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R. ŠTUKOVSKY

Endokrinologisches Institut der Slowakischen Akademie der Wissenschaften, Bratislava (Tschechoslowakei), 22. August 1958.

Summary

A nomograph is presented, which gives (according to FISHER's formula) the pooled probabilities for the combination of two, three, and four independent experiments respectively.

PRO EXPERIMENTIS

A Method for the Separation and Estimation of Catechol Amines in Urine

Adrenaline, noradrenaline, and a third catecholamine, namely, hydroxytyramine are constituents of normal urine[1–3]. While biological methods are available for determination of catecholamines in blood and urine, these methods have obvious limitations for routine work and can only be employed for the determination of the pharmacologically active hormones, adrenaline and noradrenaline. A differential fluorometric method[4] has been used in the past for determination of all three catecholamines. In this method the catecholamines are estimated by a combination of the fluorometric methods of WEIL-MALHERBE and BONE[5] and of VON EULER and FLODING[6]. The former method estimates the sum of the three catecholamines. Adrenaline and noradrenaline are estimated separately by the latter, and hydroxytyramine is obtained by the difference in results between the two methods.

It seemed desirable to develop a method for simultaneous estimation of all three catecholamines. In previous experiments[7,8], it was shown that it is possible to acetylate catecholamines quantitatively in biological tissue and urine. These acetylated derivatives of adrenaline, noradrenaline, and hydroxytyramine have been shown to be stable, whereas the free compounds are known for their instability. In the procedure described below we acetylated the catecholamines in urine and separated the acetylated derivatives by paper chromatography.

Urine samples were acidified to pH 2 and hydrolyzed by refluxing at 100°C for 30 min. After cooling to room temperature, the urine sample was extracted four times with an equal volume of ethylacetate. The organic phase contained all the acidic catechols which could interfere with the separation of the catechol amines. The aqueous urine extract was adjusted to pH 4 by adding 0·5 *n* NaOH while stirring. The catechol amines in the extract were acetylated by adding 5 g $NaHCO_3$ and 2·5 cm^3 acetic anhydride in four equal portions with constant stirring until foaming ceased. Methylene chloride (50 ml) was added and stirring was continued for another 10 min. The mixture was transferred to a separatory funnel and the organic phase removed. The water was extracted three times with 50 ml of methylene chloride and the combined methylene chloride extracts were counter washed with 50 ml of water filtered through Na_2SO_4 and evaporated in a vacuum. The dry extract was dissolved in 0·5 ml of methyl alcohol and applied to Whatman No. 1 paper for chromatographic separation in the Bush system 'C'[9] using as solvents: toluene, ethyl acetate, methyl alcohol, and water in the ratio 10:1:5:5.

Table I
Comparison of R_f Values obtained after Paper Chromatography of Free Catechol Amines and their Triacetate Derivatives

Catecholamine	R_f Values	
	Free Compound*	Triacetate Derivative**
Noradrenaline	0·15	0·12
Adrenaline	0·28	0·50
Hydroxytyramine	0·33	0·82

* Solvent System: Phenol/HCl.
** Solvent System: Toluene: ethyl acetate: methyl alcohol.

Ascending chromatograms were developed after 24 h. The control area, which contained 100 γ of adrenaline acetate, noradrenaline acetate, and hydroxytyramine acetate, was developed by spraying first with 10% KOH in MeOH:H_2O (1:1) and then with a freshly prepared mixture of 1% $FeCl_3$ and 2% $K_3Fe(CN)_6$. Adrenaline acetate, noradrenaline acetate, and hydroxytyramine acetate were assumed to be opposite their control spots. These areas were cut out and a mixture of ethylenediamine and ethylenediamine hydrochloride was added to corresponding paper strips. A one step reaction was carried out by shaking and heating this mixture at 50°C, resulting in hydrolysis of the acetate derivative and condensation of the free compound with ethylenediamine. The fluorescent condensation product was extracted into isobutanol and a quantitative fluorometric determination using the WEIL-MALHERBE[5] method was carried out.

A comparison of the R_f values (see Table I), obtained from paper chromatography separation of the acetylated catecholamine derivatives and the free compounds, shows that an effective separation can be carried out by the use of this method. Another advantage of the method is the elimination of the adsorption of the catecholamines on alumina. Adsorption on alumina, which was an essential step in previous procedures, is known to be only a semi-quantitative operation.

A preliminary study of the acetylation method for the separation and determination of catecholamines has been completed on the urines of ten psychiatric patients with various diagnoses who were receiving different treatments. The results (see Table II) demonstrate a wide inter-subject variability among the psychiatric patients studied. Values obtained by this method are higher than those obtained by bioassay[10] or by specific fluorometric methods[6]. This may be the result of the elimination of alumina adsorption or may occur because of non-specificity of the condensation with ethylenediamine.

[1] P. HOLTZ *et al.*, Arch. exp. Path. Pharmakol. *200*, 356 (1942).
[2] U. S. VON EULER *et al.*, Biochem. J. *49*, 655 (1951).
[3] M. GOLDSTEIN and I. ABELIN, Helv. chim. Acta *39*, 158 (1956).
[4] H. WEIL-MALHERBE and A. D. BONE, J. clin. Path. *10*, 138 (1957).
[5] H. WEIL-MALHERBE and A. D. BONE, Biochem. J. *51*, 311 (1951).
[6] U. S. VON EULER and I. FLODING, Scand. J. clin. Lab. Invest. *8*, 288 (1956).
[7] M. HOGOPIAN, to be published.
[8] M. GOLDSTEIN, M. HOGOPIAN, and R. I. DORFMAN, IV. Int. Biochem. Congress, Vienna 1958.
[9] I. E. BUSH, Biochem. J. *50*, 370 (1951).
[10] U. S. VON EULER, Acta physiol. scand. *19*, 207 (1949).

Table II
Excretion of Noradrenaline, Adrenaline, and Hydroxytyramine in Various Psychiatric Patients

Subject	Noradrenaline (µg/100 mg creatinine)	Adrenaline (µg/100 mg creatinine)	Hydroxytyramine (µg/100 mg creatinine)
1	2·80	0·60	39·00
2	4·90	1·10	28·90
3	2·20	0·30	24·50
4	1·90	0·50	26·00
5	2·45	0·38	24·80
6	7·90	1·32	39·30
7	6·85	0·95	31·00
8	8·00	1·67	46·00
9	6·25	1·50	52·50
10	4·10	0·02	36·00

Further specificity can be obtained by using a double-label technique. Urine extracts can be acetylated with tritium labeled acetic anhydride to convert adrenaline, noradrenaline, and hydroxytyramine quantitatively to the tritium labeled triacetate. A measured amount of authentic triacetate adrenaline-C^{14}, triacetate noradrenaline-C^{14}, and triacetate hydroxytyramine-C^{14} is added to each sample and the double labeled catecholamines are identified and purified by paper chromatography. The tritium and carbon^{14} content of the purified catecholamines is assayed by simultaneous counting in a liquid scintillation spectrometer. The amount of adrenaline, noradrenaline, and hydroxytyramine in the original extract can be calculated from the determination of the amount of carbon^{14} indicator lost during the purification, the yield of tritium radioactivity, and the specific activity of the tritium labeled acetic anhydride.

M. Goldstein, A. J. Friedhoff, and C. Simmons

New York University College of Medicine, Department of Psychiatry and Neurology, Psychopharmacology Research Unit, New York, November 3, 1958.

Zusammenfassung

Es wird eine neue Methode zur Bestimmung der Katecholamine im Harn beschrieben. Die Katecholamine werden im Harn acetyliert und die Acetylderivate papierchromatographisch im Bush-«C»-System getrennt. Nach erfolgter Trennung werden die einzelnen Katecholamine mit Äthylendiamin kondensiert und fluorimetrisch untersucht. Eine Spezifitätserhöhung der Methode durch Anwendung doppelt markierter Isotopen wird in Betracht gezogen.

Informations - Informationen - Informazioni - Notes

THEORIA

Thermal Polymerization of Amino Acids and a Theory of Biochemical Origins[1]

By S.W. Fox, Kaoru Harada, and A. Vegotsky[2]

The possibility of synthesizing peptides and perhaps protein (Fox and Middlebrook[3]) by simple heating of unsubstituted amino acids is one which has not been favored by chemical experience (Katchalski[4], Noguchi and Hayakawa[5], Curphey[6], Meggy[7], Bamford, Elliott, and Hanby[8]). When this goal was approached as a theoretical problem in prebiological molecular evolution, it became possible to visualize how the energetic barrier for the synthesis of protein could be overcome thermally (Fox, Johnson, and Vegotsky[9]), and how precise ordering of residues might result from selective influences of the reactant amino acids themselves (Fox[10]). The experiments which have resulted indicate that it is possible to prepare copolymeric peptides thermally if aspartic acid or glutamic acid is a reactant. It is furthermore possible, by using a considerable molar excess of both aspartic acid and glutamic acid, to copolymerize the eighteen common amino acids into a *proteinoid* which, after being hydrolyzed, reveals on paper chromatography the same qualitative composition as casein and other proteins.

In considering this knowledge in detail, attention should first be focussed on the chemistry of each of the dicarboxylic amino acids. It has long been known that heating glutamic acid alone yields the inner lactam, pyrrolidonecarboxylic acid (pyroglutamic acid). In this research it has been learned that heating glutamic acid with each of a number of other amino acids produces a copolymeric linear peptide when the temperature of reaction is 170°C for 3 h (Harada and Fox[11]). The polymer of glutamic acid and glycine has a mean chain weight of 10,000 to 20,000 after dialysis. During such reactions, the glutamic acid is converted to pyroglutamic acid which is molten in the presence of other materials at 170°C, and functions therefore as a solvent. The glutamic acid and pyroglutamic acid probably contribute also as acid catalysts. In addition, comparative yields suggest that the pyroglutamic acid is the more reactive species.

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[2] The Oceanographic Institute and Department of Chemistry of The Florida State University, Tallahassee.

[3] S. W. Fox and M. Middlebrook, Fed. Proc. *13*, 211 (1954).

[4] E. Katchalski, Adv. Protein Chem. *6*, 123 (1951).

[5] J. Noguchi and T. Hayakawa, J. Amer. chem. Soc. *76*, 2846 (1954).

[6] E. G. Curphey, Chem. & Ind. *1956*, 783.

[7] A. B. Meggy, J. chem. Soc. *1956*, 1444.

[8] C. H. Bamford, A. Elliott, and W. E. Hanby, *Synthetic Polypeptides* (Academic Press, New York 1956).

[9] S. W. Fox, J. E. Johnson, and A. Vegotsky, Science *124*, 923 (1956).

[10] S. W. Fox, Amer. Scientist *44*, 347 (1956).

[11] K. Harada and S. W. Fox, J. Amer. chem. Soc. *80*, 2694 (1958).