the deproteinization of the tissue with either perchloric acid or ethanol, adsorption of the amines on the weak cation exchange resin Bio-Rex 70, elution and spectrofluorometric determination of the amines. The advantages of the method described in this paper over other published ones are: (a) the purification steps of the amines are greatly reduced, requiring only two-and-one-half hours to run up to 50 samples through the columns, (b) the recovery of the amines is higher, (c) one can determine quantitatively 20 ng of each of the four amines in one tissue sample, and (d) the method is highly reproducible.

MATERIALS AND METHODS

REAGENTS AND EQUIPMENT

Disodium (Ethylenedinitrilo) tetra-acetate (EDTA) - 10% (w/v) (Baker).

Perchloric Acid ($HClO_4$) - 0.4 N (Baker).

Hydrochloric Acid (HCl) - (Baker).

Acetic Acid (HAc) - 0.5 N (Baker).

Phosphoric Acid (H_3PO_4) - 3 M (Baker, 85.3%).

Sodium Hydroxide (NaOH) - 10 N (Fisher).

Potassium Hydroxide (KOH) - 2 N (Baker).

Alkaline Ascorbate - 100 mg L-ascorbic acid (Baker) is dissolved in 1.0 ml quartz distilled water and 50 ml 10 N NaOH added.

Alkaline Sulfite - 1.25 gm Na_2SO_3 (Baker) is dissolved in 5 ml quartz distilled water and 45 ml 5 N NaOH added.

Sodium Periodate ($NaIO_4$) - 0.5% (w/v) (Baker).

Potassium Ferricyanide ($K_3Fe(CN)_6$) - 0.25% (w/v) (Baker).

Resin - Bio-Rex 70, 200-400 mesh, sodium form (Bio-Rad Laboratories).

Potassium Carbonate (K_2CO_3) - 1 N (Baker).

Buffers:

0.1 M phosphate, 0.1% EDTA, pH 6.5 - 8.28 gm $NaH_2PO_4 \cdot H_2O$, 5.68 gm Na_2HPO_4, 10 ml - 10% EDTA/liter quartz distilled water.

0.02 M phosphate, 0.2% EDTA, pH 6.5 - 1.66 gm $NaH_2PO_4 \cdot H_2O$, 1.14 gm Na_2HPO_4, 20 ml - 10% EDTA/liter quartz distilled water.

0.5 M phosphate, pH 7.0 (norepinephrine-epinephrine determination) - 70.98 gm Na_2HPO_4/liter quartz distilled water. Mix 61.0 ml Na_2HPO_4 solution with 39.0 ml KH_2PO_4 solution.

0.5 M phosphate, pH 7.0 (dopamine determination) - Adjust a 1 M solution of KH_2PO_4 (Baker) to pH 7.0 with 1 N NaOH and dilute with quartz distilled water to 0.5 M.

Citrate, 0.5 M, pH 4.0 - Adjust a 1 M citric acid (Baker) solution to pH 4.0 with NaOH and dilute with quartz distilled water to 0.5 M.

5-Hydroxytryptamine, Dopamine, Norepinephrine, Epinephrine (Regis Chemical Co.) - Made up in 1.0 mg/ml 0.01 N HCl stock solutions and stored up to three months at 4°C. Stock solution of 5-hydroxytryptamine is in quartz distilled water.

5-Hydroxytryptamine-2-^{14}C, 3,4-Dihydroxyphenylethylamine-1-^{14}C·HBr, DL-Epinephrine-7-^{14}C, DL-Norepinephrine-7-^{14}C (New England Nuclear).

All chemicals were reagent grade. Reagents and quartz water (double distilled) were stored in hard glass bottles with glass stoppers. Tissues were homogenized with a Polytron homogenizer, obtained from Brinkmann Instruments. Fluorescence was measured in a Turner Model 210 Spectrophotofluorometer.

Preparation of Resin

The resin is washed several times with distilled water to remove the fine particles. It is stirred with two volumes of water, allowed to settle and the supernatant suspension is decanted. The process is repeated 10 to 15 times until the supernatant liquid is clear. The resin is transferred to a sintered glass Buchner funnel and 5 volumes of 3 N HCl is passed through the filter over a one hour period per 200 ml of resin. It is then washed with quartz distilled water (10 volumes) until the effluent is neutral. The resin is buffered at pH 6.5 by passing through 20 volumes of 0.1 M phosphate buffer containing 0.1% EDTA over a period of one hour per 100 ml of resin, or until the pH of the final filtrate is identical to the inflowing buffer. The resin is stored in the refrigerator in 0.1 M phosphate buffer.

Preparation of Samples

Whole rat brain, dissected cat brain, hearts and spleens are homogenized in 0.4 N $HClO_4$ (5 ml/gm tissue containing 0.1 ml 10% EDTA/gm tissue). The samples are homogenized for 20 sec/2 gm of tissue by means of a Polytron homogenizer. For tissues heavier than 3 gm, 15 ml aliquots of the homogenate are taken. The homogenates are centrifuged at 30,000 x g at 4°C for 15 minutes in a Sorvall centrifuge. The supernatants are decanted and adjusted to pH 5.5-6.0 with 2 N KOH. For small amounts of tissues, the volume is brought to at least 7 ml by adding 0.4 N $HClO_4$. For final adjustment 0.1 N HCl and 0.1 N NaOH are used. After the pH adjustment, the samples are centrifuged again for 5 minutes to remove the $KClO_4$ or the adjusted

supernatant is frozen overnight before putting on the columns. Mouse and rat adrenals are homogenized in 1 ml of 0.4 N $HClO_4$ containing .05 ml of 10% EDTA with a small glass homogenizer. The homogenizer is rinsed three times with 1.0 ml of 0.4 N $HClO_4$. Homogenates and rinses are combined and centrifuged. One tenth of the supernatant is diluted to at least 7 ml with $HClO_4$. pH is adjusted to 6 and centrifuged again before putting on the columns.

If ethanol is used for precipitating the protein, the tissues are homogenized in ice-cold 75% ethanol (5 ml/gm tissue containing 0.1 ml 10% EDTA/gm of tissue) and then centrifuged at 30,000 x g at $4^{o}C$ for 15 minutes. The supernatants are decanted and diluted with an equal volume of quartz distilled water and passed through the columns.

The ethanol extraction is particularly suitable for pineals and tissues weighing less than 50 mg. They are homogenized in 0.5 ml of 75% ethanol with a small glass homogenizer. The homogenizer is rinsed three times with 0.5 ml of 75% ethanol. Homogenates and rinses are combined and centrifuged at 30,000 x g for 15 min. The 2 ml supernatant is mixed with 2 ml of cold distilled H_2O and put on a 0.4 x 2 cm column. Before passing samples through the columns, ^{14}C-labeled amine is added routinely to each supernatant in order to monitor column recovery.

Column Procedure

The columns used were obtained from Kontes Glass Co. (0.6 cm I.D., 25 cm overall length with a top reservoir of 25 ml) and modified by glass welding a teflon stopcock to it. The addition of the stopcock facilitates preparation of the columns the day prior to assay and also permits control

of sample and eluant flow rate. For resin support a small amount of glass wool is used. The resin is stirred and, for routine determination of 5-HT and the catecholamines in a tissue sample, the columns are packed to 3 cm. When the resin is previously equilibrated with the buffer, 3-4 ml 0.1 M phosphate buffer, pH 6.5, then 5 ml quartz distilled water containing 0.1% EDTA are passed through before putting on the samples. If the resin is not completely buffered before use, more buffer is run through until the effluent reaches pH 6.5. After the samples are passed through, the columns are washed with 10 ml 0.02 M phosphate buffer pH 6.5, then with 3 ml quartz distilled water. The columns are allowed to run dry and routinely eluted with 5 ml 0.5 N HAc. Column blanks are run along with the samples. For the determination of about 10-20 ng of each amine in a tissue, columns of 0.4 x 2.0 cm are used washed with 3 ml 0.02 M phosphate buffer followed by washing with 2 ml of distilled H_2O and eluted with 2.5 ml of 1.0 HAc. To determine low levels of 5-HT, the columns can be eluted with 1 ml of 1.0 N HCl.

Fluorometric Analysis

5-Hydroxytryptamine (5-HT) - To a 1 ml aliquot of eluate is added 0.3 ml concentrated HCl. The fluorescence is read at activation wavelength 2780 Å, emission 5450 Å. External standards in the appropriate concentrations and reagent blanks are run each time samples are read. These are serially diluted with the eluting acid from a 1 mg 5-HT/ml H_2O stock solution which is kept frozen and remains stable for at least 2 months.

Norepinephrine (NE), Epinephrine (E) - To form the fluorescent trihydroxyindole derivative, a modification of the procedure of Anton and Sayre (4) is used. To analyze samples for NE and E at pH 7.0, 0.6 ml 0.5 M phosphate buffer pH 7.0 is added to a 0.6 ml aliquot of eluate. The pH of sample and external standards is adjusted to 6.5-7.0 with 1 N K_2CO_3. To this sample is added 0.06 ml 0.25% $K_3Fe(CN)_6$ (freshly made). Exactly 1 minute later 0.6 ml alkaline ascorbate is added. Within 15-30 seconds 1.5 ml of H_2O is added. The sample is read at activation wavelength 3306 Å, emission 5028 Å within the 3-6 minute period after the oxidation has begun.

Epinephrine - A 0.6 ml aliquot of sample is adjusted to pH 2.5 with 0.1 N HCl. The standards are brought to the same volume as the samples with 0.5 N HAc. Samples and standards are then oxidized as described above. Fluorescence is read at activation wavelength 3360 Å, emission 5074 Å.

Dopamine (DA) - To a 0.6 ml aliquot of eluate is added 0.3 ml 0.5 M phosphate buffer pH 7.0. The sample is adjusted to pH 6.5-7.0 with 1 N K_2CO_3. External standards are run with the samples.

To oxidize, 0.06 ml 0.5% $NaIO_4$ is added to the sample. After exactly one minute, 0.3 ml alkaline sulfite solution is added. Both of these reagents are prepared just before using. Then 0.3 ml 0.5 M citrate buffer pH 4.0 and 0.51 ml 3 M H_3PO_4 are added as quickly as possible. The samples and standards are read at activation wavelength 3160 Å, emission wavelength 3700 Å within 10 minutes.

RESULTS AND DISCUSSION

The concentrations of each amine found in the various tissues using the procedure described in the Methods section are given in Table 1. The levels of each amine found in our laboratory are comparable to literature values (4, 6, 14, 15, 16, 17).

The recoveries that were obtained with both the perchloric and ethanol extraction methods are shown in Table 2. For the recovery study, tissues were homogenized in either perchloric acid or ethanol. Aliquots of the homogenates were taken and either ^{14}C-labeled or unlabeled amines were added. After centrifuging, the pellets were re-extracted again with half the volume of acid or ethanol originally used, the extracts combined with the original supernatant and also run separately on the columns. For routine application of the procedure, re-extraction of the pellets is omitted and the recovery correction made using the data in Table 2.

Several investigators (6, 10, 18) have shown that 5-HT stability in perchloric acid rapidly decreases. Great care is taken to process samples quickly and keep them ice cold. The samples are left no longer than one half hour to one hour at the acid pH.

In order to chelate any contaminating heavy metals during homogenization, EDTA is added. Several authors (6, 10, 18) used ascorbic acid also to protect the catecholamines and serotonin during extraction. In our laboratory, ascorbic acid added to rat brain tissues did not affect the recovery of the amines (Table 3).

If samples are homogenized in ethanol they should be run through the column the same day due to rapid destruction of the samples. Fig. 1 shows

the drop in recovery of all amines except dopamine when stored at 4°C for a period of time indicated on the graph. Yet perchloric acid extracts, when adjusted to pH 6.0, can be stored for at least 5 days without an appreciable loss of amine recovery (2, 10, 18).

The pH of the ethanol extracts when diluted 1:1 with water before passing through the column is between 6.0-6.5 and they can be applied directly to the columns. In the case of perchloric acid extracts, it is necessary to adjust the pH between 5.5-6.0 for maximum recovery. Fig. 2 shows the percentage recovery of each amine at various pH's.

For the adjustment of the pH of the perchloric acid extracts, KOH was used instead of K_2CO_3 used by others (2, 10). There was no destruction of 5-HT due to the KOH, as noted in other studies (2) (which may be explained by the purity of the KOH) and levels of 5-HT were the same whether KOH or K_2CO_3 was used. However, when K_2CO_3 was used the gas evolving from the columns caused channeling or an air-block in the resin bed, stopping the flow.

Extracting tissues in ethanol has the advantage of eliminating the pH adjustment of the samples. The ethanol extracts have a slower flow through the column. For small tissue samples like adrenal or hypothalamus which require less volume to precipitate the protein, the ethanol procedure is ideal. However, for tissue samples of a larger volume, it takes considerable time for the samples to pass through the column. In order to increase flow rate and yet retain good recovery the following were tried:

(A) Passing through ethanol extracts without diluting with water: This resulted in disrupting the resin bed; channeling occurred and recovery was very low.

(B) Homogenizing tissues in 80% or 85% ethanol and diluting with water in various ratios: The supernatants were very cloudy due to the lipid emulsification, therefore the flow rate was low. However, homogenizing in 75% ethanol and diluting with an equal volume of water gave a rather clear supernatant resulting in a flow rate of 0.2-0.25 ml/min. The recovery of the amines ranges from 87-94% and is shown in Table 2.

(C) Addition of Tween 80 to disperse the lipid: This technique somewhat increased the flow rate (2 drops/10 ml of homogenate increased by 0.04 ml/min), but great care had to be taken in washing the columns with water since Tween 80 fluoresced at the wavelength of the amines. Even when the columns were extensively washed, dopamine still had a fluorescence 4% higher than in the control sample. Thus, none of the procedures for increasing flow rate are satisfactory for samples of large volume homogenized in ethanol.

It is claimed by a few authors (4, 6) that recovery using columns compared to recovery using organic extraction is about 10-20% more variable. To investigate that question under the conditions used, studies of recovery were performed. For example, 37 columns were run with 5-HT-^{14}C added; mean recovery: 93%, S.D. 4.15, S.E. ± 0.68. In order to minimize the variation from one column to another, the resin is constantly stirred while packing the column to assure uniform distribution of the resin

particles. ^{14}C-labeled amines are chosen to monitor column recovery since there was variability in the recovery of tritium-labeled 5-HT available to us. The ^{14}C-labeled amine should not be kept more than a month at $4^{\circ}C$ and is most stable when stored in liquid nitrogen. The columns can be packed the night before use; if some of them become dried, a few ml of 0.1 M phosphate buffer and 5 ml of water run through prior to use results in 94% recovery of the amines.

The resin is a weakly acidic cation exchanger which in the form available commercially is partially purified. When the resin was not recycled through the hydrogen and sodium form, there was negligible interfering fluorescence at the wavelengths investigated for the assay. The resin was washed with acid since it was found that if the acid wash was omitted, there was a 7% drop in 5-HT recovery and a 20% drop in the dopamine recovery. Since the resin has the carboxylic group on it, washing with acid is quite fast due to its greater affinity for the hydrogen ion, but buffering it takes considerable time.

Columns can be reused after elution by washing with 5 ml water, 10 ml 0.1 M phosphate buffer containing 0.1% EDTA, and 5 ml water with 0.1% EDTA. However, the time involved to regenerate the columns is greater than that required to replace them with fresh resin and after the second or third use of the column, depending on the amount of tissue run on it, the top layer (1-2 mm) should be removed and replaced with fresh resin. After recycling the column up to 5 times we found that the recovery was 90 ± 5%.

The amount of the resin to be used is found to be important, especially in the case of the perchloric extracts. Fig. 3 shows the

recovery of each amine at various column lengths. The optimum recovery was found at 3 cm for perchloric acid extracts. Because of the slower flow rate with ethanol extracts 2.0-2.5 cm can be used, as Fig. 4 shows.

Several acid concentrations and volumes were used for eluting the 0.6 x 3.0 cm column. 5.0 ml acetic acid, 0.5 or higher normality, eluted all the four amines of interest off the columns. Eluting with acetic acid of a lower concentration but the same volume resulted in a 90-95% recovery of NE, E, and DA but only 75% recovery of 5-HT. Elution with 3.0 ml of 1 N HCl showed similar recoveries to elution with 0.5 N HAc; however, for the spectrophotofluorescence (SPF) determination of NE, E, and DA, quenching was a problem. Therefore, for routine analysis of the four amines, elution with 5 ml of 0.5 N HAc resulted in maximum recovery and minimum quenching. 0.5 N HAc was also desirable to use because, according to literature (6, 18) and in our experience, 5-HT and the catecholamines are most stable at pH 4-5 when stored. The eluted sample pH falls within this range.

Eluting with 0.5 N HAc and 1.0 N HCl (0.6 x 3 cm column), the amines listed in Table 4 were eluted from the column along with NE, E, DA and 5-HT. Their interference in fluorescence assay is shown in Table 5. Concentrations up to 100 µg of 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, or DOPA when run through the column showed no interfering fluorescence.

If only one amine is of interest, specific elution volumes and acid concentration may be selected by knowing in which fraction of the eluate the amine comes off the column (see Fig. 5). For example, if only 5-HT is assayed, particularly if low levels are suspected, the first two mls

of the eluate may be discarded and the 5-HT concentrated into 3 mls. As seen on the graph, about 10% will be lost in the second ml, but if 5-HT-^{14}C has been added, recovery is monitored and the loss may be corrected.

The stability of the column eluates for various time periods is shown in Fig. 6.

Fluorescence

Instrument - The Turner Model 210 is designed to function as an ordinary, highly sensitive SPF and as an absolute instrument by automatically correcting excitation and emission spectra. The settings used are indicated in Table 6.

Table 6 shows the ranges of relative fluorescence for a 250 ng amine/ml standard solution, the "column blanks," standard reagent blanks, and the four amines in the tissues studied. The fluorescence intensity of each amine represents a reading from an aliquot of the 5 ml eluate; 1 ml for 5-HT determination and 0.6 ml for NE, E, and DA.

Column blanks are duplicate reagent blanks carried through the entire column procedure. For NE, E and DA determination it was found that tissue blanks prepared by omitting the oxidizing agent, or by reversing the order in which the oxidizing and reducing agents were added, showed identical fluorescence intensity to column reagent blanks.

5-HT - 5-HT is quite stable in 3 N HCl as can be seen in Fig. 7, which summarizes the stability of the fluorophores of the four amines. Rapid destruction of serotonin will result, however, if the HCl used contains

metallic ions, notably Fe^{+++} (10). Baker HCl, reagent grade, is always used.

The fluorescence of NE, E and DA in concentrations up to 0.5 µg was studied at 5-HT wavelengths, and interference was found to be negligible at the concentrations found in the tissue samples (see Table 5). 5-Hydroxytryptophan and 5-hydroxyindoleacetic acid fluoresce at 5-HT wavelength in strong acid but these compounds are not retained on the weakly acidic cation exchange resin.

NE, E - Both NE and E fluoresce maximally at pH 6.5-7.0, and it has been determined experimentally that the fluorescence of E is 1.35 times that of NE at this pH. As can be seen in Table 5, there is interfering fluorescence at NE wavelengths and at pH 6.5-7.0 from DA (4%) and 5-HT (1%) when in the concentrations one would find these amines in tissue. This may be corrected for when the calculation is made. When the pH of the eluate is lowered to 2.5 the fluorescence of NE is reduced to 4% and the following calculation may be made:

pH 2.5 Net Fluoresc. units of sample X µg E/fl unit - 4% = µg E

pH 6.5-7.0 Net Fluoresc. units of sample X µg NE/fl unit - 5% - µg NE + E

µg NE + E - µg E X 1.35 = µg NE

There is no interference from the other amines studied so far (Table 5). When cat hypothalamus was assayed for all four amines we found 0.22 µg/g E (Table 1) after a 4% correction for NE fluorescence interference. These data would justify further studies dealing with E.

When oxidizing the NE and E samples, two samples may be done at 30 sec intervals. The timing as outlined in Methods is precisely followed to insure consistent results and avoid variations in fluorescence because of reading at different time intervals. As shown in Fig. 7, the fluorophores are stable for ten minutes.

Dopamine - Several methods for developing the fluorophore of DA were tried but a modification of the periodate oxidation procedure of Anton and Sayre (19) gave the most reproducible results. As will be noted, there is interfering fluorescence by NE at DA wavelengths if NE is present in sufficient concentration. However, in the 0.6 ml aliquot assayed for DA, the concentration of NE present is not high enough to interfere. Although the fluorescence properties of DOPA are similar to those of dopamine, there is no interference, as DOPA is not adsorbed on the column.

The fluorescence of dopamine is greatly influenced by the pH at which it is oxidized as seen in Fig. 8. Therefore, care must be taken that the pH of the sample or the standard does not differ by more than 0.1. To assure this uniformity, a repipet is used to dispense the K_2CO_3. The timing timing for oxidation outlined in Methods is followed closely, and the samples are read on the SPF within ten minutes after oxidation has begun, since the fluorophore is stable during this period as shown in Fig. 7.

Because fluorescent light is employed to develop the DA fluorophore in many methods, we felt it was important to determine the influence of the overhead fluorescent lights in our laboratory upon fluorescence intensity of DA. The relative fluorescence was reduced by 5% when the

oxidation procedure was carried out with the lights out and only daylight in the room.

It must be noted that scrupulously clean glassware is a necessity when assaying for DA. It has consistently been found that certain cleaning agents, notably synthetic detergents, fluoresce at DA wavelengths. An inorganic cleaning agent, Calgonite, is now being used routinely to wash glassware and care is taken to rinse thoroughly.

CONCLUSIONS

The procedure outlined using a weak cation exchange resin has particular advantages under certain conditions: (1) to analyze tissues for both indoleamines and catecholamines, (2) to separate the desired amine from its precursor amino acids or metabolites, since neither precursors nor major metabolites adsorb onto the resin. In addition, the sensitivity, rapidity and reproducibility of the method make it ideal for assay of large numbers of tissue samples.

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FOOTNOTES

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[2]A preliminary report of this work was presented at the 1970 Fall Meeting of the American Society of Pharmacology and Experimental Therapeutics at Stanford, California.

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TABLE 1.

Concentration of amines in tissues

	5-HT µg/g	DA µg/g	NE µg/g	E µg/g
Cat brain parts:				
Cortex	0.17 ± 0.05[a]	0.29 ± 0.13	0.19 ± 0.01	
Hypothalamus	0.78 ± 0.07	0.42 ± 0.01	1.90 ± 0.25	0.22 ± 0.04
Cerebellum	0.09 ± 0.03	0.15 ± 0.08	0.12 ± 0.03	
Brain Stem	0.53 ± 0.14	0.19 ± 0.09	0.32 ± 0.03	
Midbrain	0.51 ± 0.07	1.20 ± 0.33	0.45 ± 0.13	
Adrenals			583.00 ± 104.00	607.00 ± 64.00
Rat: Whole brain	0.42 ± 0.01	1.00 ± 0.08	0.45 ± 0.01	
Brain Stem	0.44 ± 0.03		0.57 ± 0.01	
Telencephalon	0.39 ± 0.01	1.20 ± 0.60	0.46 ± 0.01	
Raphe Nuclei	0.644± 0.03			
Heart	0.37 ± 0.06		0.69 ± 0.04	
Spleen	5.40 ± 0.51		1.40 ± 0.18	
Adrenals			254.00 ± 16.00	951.00 ± 50.00
Pineal	87.00 ± 7.0			
Mouse: Whole brain	0.58 ± 0.09	0.96 ± 0.03	0.41 ± 0.02	
Adrenals			106.00 ± 8.00	316.00 ± 16.00
Hamster brain stem	0.69 ± 0.03			

[a] S.E. is based on at least 10 samples.

TABLE 2.

Recovery[a]

	5-HT		NE		E		DA	
	$HClO_4$ %	Ethanol %	$HClO_4$ %	Ethanol %	$HClO_4$ %	Ethanol %	$HClO_4$ %	Ethanol %
.25 µg of amine added to rat brain homogenate	90	87	95	94	92	88	90	92
^{14}C labeled amine added to homogenate	89	85	84	85	85	90	89	89
.25 µg of amine added to rat brain supernatant	94	95	97	97	98	94	90	89
^{14}C labeled amine added to supernatant	95	92	96	99	96	97	93	99

[a]The mean recovery of each amine with a standard deviation of 1 - 6% is based upon three determinations (5 samples each time).

TABLE 3.

Effect of ascorbic acid on recovery of 5-HT, NE and DA

	5-HT µg/g	NE µg/g	DA µg/g
[a]Ascorbic Acid + EDTA	0.37 ± 0.01	0.40 ± 0.02	1.2 ± 0.1
[a]Only EDTA	0.37 ± 0.01	0.41 ± 0.01	1.3 ± 0.1

[a]Number of samples (N) = 5.

TABLE 4.

Percent elution of some other amines

Compound	HAc Elution (5 ml)	HCl Elution (3 ml)
Spermidine	6 %	64
Histamine	56	78
Tyramine	92	92
Tryptamine	50	82

TABLE 5.

Percent fluorescence interference of each amine at other amines' wavelengths

AMINE	5-HT 2780, 5450Å	DA 3160, 3700Å	NE 3306, 5028Å	E 3360, 5074Å
5-HT	100	0	1	0
DA	0	100	4	0
NE	0	<1	100	4
E	0	0	135	100
HISTAMINE	0	<1	0	0
SPERMIDINE	0	0	0	0
TYRAMINE	0	0	0	0
TRYPTAMINE	0	<1	0	0

Range of relative fluorescence intensities at indicated instrument settings of Turner spectrofluorometer

	5-HT		DA	NE		E
	0.6 X 3[a]	0.4 X 2	0.6 X 3	0.6 X 3	0.4 X 2	0.6 X 3
INSTRUMENTATION SETTINGS						
Excitation Bandwidth (Å)	250	250	250	250	250	250
Emission Bandwidth (Å)	150	150	150	150	150	150
Excitation Wavelengths (Å)	2780	2780	3160	3306	3306	3360
Emission Wavelengths (Å)	5450	5450	3700	5028	5028	5074
Fluorescence Sensitivity Range	X3	X10	X3	X3	X10	X10
ELUTION VOLUME (ml)	5.0	1.5	5.0	5.0	1.5	5.0
ALIQUOT VOLUME (ml)	1.0	0.5	0.6	0.6	0.4	0.6
RELATIVE FLUORESCENCE INTENSITY[b]						
250 ng Free base/ml	48.0-53.0	---	48.0-55.0	48.0-53.0	---	48.0-53.0
Standard Blank	0.6- 0.8		2.0- 4.0	1.0- 2.0		1.0- 2.0
Column Blank	0.8- 1.0		2.5- 4.5	1.2- 2.0		1.2- 2.0
Whole Rat Brain	22.0-27.3	---	35.4-49.6	23.0-29.0	---	---
Whole Mouse Brain	9.2-11.6	---	16.0-20.6	5.9- 8.9	---	---
Cat Hypothalamus	5.2-11.6	---	1.3- 5.4	14.4-23.2	---	1.3- 4.2
100 ng Free base/ml	---	67.5	---	---	63.1	---
Standard Blank	---	1.0	---	---	4.3	---
Column Blank	---	1.3	---	---	5.5	---
Hamster Brain Parts						
Medulla & Pons	---	17.1	---	---	21.1	---
Midbrain	---	11.2	---	---	10.5	---
Basal Ganglia	---	3.6	---	---	9.3	---
Cortex	---	27.8	---	---	44.0	---
Rat Pineal (Single)	---	25.1	---	---	---	---

[a] Column size (in cm)

[b] Fluorescence of tissue samples represents the aliquot of the total elution volume indicated in the table.

FIGURE LEGEND

FIGURE 1. Ethanol supernatant storage

FIGURE 2. Percent recovery of eacn amine at various pH's

FIGURE 3. Perchloric extracts

FIGURE 4. Ethanol extracts

FIGURE 5. Fractionation of each amine on a column size of 0.6 x 3 cm

FIGURE 6. Stability of the column eluates

FIGURE 7. Stability of the fluorophores

FIGURE 8. Influence of oxidation pH upon fluorescence intensity of dopamine

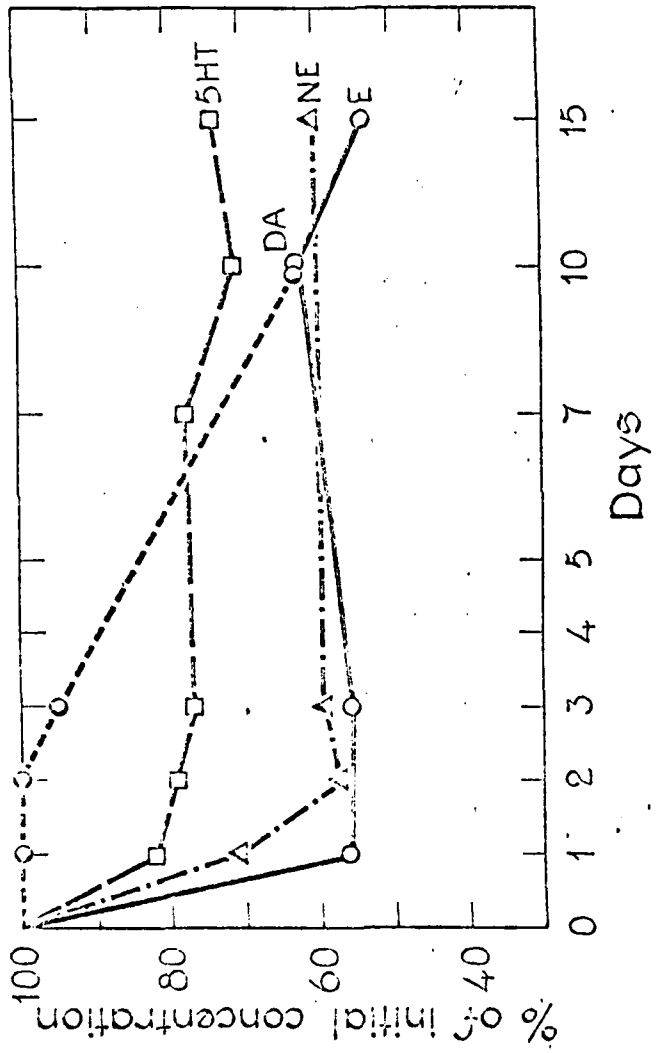

FIGURE 1. Ethanol supernatant storage

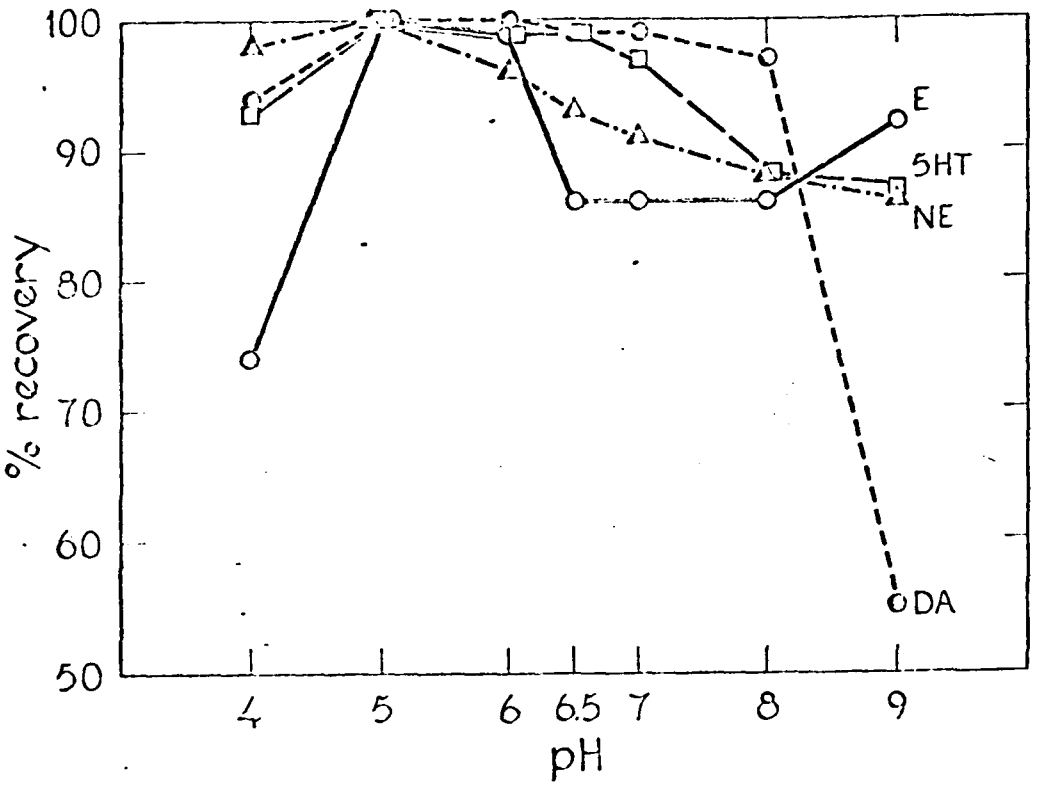

FIGURE 2. Percent recovery of each amine at various pH's

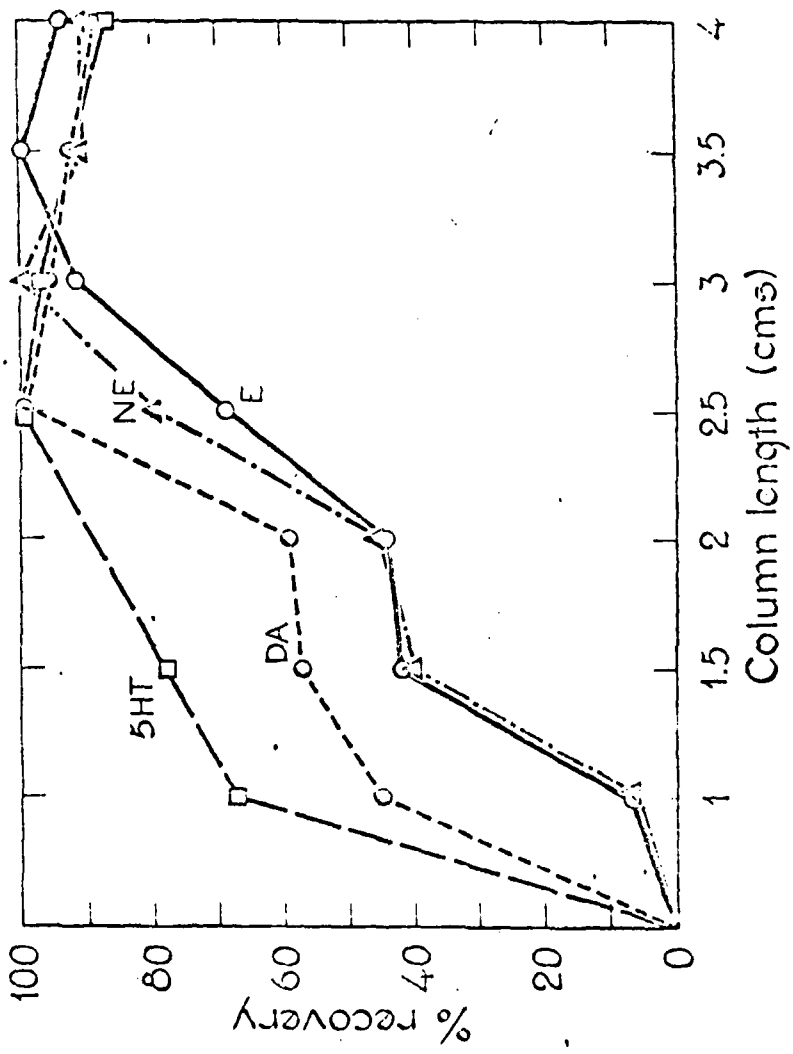

FIGURE 3. Perchloric extracts

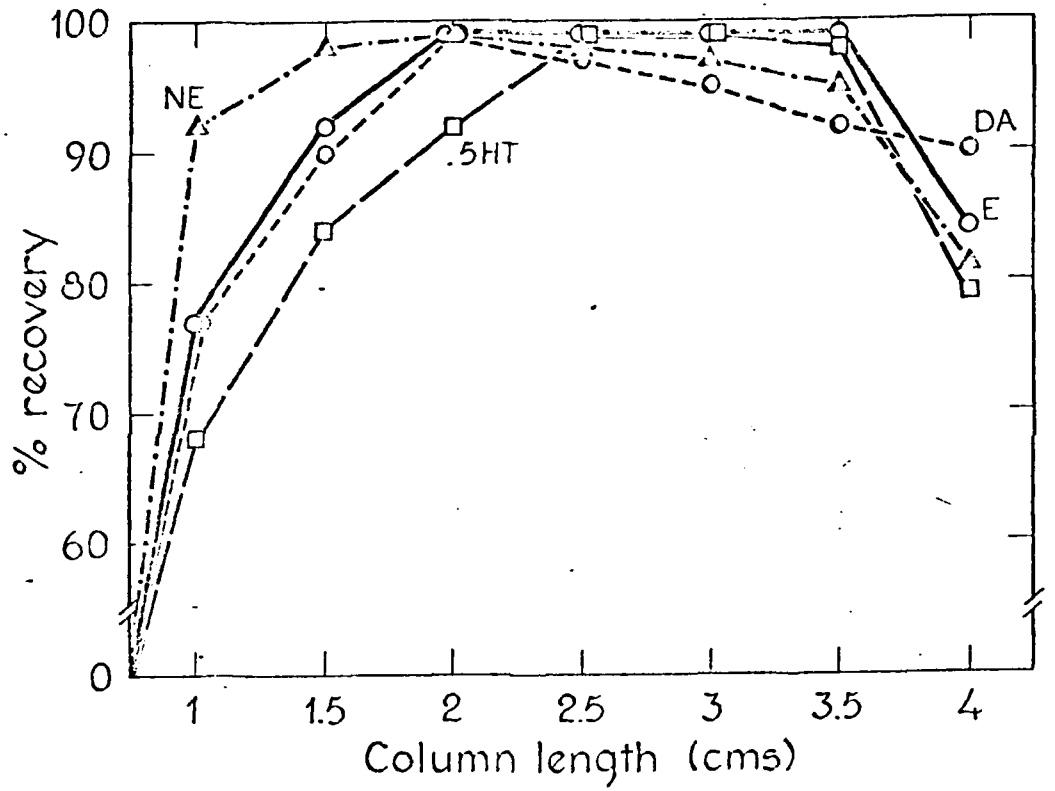

FIGURE 4. Ethanol extracts

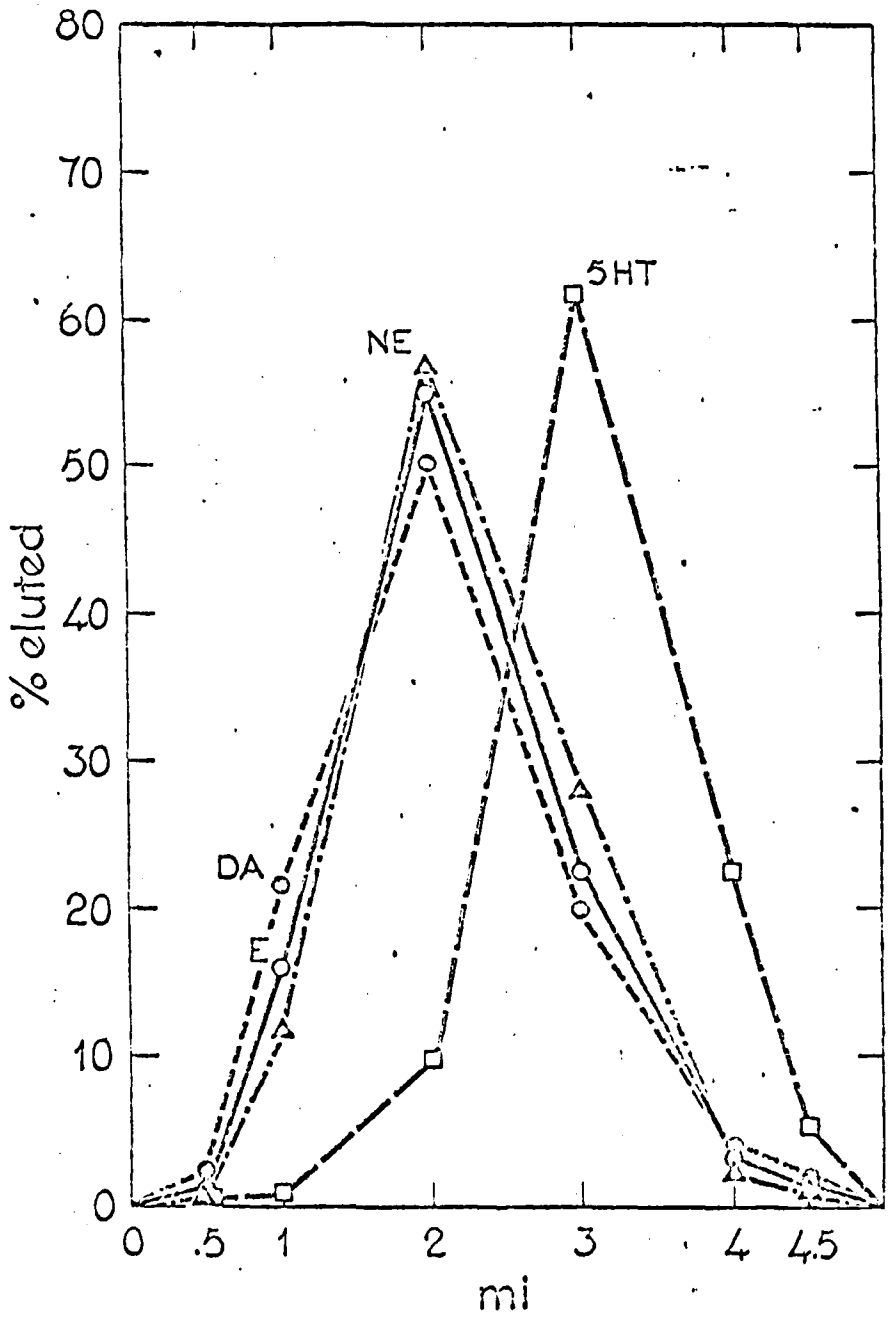

FIGURE 5. Fractionation of each amine on a column size of 0.6 X 3 cm

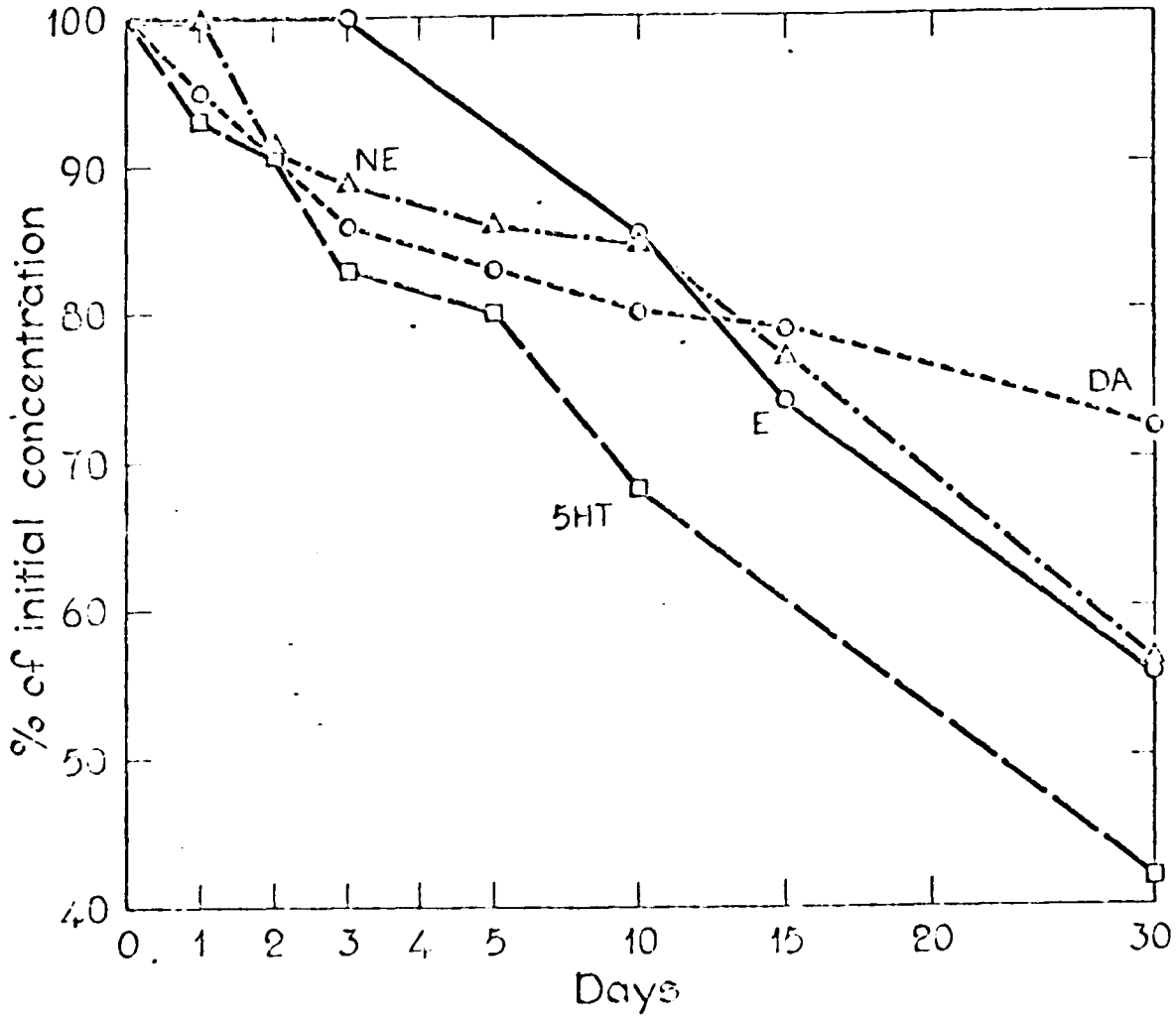

FIGURE 6. Stability of the column eluates

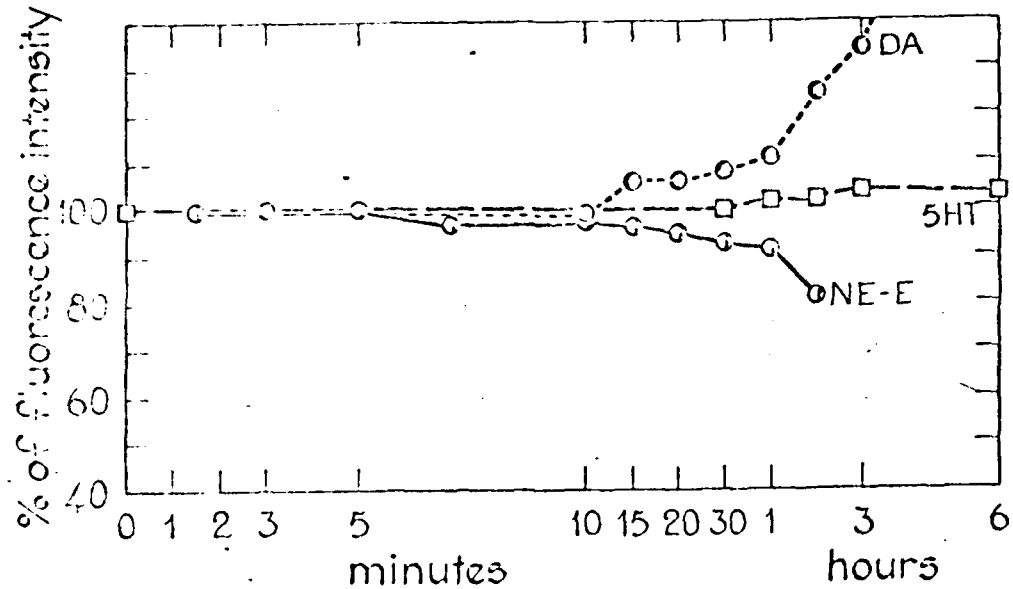

FIGURE 7. Stability of the fluorophores

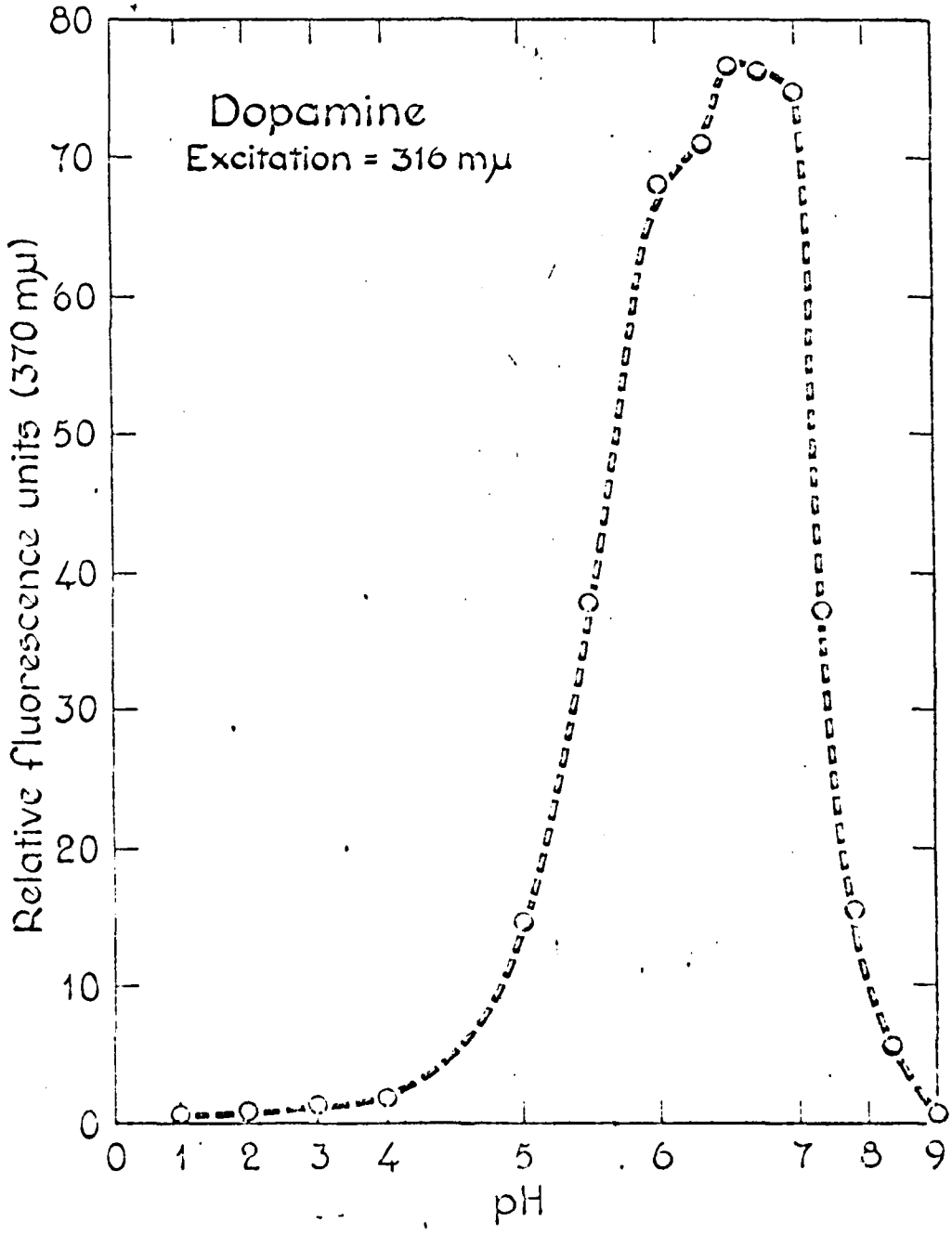

FIGURE 8. Influence of oxidation pH upon fluorescence intensity of dopamine


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