Betaninuria in Childhood

By

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A few hours after the consumption of beetroot, certain persons excrete violet pigment with the urine [9, 2], a phenomenon often mistaken for haematuria. Beetroot *(Beta vulgaris* var. *esculenta)* contains betanin, a red pigment occurring in Centrospermae, which is chemically different from anthocyanins, the reddish-violet-blue pigments of most plant species. Betanin is an alkaloid, containing a di-hydro-indole ring linked through a C_2-chain, further a partially hydrated pyridine ring [6, 7] (Fig. 1).

Even the few existing reports on betaninuria are contradictory as regards the responsible factors. According to certain authors, everybody may excrete betanin; some persons require smaller, others larger doses [13]. Zindler and Colovos [15] attribute the phenomenon to food allergy. Allison and McWhirter [1] then stated that, genetically, a healthy human population falls into two parts, excretors (+) and non-excretors (—); in excretors the betaninuria is caused by the presence of a
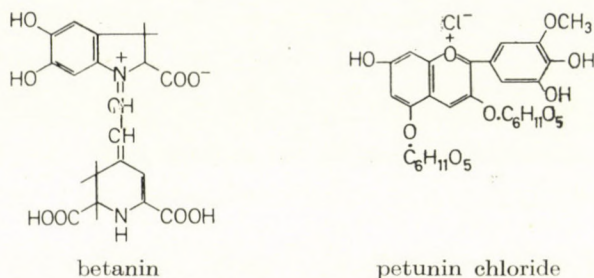

betanin   petunin chloride

Fig. 1. Structure of betanin, the most important betacyanin; and that of petunin chloride, a characteristic anthocyanin

Excretion of betanin following ingestion of beetroot is called betacyaninuria or beeturia; we suggest the more accurate term betaninuria.

single autosomal recessive gene so that such persons are homozygous for the so-called *bt* gene. Relying on the simple inspection of urine,

among 104 persons these authors found 10 patients positive for the character at issue, and supported their theory by the examination of parents and offsprings in 11 families. An editorial of the British Medical Journal, published in 1957 [3], regarded this report so important that it expatiated upon the role of the hypothetical gene in the matter of selection and its possible significance in evolution. PENROSE [8] refused to draw such far-reaching conclusions. Relying on the genetic theory SALDANHA et al. [10, 11] examined Dutch immigrant families in Brazil, and it is only with certain reservations that their results can be reconciled with the hypothesis of simple recessive inheritance. The frequency of betaninuria among 118 persons amounted to 8 or 39 per cent according to which shade of urinary colour was classified as positive by visual estimation; there was no significant difference between males and females. In a subsequent study, SALDANHA et al. [12] examined pigment excretion among a Japanese, a Negro and a white population. The number of positive individuals among the Japanese was significantly larger than among white people, although as regards age the examined groups were inhomogeneous (extreme values, 7 and 53 years), while in mean age the groups showed likewise great differences (14.6, 31.9 and 23.9 years, respectively).

Current theories on the development of betaninuria may be divided into two groups.

(i) Disturbed denaturalization. Ac-

cording to this theory, betaninuria is a congenital disturbance of pigment decomposition [1, 12].

(ii) Inhibited absorption. According to this theory, urinary excretion of betanin depends on the degree of intestinal absorption. This view is shared by WATSON et al. [13, 5, 14] who tried to justify it by the results of HORWITT's [4] earlier animal experiments; this author administered anthocyanin to animals by the intravenous route and observed its unaltered excretion with the urine.

The influence of environmental factors was also emphasized by WATSON et al. [13] who studied the effect of iron deficiency on the development of betaninuria. It is evident that the aetiology of betaninuria and the mechanism of the phenomenon are still controversial.

Since the methods employed so far were unreliable and contained a number of subjective elements (the amount of ingested beetroot varied, and with a few exceptions estimations were based on the mere inspection of urine, results were qualitative and therefore incomparable. The present study was undertaken to elaborate a method which would yield reliable quantitative data.

MATERIAL AND METHOD

To eliminate sources of error inherent in age and weight of the examined individuals, we studied betaninuria in kindergarten children between 3 and 6 years of age.

Instead of feeding whole beetroot with a variable pigment content, we made the children drink the juice of cooked beetroots which was always prepared in the same
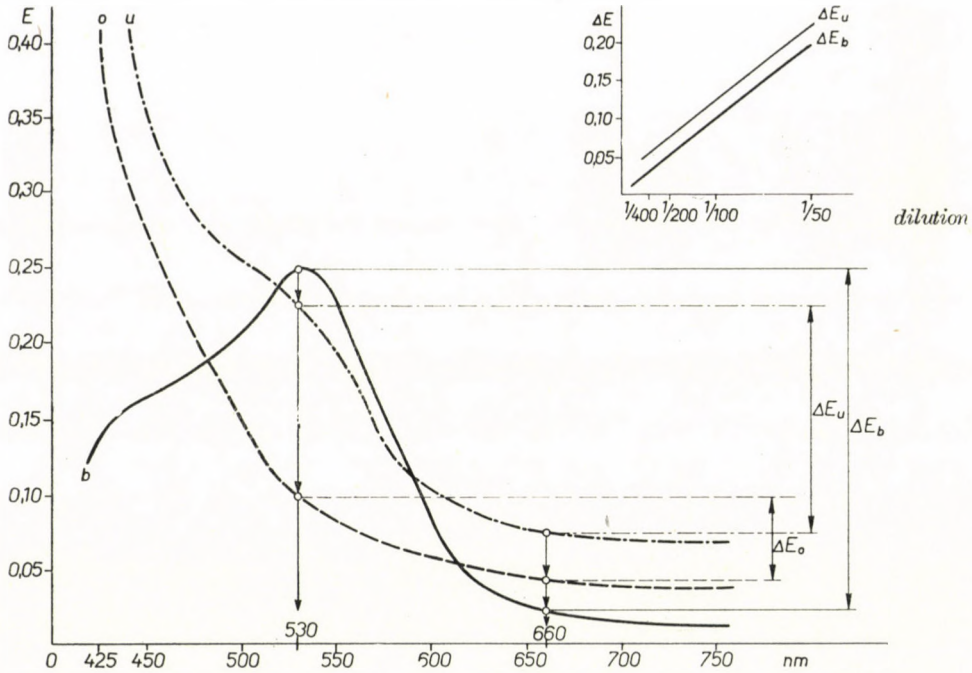
FIG. 2. Schematic illustration of the photometric determination of Δ E-values. b = diluted beetroot juice; o = extinction curve of a negative urine sample; u = curve of beetroot juice-urine mixture

FIG. 2a. Δ E-values (ΔE_b) of pure beetroot juice (dilutions from 1 : 50 to 1 : 400) and equally diluted beetroot juice mixed with urine (ΔE_u)

place and in the same manner. This allowed a standard dosage and the pigment was found to appear quicker in urine than after ingestion of whole beetroot.

Beetroots were cooked, cut into small pieces and soaked in sugared vinegar for 24 hrs which ensured a satisfactory elution of the pigment. The pigment contents of juices so prepared showed not more than 10% fluctuation.

Urine samples were collected immediately before the test. In order that the differences between the examined children should be as small as possible in respect of the degree of hydration, they received identical quantities of fluid for breakfast. Two hours later they were made to drink 100 ml of the juice and then they were allowed no food or fluid until the collection of samples. As the age limits were narrow in the

examined population, it seemed unnecessary to adjust the doses individually.

In the first informative experiments, urine samples were collected 150 and 300 min. after the ingestion of beetroot juice. As fractions sampled at or around 150 min. were optimal, subsequent collections were made between 120 and 150 min. Volume and pH of the discharged urine were determined and 20 ml samples were used for pigment estimation. If this did not take place within an hour, the samples were stored at $+5\,^\circ$C and placed into a water bath of 35 to $40\,^\circ$C immediately before estimation. Turbidity ceased in the water bath without damage to the betanin; those few samples which had still remained turbid were discarded.

Visual estimation was also made in some cases in test tubes filled to identical

heights. Those — seen from above — with a violet tint were classified doubly positive $(++)$, those with an orange red colour were regarded as positive $(+)$; yellow samples counted as negative $(-)$. A Pulfrich photometer was used, with 1 cm cuvettes.

RESULTS AND DISCUSSION

Betanin shows maximum extinction at about 535 nm. Estimation was, however, made difficult by the proper colour of urine. It was attempted to recover betanin from urine by means of solvents which do not mix with water, but none of the ten solvents with different polarities dissolved the betanin out of the aqueous phase. It was therefore necessary to determine the extinction of betanin at different wavelengths, i.e. in an indirect manner. Fig. 2 is a schematic illustration of the procedure.

In Fig. 2 it can be seen that curve (b) with a peak at about 530 nm, has a character quite different from that of curve (o) which shows exponentially decreasing E-values, while curve (u) at about 530 nm shows an inflection owing to the two other curves. The technique of estimation was then as follows. We determined E at 530 nm for the indication of betanin concentration, then at 660 nm, where betanin practically ceases to absorb light. This latter value was used for correction: $\varDelta E = E_{530} - E_{660}$. With pure beetroot juice, $\varDelta E$ is in linear correlation with the concentration of betanin (Fig. 2a) and can therefore be used for direct quantitative measurement. On its basis was compared the concentration of juices to be ingested.

In this respect it has, however, to be borne in mind that the value E_{660} used for correction is not identical with, but only proportional to, the value for the urinary colour determined at 530 nm ($\varDelta E = E_{530} - E_{660} > 0$ even in cases of negative urine). Value $\varDelta E$ is, thus, composed of two factors; one is the E of urinary pigmentation which cannot be subjected to correction (E_o), the other the E of betanin (E_b), so that $\varDelta E_u = \varDelta E_o + \varDelta E_b$. The ratio of the two factors varies; with negative urines $\varDelta E_b = 0$, hence $\varDelta E_u = \varDelta E_o$ which gives a value of 0.01 to 0.03 in the case of light-coloured urines, while it may amount to 0.10 and even more with intensively pigmented orange-yellow urines. Although the colour of negative urines varies from individual to individual (different $\varDelta E_o$ values), this variation shows a normal distribution and can be evaluated statistically. Since the analysis of a sufficient number of urine samples collected after the ingestion of beetroot juice yields statistically evaluable figures, it is possible to determine whether the distribution of the $\varDelta E$ values corresponds to the norm, i.e. whether it is the same as that of negative urines or different from the normal distribution.

In 244 children aged 3 to 6 years after the ingestion of beetroot juice the empirical frequency distribution of $\varDelta E$-values at the 1 % level showed a significant deviation from the normal distribution ($\chi^2 = 29.98$; $n = 244$; $\chi^2_{P=1\%} = 27.7$). It can be seen from Fig. 3 that the empirical frequency distribu-

No. of samples

$$\bar{x} = 0.077\,E$$
$$s = 0.0383$$
$$x^2 = 29.98\ (x^2_{p=1\%} = 27.7)$$
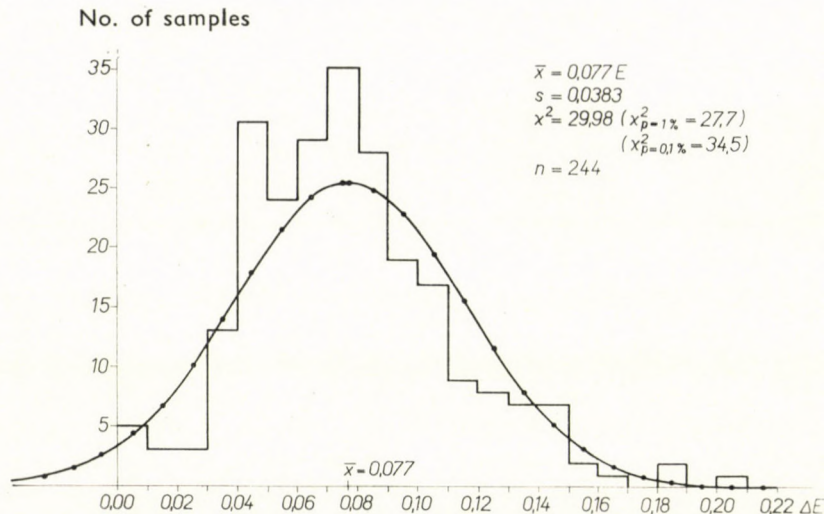$$(x^2_{p=0.1\%} = 34.5)$$
$$n = 244$$

$$\bar{x} = 0.077$$

FIG. 3. Empirical frequency distribution of Δ E-values, and χ^2-test of normal distribution. Diagrammatic illustration

tion had two maxima, one at E = = 0.045 and one at E = 0.075. That the deviation from normal distribution was due to this bimodality is proved by the fact that by subtracting the value χ^2 (i.e. 9.59) of the class which corresponds to the first maximum, from the value of χ^2 as computed in the aforesaid manner, we obtain the χ^2-value of a modified population (20,39), which is, in this way, smaller than the χ_2-value at the
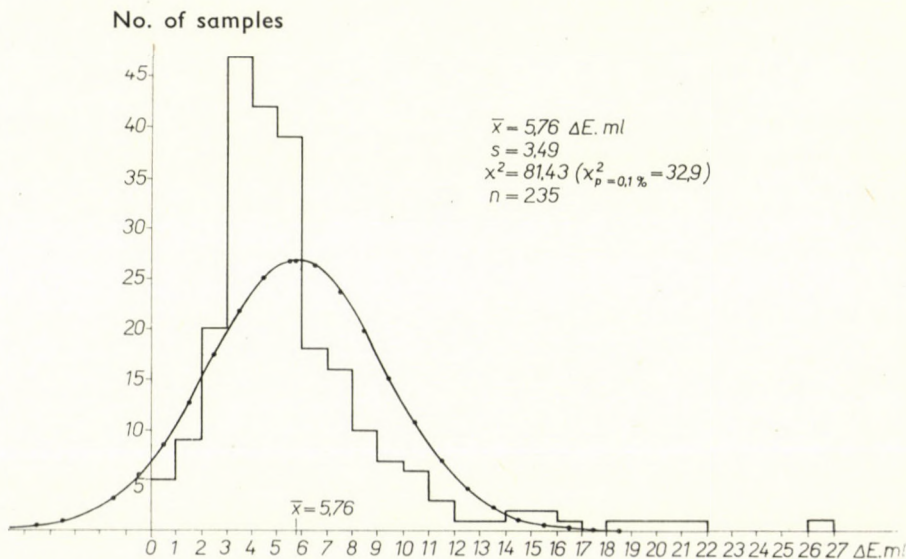
No. of samples

$$\bar{x} = 5.76\ \Delta E.\,ml$$
$$s = 3.49$$
$$x^2 = 81.43\ (x^2_{p=0.1\%} = 32.9)$$
$$n = 235$$

$$\bar{x} = 5.76$$

FIG. 4. Diagrammatic illustration showing the empirical frequency distribution of ΔE· ml-values, and the χ^2-test of normal distribution

P=5% level (i. e. 22.4) so that the population without this maximum would show a normal distribution.

We studied three of the factors affecting the concentration and the light absorption of urinary betanin, *viz.* the time factor, the volume and the pH of urine. In the first series of examinations urine samples were collected from 45 children 150 min. and 300 min. after the ingestion of beetroot juice. In spite of considerable individual differences in ΔE at 150 min. and at 300 min., the average $\overline{\Delta E}$-values for the entire examined group were not significantly different; the values for the earlier samples were even somewhat higher than those of the later ones ($\overline{\Delta E}_{150 \text{ min.}} = 0.077$ and $\overline{\Delta E}_{300 \text{ min.}} = 0.070$). It is thus clear that under the given experimental conditions betanin appeared in the urine already 2 1/2 hrs after the consumption of juice, and excretion showed no significant change after another 2 1/2 hrs. The few urine samples collected before 150 min. admitted of no statistical evaluation, neither did we find any correlation between the value for pH and that for ΔE in the examined 32 samples. In subsequent examinations urine was therefore sampled between 2 and 2 1/2 hrs. and evaluations were made without regard to the time factor within these limits or the pH-value.

There was a significantly negative correlation between urinary volume of 236 individuals and the value for ΔE above the $P = 0.1\%$ level. The correlation coefficient r was, thus, a reliable indicator of the correlation in question, in spite of its absolute value having been as low as $r = 0.406$ ($n = 236$; $r_{P = 0,1\%} = 0.321$). This follows from the fact that the value for ΔE depends not solely on urinary volume. However, the mere existence of the correlation justified its closer examination.

We computed in 235 children the product of the urinary output in ml and ΔE, i.e., $\Delta E \cdot$ ml, a value proportional to the amount of betanin excreted before the 150 min. sampling. ΔE, in itself, is proportional to the concentration of betanin. The χ^2-test proved that the empirical frequency distribution of the examined population was very significantly (much above the $P = 0.1\%$ level) different from the normal value ($\chi^2 = 81.43$; $n = 235$; $\chi^2_{P=0.1\%} = 32.9$). (See Fig. 4.)

With a view to elucidating the reason for this great deviation from the normal distribution we prepared a diagram showing the empirical frequency distribution of urinary volumes of the population. It can be seen in Fig. 5 that the distribution is obviously skew to the right. It follows that the volume factor was responsible for the peculiar distribution in the case of $\Delta E \cdot$ ml.

Since the value $\Delta E \cdot$ ml, indicating the amount of betanin, depended rather on the volume of urine than on the value ΔE, the product did not truly characterize the betaninuria. ΔE alone (indicating the concentration of betanin) was a more suitable index.
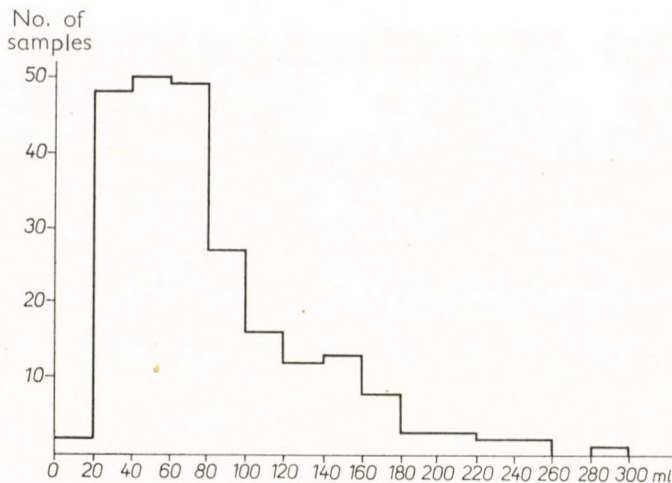
FIG. 5. Empirical frequency distribution (ml) of urinary volume

To clarify whether betaninuria is an individual trait or whether its occurrence is determined by various factors, beetroot juice was administered twice and the urine analysed twice in 62 cases, and three times in 28 cases, at one-month intervals. With these two or three ΔE-values there were none yielding a mean standard deviation which would have been significantly less than that in the entire examined population. In view of the few repetitions, this was not to mean that there was no consistency as regards betaninuria in individual children. It will therefore be necessary to subject the same individuals to still more examinations in order to gain a more faithful picture of individual fluctuations.

In the last series of examinations, the reliability of visual examination was compared with the photometric procedure in 66 children. $\overline{\Delta E}$ amounted to 5.9 in 23 visually negative, to 7.5 in 23 visually simply positive (+) and 13.0 in 20 doubly positive (++) samples. The difference between the $\overline{\Delta E}$ of the negative and that of the simply positive samples was significant at the P = 5% level while with the + and ++ samples at the P = = 0.1% level. This means that, relying on visual estimation under standardized conditions, we actually succeeded in forming the groups according to the different colours of the samples. It was subsequently found that a further group could have been inserted between the + and the ++ groups whose $\overline{\Delta E}$, too, would have significantly differed from those of the others. The real problem, the same which SALDANHA et al. [11] had to face, consisted in defining the groups which have to be accepted as positive, i.e. to tell which shade of pigmentation indicates the presence of betanin in urine. The grouping had, moreover, to be such as to

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ensure that, on another occasion, the same shade should again be classified as positive both by us and by others. The current methods cannot cope with this problem because they register the combined colours of betanin and urine. The visual method classifies only according to the tint of the mixed colour of yellow and violet; although the ΔE value allows for a certain correction which depends on the urinary colour, this, too, does not show the concentration of betanin alone. This would require a separation of the two types of pigment; a sample could be classified as undoubtedly positive only if, after the separation, it would show extinction at 530 nm even if it did not contain a component absorbing light at e.g. 420 nm: Again, a sample could be classified as undoubtedly negative only if, after separation and concentration, it had practically no extinctive power at any wavelength (including that of 530 nm), i.e. if it were water clear. No method of this kind has so far been elaborated. As it is, we are at present unable to solve the problem in a direct manner, and we are free to draw the dividing line between positive and negative samples and to set up any number of groups at discretion. It seems, therefore, more expedient to work with quantitative characteristics (e.g. the value ΔE as employed by us); they allow mathematical-statistical evaluation on the evidence of an objective index.

Visually obtained data, too, can be evaluated mathematically; this method can, however, be applied to an entire population only and not to individuals separately and is less reliable than our method; while it sets up classes on visual evidence, our procedure is based on instrumental recording differentiating about 20 classes if the accuracy of measurements (E) is 0.01.

Even our method is unable to show the distribution of betaninuria-positive and negative individuals in a given population, although the bimodal frequency histogram, as shown in Fig. 3, indicates the probable distribution. As long as the problem is not settled it is impossible to tell whether the phenomenon at issue is determined by genetic or environmental factors. More advanced methods are required for the solution of this problem.

SUMMARY

A method for the quantitative determination of betaninuria has been worked out and applied in a population of 3 to 6-year-old children who received a predetermined amount of betanin in beetroot juice. To eliminate the error due to the colour of urine, the

value ΔE, i.e. the difference of extinctions at 530 nm and 660 nm, has been introduced, which characterizes the concentration of urinary betanin.

Urinary ΔE-values, observed in 244 children, showed a bimodal distribution significantly differing from the normal. Excretion of betanin was measurable at 150 min. after the ingestion of beetroot juice.

No correlation was found between urinary ΔE and the pH, whereas a significant negative correlation was obvious between urinary ΔE and the output, at the $P = 0.1\%$ level. The value for $\Delta E \cdot ml$, i.e. the product of urinary volume and ΔE, showed a significant deviation to the right from the normal distribution, a phenomenon due to the still more pronounced skew distribution of the volume factor. Therefore, ΔE, in itself, is more characteristic of betanin excretion than the product $\Delta E \cdot ml$.

After the repeated ingestion of beetroot juice by the same children, urinary ΔE-values showed a scatter not significantly different from that estimated in the entire population.

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