EXPERIENCES WITH THE FLUOROMETRIC METHOD FOR DETERMINATION OF THE PYRIDINE NUCLEOTIDES IN BLOOD

WITH SOME DETERMINATIONS ON BLOOD FROM PSYCHIATRIC PATIENTS

by

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In 1947 LEVITAS *et al.*[1] described a rapid and elegant method for the determination of the total pyridine nucleotides in blood. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) when treated with alkali and acetone at room temperature and subsequently heated with acid form a fluorescent compound, the fluorescence of which is within a certain range proportional to the amount of pyridine nucleotide. The method is more rapid than the commonly employed procedures (microbiological, fermentative, spectrophotometric), and the authors state that in duplicate determinations the method gives uniformly reproducible results, but they do not give figures for the degree of this reproducibility. By following closely the procedure of LEVITAS *et al.*, it has not been possible to attain a satisfactory agreement between duplicate determinations. Introduction of some minor alterations in the procedure, however, led to a considerable decrease of the experimental error.

APPARATUS AND CHEMICALS

A Beckman Model DU Quartz Spectrophotometer with Fluorescence Accessory Set was used. Diaphragm from tungsten lamp housing 10 mm diameter. The exciting radiation was filtered through a Corning glass filter No. 5860 (old glass code 586) with maximum transmission at 3650 Å. The fluorescent light passed through a combination of Corning glasses No. 3389 and 4308 (old glass codes 038 and 428) with maximum transmission at 5000 Å. The fluorescence of the unknown sample was read at the transmission scale as per cent of the standard quinine sulphate fluorescence, in the following indicated as r.u. (relative units).

The reagents were prepared exactly as prescribed*. The saturated solution of NaOH was made from EKA-reagent sodium hydroxide pellets, with max. contamination of Fe 0.001% and of other heavy metals 0.000%. Hematocrit-determinations were made in duplicate and gave an average of 46% for men and 42% for women.

RESULTS

By addition to blood filtrate of DPN (of known purity), resp. of N'-methylnicotinamide, LEVITAS *et al.* found the ratio: Galvanometer divisions per γ DPN/galvanometer divisions per γ N'-methylnicotinamide ion, to be $15/38 = 0.395$. With the apparatus and chemicals employed here the corresponding ratio: r.u. per γ DPN/r.u. per γ

* The N'-methylnicotinamide chloride was placed at my disposal by Dr. W. A. PERLZWEIG, to whom I am most grateful.

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N'-methylnicotinamide ion was: 0.654*. In blood filtrates the fluorescence produced was a linear function of the added N'-methylnicotinamide in at least the range 0.2 to 3.0 γ.

The calculations were carried out as given by LEVITAS *et al.*:

Hematocrit = H%

0.5 ml blood filtrate (= 0.1 ml blood = 0.1·(H/100) ml erythrocytes)	F r.u.
Recovery (0.5 ml filtrate + 0.5 γ N'-methylnicotinamide)	R r.u.
Blank	B r.u.

$$\frac{(F-B)\cdot 0.5\cdot 100}{(R-F)\cdot 0.654\cdot 0.1\cdot H}\ \gamma \text{ total PN per ml erythrocytes}^{**} \qquad (1)$$

For normal blood R and F were found to be about 50–60 and 40–50 r.u. B always turned out to be zero r.u. It was, however, necessary to introduce another blank, determined in a sample containing 1.0 ml of water and 0.5 ml of acetone. Even after redistillation over $KMnO_4$ this blank, A, was never found below 1 r.u. and seldom above 3 r.u. A is introduced in formula (1) in the place of B. It has only to be determined for each new preparation of permanganate-destilled acetone.

As will be seen from (1) it is not the absolute values of F, R and A, but their differences, (F — A) and (R — F), that are applied in the calculations. Since F and R are rather close to each other, small variations in these will result in great variations in the final value. For that reason it is useful to add 2.0 γ of N'-methylnicotinamide instead of 0.5 γ in the recovery (now marked R'). This changes (1) to:

$$\frac{(F-A)\cdot 2.0\cdot 100}{(R'-F)\cdot 0.654\cdot 0.1\cdot H}\ \gamma \text{ total PN per ml erythrocytes} \qquad (2)$$

In an experiment with ten parallel determinations on the same blood filtrate addition of 0.5 γ, resp. 2.0 γ N'-methylnicotinamide in the recovery resulted in relative standard deviations (coefficients of variation) of 14, resp. 7% of the mean.

When addition of 2.0 γ in the recovery is used it may occasionably happen — if pyridine nucleotide concentrations or hematocrit values are exceptionally high — that the recovery fluorescence exceeds the range of the transmission scale (110%). The only thing then necessary is to adjust the sensitivity to 90 instead of 100% for the quinine standard. The absolute values of F, A and R' are thus multiplied by 0.9, while the final result remains the same, with only a negligible decrease of accuracy.

It was observed that great care is necessary in timing the condensation at alkaline reaction ('5 minutes at room temperature'). As will be seen from Fig. 1 A this is due to the slope of the curve in that region. It is therefore advisable to extend the 'alkali-time' to 15.0 minutes, as an approximate plateau is then reached; hence small offences against the timing are of less importance. It is peculiar that the fluorescence yield is greatest at room temperature (20–22° C) and lower at 0° and 30° (Fig. 1 B and C).

LEVITAS *et al.* state that the fluorescence remains stable in the daylight or in the dark for at least 3 days. This has not been the case with the reagents employed here. As Fig. 2 shows there is a considerable decrease of fluorescence in diffuse daylight and

* The DPN-preparation, kindly supplied by Dr. OLOV LINDBERG and analysed by Dr. A. KORNBERG by the method of HORECKER AND KORNBERG[3], was of 28% purity.

** For convenience DPN + TPN + possible other reacting substances in the blood (*e.g.*, N'-methylnicotinamide) will be designated as 'total PN' (total pyridine nucleotides).

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a slight increase in dark. The fluorescent samples should be kept in dark and read within 24 hours. It was confirmed that the blood filtrate is stable for about a week if kept in the refrigerator.

Heparin was used as anticoagulant; even in amounts many times those commonly used this compound does not influence the hematocrit or the fluorescence values. The tubes should be closed with glass stoppers, because corks influence the fluorescence to a moderate and rubber stoppers to an appreciable extent. With the procedure outlined above an average relative standard deviation of 5.6% was obtained in the daily routine parallel-determinations. With triplicate determinations this gives an average relative standard error of $5.6/\sqrt{3} = 3.2\%$ of the mean.

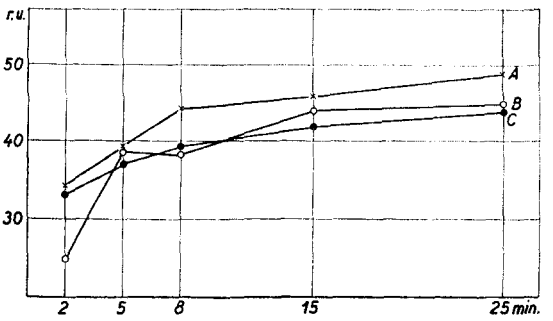

Fig. 1. Dependence of fluorescence on condensation-time in alkali. Ordinate: Fluorescence in r.u. Abscissa: 'Alkali-time' in minutes. *A*: Room temperature (20–22°); *B*: 0°; *C*: 30°

The results of 35 analyses performed on blood from 20 adult persons are shown in Table I. The subjects were physically and mentally healthy and, in particular, did not present symptoms of pellagra. They were all in the postabsorptive state, and none of the women were menstruated at the time of drawing the sample. The table reveals rather great variations, both between different individuals and in the same individual at varying times, even with few hours' interval. The inter-individual standard deviation is somewhat higher than that of LEVITAS *et al.* (9 γ, resp. 7 γ)*. No relation is found to sex and no dependence on age in the range investigated (23 to 50 years). Total pyridine nucleotides were determined in the blood from 7 patients suffering from endogenous depression, 2 patients with mania and 8 schizophrenics. Their values were in close accordance with the normal values. (Ranges: 59–87 γ, 68–71 γ, and 57–78 γ total PN per ml erythrocytes).

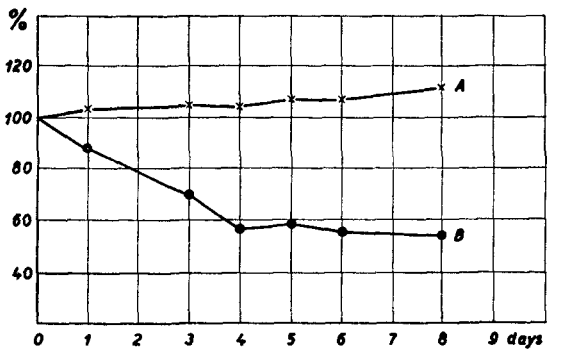

Fig. 2. Stability of fluorescent condensation-product at room temperature. Ordinate: Per cent of initial fluorescence. Abscissa: Time in days. *A*: Sample kept in dark. *B*: Sample kept in diffuse daylight

Analyses on the blood from 3 schizophrenics before and during insulin coma did not reveal reliable changes (67 and 51 γ, 78 and 85 γ, resp. 74 and 80 γ); neither did the level change in a normal subject after ingestion of 100 g of glucose dissolved in 200 g of water (before: 74 γ, after: 74 γ).

* When computing the inter-individual statistics only one single value for each individual should be used (the average value for those with more than one determination). If all determinations were taken into account a bias would be introduced when several analyses were made on a subject with excessively high or low level (*e.g.*, E.H. in this material). Calculation of the figures of LEVITAS *et al.* in this way gives a mean of 78 γ and a standard deviation of 7 γ.

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TABLE I

TOTAL PYRIDINE NUCLEOTIDES (EXPRESSED AS DPN) IN ERYTHROCYTES (γ PER ml) OF 20 NORMAL INDIVIDUALS

Subject	Sex	Age	Date	Total PN	
E.S.	M.	38	Sept. 29	71	
A.P.	M.	36	Sept. 30	71	
M.S.	M.	29	Oct. 11, 9 a.m.	77	
—	—	—	Oct. 11, 11 a.m.	74	
—	—	—	Oct. 11, 5 p.m.	84*	
—	—	—	Oct. 11, 11 p.m.	70*	
—	—	—	Oct. 12, 4 a.m.	78	75
—	—	—	Nov. 18	71	
—	—	—	Dec. 2, 9 a.m.	74	
—	—	—	Dec. 2, 10 a.m.	74**	
O.S.	M.	34	Nov. 12	95	
E.H.	F.	27	Oct. 4	49	
—	—	—	Oct. 11	73	
—	—	—	Oct. 18	42	56
—	—	—	Nov. 15	59	
J.M.L.	M.	41	Nov. 12	83	
W.Z.	F.	29	Nov. 15	68	
E.C.	F.	39	Nov. 19	71	
L.E.	F.	48	Nov. 20	61	
E.G.	M.	34	Nov. 20	78	
H.O.	M.	43	Nov. 21	65	
A.M.E.	F.	49	Nov. 26	66	
K.A.	F.	50	Nov. 26	68	
H.P.	M.	49	Nov. 27	71	
E.F.	F.	47	Nov. 27	62	
A.W.	M.	23	Dec. 3	74	
S.H.	F.	34	Dec. 3	72	
E.S.	M.	49	Dec. 4	54	
O.G.	M.	42	Dec. 4	66	
K.E.	M.	38	Oct. 12	69	
—	—	—	Oct. 13	65	
—	—	—	Oct. 14	68	70
—	—	—	Oct. 15	71	
—	—	—	Oct. 16	81	
—	—	—	Oct. 17	68	

* Has eaten common meals.
** ½ hour before ingested 100 g of glucose.

Range	42–95
Average (inter-individual)	70
Standard Deviation (—)	9

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SUMMARY

The fluorometric method of LEVITAS *et al.* for the determination of the pyridine nucleotides in blood is adapted to the Beckman fluorometer. In order to enhance the reproducibility it is proposed to extend the time for condensation with acetone in alkali to 15.0 minutes and to increase the amount of N'-methylnicotinamide in the recovery to 2.0 γ. With these and some other modifications it is possible to reduce the relative standard deviation in parallel determinations to 5.6%.

Analyses on blood from psychiatric patients did not show deviations from the normal level.

RÉSUMÉ

La méthode fluorométrique de LEVITAS *et col.* pour la détermination des pyridine-nucléotides dans le sang est adaptée pour le fluoromètre de Beckman. Afin d'augmenter la reproductibilité, il est proposé de porter le temps de condensation avec l'acétone en solution alcaline à 15.0 minutes, et d'utiliser pour la reprise une quantité de N'-méthylnicotinamide égale à 2.0 γ. Au moyen de ces modifications et de quelques autres, il est possible, dans des déterminations parallèles, de ramener la déviation standard relative à 5.6%. Des analyses faites sur le sang de malades mentaux montrent que dans ce domaine celui-ci est identique au sang normal.

ZUSAMMENFASSUNG

Die fluorometrische Methode von LEVITAS *et al.* zur Bestimmung der Pyridinnukleotide im Blute wurde an das Bechman'sche Fluorometer angepasst. Um die Reproduzierbarkeit der Ergebnisse zu verbessern soll die Dauer der Kondensation mit Aceton in Alkali auf 15.0 Minuten ausgedehnt und die Menge von N'-Methylnikotinamid auf 2.0 γ erhöht werden. Mit Hilfe dieser und einiger anderer Abänderungen gelingt es, die durchschnittliche relative Abweichung auf 5.6% herabzudrücken.

Blutanalysen von Geisteskranken zeigten hier keine Abweichungen von normalem Blut.

REFERENCES

1 N. LEVITAS, J. ROBINSON, F. ROSEN, J. W. HUFF, AND W. A. PERLZWEIG, *J. Biol. Chem.*, 167 (1947) 169.

2 B. L. HORECKER AND A. KORNBERG, *J. Biol. Chem.*, 175 (1948) 385.

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