lumichrome (6:7-dimethyl-isoalloxazine) is produced by the irradiation of riboflavin, while Warburg & Christian (1933) showed that lumiflavin (6:7:9-trimethyl-isoalloxazine) is formed in alkaline solution. Both these compounds exhibit a blue fluorescence. Karrer *et al.* (1934) showed that the nature of the solvent had a considerable influence on the yield of the decomposition products, but did not investigate the products of decomposition in butanol-pyridine-acetic acid solution. The decomposition in this solution is so much more rapid than in aqueous solution that it is quite possible that the decomposition takes a different course. We found that the pH of the butanol-pyridine-acetic acid solution, diluted fourfold with water and ethanol, was 4.6.

The method is sufficiently sensitive for most purposes. The smallest amount of riboflavin which can be measured with an accuracy of 10% is about 0.1 μ g. The limit of sensitivity of the method, i.e. the smallest amount which can be regarded as significantly greater than zero, is about 0.01 μ g. The smallest concentration which can be measured depends upon the size of the aliquot taken, which in turn depends upon the amount of interfering substances present.

SUMMARY

1. A modification of the fluorimetric method of determining riboflavin in biological materials, based on the method of Najjar (1941) but differing in several important respects, is described.

2. The method differs from those previously described by including a test for specificity.

3. The photochemical destruction of riboflavin is not a simple exponential function of time, but is dependent upon the concentration of the decomposition products, i.e. a reversible reaction is involved.

4. High concentrations of KMnO_4 rapidly destroy riboflavin.

5. The introduction of an additional purification step involving adsorption on Florisil did not increase the specificity of the method.

6. In general, figures obtained by the proposed method agreed closely with those obtained by a microbiological method.

7. The accuracy of the method is about 10% and the limit of sensitivity about 0.01 μ g.

We wish to thank Miss C. E. Francis for some of the analyses in Table 9 and Dr C. White for the analyses made by the microbiological method in Table 8.

REFERENCES

- Barton-Wright, E. C. & Booth, R. G. (1943). *Biochem. J.* **37**, 25.
DeMerre, L. J. & Brown, W. S. (1944). *Arch. Biochem.* **5**, 181.
Ephraim, F. (1934). *Text Book of Inorganic Chemistry*, 2nd English ed. by P. C. L. Thorne. London: Gurney and Jackson.
Euler, H. & Adler, E. (1934). *Hoppe-Seyl. Z.* **223**, 105.
Karrer, P., Saloman, H., Schopp, K., Schlittler, E. & Fritzsche, A. (1934). *Helv. chim. Acta*, **17**, 1010.
Kavanagh, F. (1941). *Industr. Engng Chem. (Anal. ed.)*, **13**, 108.
Morell, D. B. (1946). To be published.
Morell, D. B. & Slater, E. C. (1946). *Biochem. J.* **40**, 652.
Najjar, V. A. (1941). *J. biol. Chem.* **141**, 355.
Price, S. A. (1945). *Nature, Lond.*, **156**, 171.
Rubin, S. H. & DeRitter, E. (1945). *J. biol. Chem.* **158**, 639.
Slater, E. C. & Morell, D. B. (1946). To be published.
Warburg, O. & Christian, W. (1933). *Biochem. Z.* **266**, 377.

The Fluorimetric Determination of Riboflavin in Urine

BY D. B. MORELL AND E. C. SLATER, *Australian Institute of Anatomy, Canberra, Australia*

(Received 17 April 1946)

In a previous paper (Slater & Morell, 1946*a*), we described a specific method for the determination of riboflavin in biological materials. The development of this method was made necessary by our finding that the methods commonly employed for the measurement of riboflavin in urine were non-specific and seriously over-estimated the riboflavin content of urines containing small amounts of the vitamin.

Since much of our knowledge of the metabolism of riboflavin (especially in man) is based upon determinations of the urinary excretion of riboflavin, it is useful to know the order of magnitude of the errors associated with the various methods of estimation. This is the purpose of the present paper, which presents the results of a systematic comparison of various methods of determining riboflavin in human and rat urine.

The following methods were compared:

Method 1. This method, like that used by Ferrebee (1940) for urines containing 0.5 $\mu\text{g./ml.}$ or more of riboflavin, involved no purification of the urine and the hydrosulphite blank was used. It differed from Ferrebee's method in that the internal standard procedure was followed.

Method 2. This was the method of Hodson & Norris (1939).

Method 3. In this method, the urine was purified by oxidation with cold KMnO_4 and H_2O_2 and the hydrosulphite blank was used. It was similar to that used by many workers, e.g. Sure (1944), with the addition, as in Method 1, of the internal standard.

Method 4. This method involved adsorption on Florisil and measurement of the fluorescence in the eluate after dilution with water. The hydrosulphite blank and internal standard were used. The method of adsorption and elution was the same as that of Barton-Wright & Booth (1943).

Method 5. This was essentially Najjar's (1941) direct method.

Method 6. This was essentially Najjar's (1941) indirect method.

Method 7. This was the method of Slater & Morell (1946*a*).

EXPERIMENTAL

Urines analyzed

The following samples of urine were analyzed: A1, A2—from rats fed a riboflavin-free diet for 5 weeks after weaning; B—from rats fed the normal diet of the colony; C1, C2, C3—from a healthy human on an ordinary diet. The rat urines were considerably diluted by the water used to wash the sides of the metabolism cage. All the urines were analyzed on the day of collection and urine C3 was again analyzed after 1 month's storage at room temperature at pH 1.0.

Methods

Method 1. Urine A or B (10 ml.) or C (5 ml.) were adjusted to pH 7.0–7.5, diluted to 200 ml. and filtered. The following measurements were taken in the fluorimeter (see Slater & Morell (1946*a, b*) for details of the fluorimeter used):

1. The fluorescence (R_1) of 20 ml. of the filtered solution. Owing to the instability of riboflavin to light, it was necessary to take a series of readings at various intervals of time (about 10 sec. apart) after the cuvette was exposed to the light beam of the fluorimeter. These readings were extrapolated to zero time in order to obtain R_1 .

2. The blank fluorescence (B) of 20 ml. of the solution, after the riboflavin was destroyed by $\text{Na}_2\text{S}_2\text{O}_4$. In order to avoid errors due to the presence of fluorescent substances which were slowly reduced by $\text{Na}_2\text{S}_2\text{O}_4$ (riboflavin is very rapidly reduced), this measurement was made in the following way: 20 mg. of $\text{Na}_2\text{S}_2\text{O}_4$ were added to a fresh solution in the cuvette and, at the same moment, the shutter of the fluorimeter was opened, thereby exposing the solution to the light beam; the solution was stirred and readings were taken at measured intervals of time and extrapolated back to the time of addition of the $\text{Na}_2\text{S}_2\text{O}_4$.

3. The fluorescence (R_2) of a solution obtained as follows and read in the fluorimeter in the same way as the first measurement. To 20 ml. of the solution in the cuvette 1 ml. of riboflavin solution (1.2 $\mu\text{g./ml.}$) was added and, after mixing, 1 ml. of the solution was withdrawn to keep the volume in the cuvette constant.

All the measurements were made in duplicate.

Calculation

Riboflavin content of urine

$$= \frac{R_1 - B}{1.05 \times R_2 - R_1} \times 0.06 \times \frac{200}{\text{Vol. of urine taken}} \mu\text{g./g.}$$

Method 2. The same volumes of urine as used in Method 1 were taken, adjusted to pH 7.0–7.5 and diluted to about 175 ml. 2 ml. ice-cold $\text{Na}_2\text{S}_2\text{O}_4$ solution (2 ml. of a solution of 1 g. $\text{Na}_2\text{S}_2\text{O}_4$ and 1 g. NaHCO_3 dissolved in 20 ml. ice-cold H_2O) and 2 ml. SnCl_2 solution (freshly diluted 1 to 200 from a stock solution containing 10 g. SnCl_2 dissolved in 25 ml. conc. HCl) were added and the solution allowed to stand for 10 min. The solution was diluted to 200 ml., transferred to a litre flask, shaken vigorously for 5 min. and finally filtered.

Measurements similar to those in Method 1 were taken.

Method 3. To samples of the same volume as used in Methods 1 and 2, 4% freshly prepared KMnO_4 was added dropwise at such a rate that the colour due to the previous drop had disappeared before the addition of the next drop, until the colour persisted for 1 min. The solution was decolorized with 3% H_2O_2 , adjusted to pH 7.0–7.5, diluted to 200 ml. and filtered. Measurements similar to those in Methods 1 and 2 were taken.

Method 4. The urine (25 ml.) (urines A and B undiluted, urines C diluted 1 to 2) was adjusted to pH 5.0 and diluted to 100 ml. Amounts of 20 ml. were transferred to four 50 ml. centrifuge tubes, A, B, C and D. Riboflavin solution (5 ml. of a solution containing 1.2 $\mu\text{g./ml.}$) was added to B and D and 5 ml. H_2O to A and C. To each tube, 0.5 g. Florisil (Floridin Co., U.S.A.) was added and the mixture stirred mechanically for 5 min. After centrifuging and pouring off the centrifugate, 20 ml. H_2O were added and the mixture stirred by hand for 2 min. and centrifuged. The centrifugate was discarded and as much H_2O drained off as possible. The eluting solution (6 ml. of a mixture of H_2O , 9 vol.; pyridine, 5 vol. and glacial acetic acid, 2 vol.) was then added, the mixture stirred mechanically for 5 min. and centrifuged. The centrifugate was added to a 100 ml. standard flask and a further 2 ml. of eluting solution added to the centrifuge tube. The contents of the tube were stirred by hand for 2 min., centrifuged and the centrifugate added to the same flask as previously and the solution made up to volume with H_2O .

Two measurements, each in duplicate, were taken in the fluorimeter on:

- (1) 20 ml. amounts of each eluate— R_A , R_B , R_C , R_D .
- (2) 20 ml. amounts of each eluate treated with $\text{Na}_2\text{S}_2\text{O}_4$ as in methods 1 to 3— B_A , B_B , B_C , B_D .

Calculation

Riboflavin content of urine

$$= \frac{\frac{R_A + R_C}{2} - \frac{B_A + B_C}{2}}{\left(\frac{R_B + R_D}{2} - \frac{B_B + B_D}{2}\right) - \left(\frac{R_A + R_C}{2} - \frac{B_A + B_C}{2}\right)} \times 1.2 \times F \mu\text{g./g., where } F \text{ is the dilution factor.}$$

Method 5. To two 50 ml. stoppered measuring cylinders (A and B) was added a 5 ml. sample of urine (urines A and B undiluted, urines C1 and C2 diluted 1 to 2 and urine C3 diluted 1 to 5) and to a third cylinder (C) was added 1 ml. riboflavin solution (1.2 μ g./ml.). To each cylinder was added in turn 2 ml. pyridine, 1 ml. glacial acetic acid, 2 drops 4% KMnO_4 and, after 1 min., 2 drops 3% H_2O_2 , followed by 3 g. anhydrous Na_2SO_4 and 10 ml. butanol. The cylinders were heated above 35° by immersion in hot water, then cooled to room temperature and shaken for 2 min. After centrifuging, 10 ml. of the butanol-pyridine layer were transferred to a test-tube containing 12 ml. butanol. The fluorescence of each solution was read in the fluorimeter, and after the solution had been exposed in the test-tubes to sunlight for 1 hr., the readings were repeated.

Calculation

A_1, B_1, C_1 = initial readings of solutions A, B, C.

A_2, B_2, C_2 = readings of solutions A, B, C, after exposure to sunlight for 1 hr.

F = dilution of urine.

Riboflavin content of urine

$$= F \times \frac{\frac{A_1 + B_1}{2} - \frac{A_2 + B_2}{2}}{C_1 - C_2} \times \frac{1.2}{5} \mu\text{g./ml.}$$

Method 6. Two 5 ml. amounts of urine diluted as in Method 5 were adjusted to pH 5.0 (except urine C1 which was kept at its natural pH, 6.5) and the volumes made up to 15 ml. with H_2O . The solutions were transferred to two 50 ml. centrifuge tubes containing freshly prepared PbS and the solutions stirred mechanically for 10 min. Each was then filtered through a washed asbestos filter with slow suction and washed with 3 lots of 10 ml. H_2O . The eluting solution (7 ml. of a mixture of H_2O , 70 vol.; pyridine, 30 vol. and glacial acetic acid, 2 vol.) was added to the moist precipitate and allowed to pass through under weak suction into a 15 ml. graduated test-tube. To the eluate was added 1 ml. glacial acetic acid followed by 0.67 ml. 4% KMnO_4 . After mixing and standing for 1 min., the solution was decolorized by the addition of 0.67 ml. 3% H_2O_2 , and diluted to 10 ml. After mixing, 8 ml. of this solution were added to a 50 ml. stoppered measuring cylinder, 3 g. anhydrous Na_2SO_4 added and the analysis continued as in Method 5, with the same standard (cylinder C). The

solutions were read with solutions A, B and C from Method 5 and exposed to sunlight at the same time as these solutions.

Calculation

D_1, E_1 = initial readings of the two solutions.

D_2, E_2 = readings of the solutions after exposure to sunlight for 1 hour.

F = dilution of urine.

Riboflavin content of urine

$$= \frac{\frac{D_1 + D_2}{2} - \frac{E_1 + E_2}{2}}{C_1 - C_2} \times \frac{10}{8} \times \frac{1.2}{5} \times F \mu\text{g./g.}$$

Method 7. The method of Slater & Morell (1946a) was followed, with 1 ml. of undiluted urines A1, A2, B and C3 and 1 ml. of urines C1 and C2 diluted twofold.

RESULTS

Riboflavin contents

In Table 1, the results obtained by Methods 1, 2, 3 and 4 expressed as μ g./rat/day or μ g./man/day, are compared with those obtained by Method 7. Methods 1, 2, 3 and 4 are grouped together because each includes the hydrosulphite blank and the figures therefore include 'apparent'* as well as true riboflavin. Assuming that the figures obtained by Method 7 are correct, the figures in brackets in Table 1 represent the 'apparent riboflavin' measured by the method.

It is clear that Methods 1, 2, 3 and 4 measure, as riboflavin, considerable amounts of 'apparent riboflavin'. Actually, we have found that the amount of 'apparent riboflavin' measured by Methods 1, 2 and 3 depends partly upon the amount of $\text{Na}_2\text{S}_2\text{O}_4$ used.

* 'Apparent riboflavin', as defined by Slater & Morell (1946a), signifies compounds, other than riboflavin, which fluoresce in the same spectral region as riboflavin and the fluorescence of which is destroyed by $\text{Na}_2\text{S}_2\text{O}_4$.

Table 1. *Urinary excretion of riboflavin as measured by different methods*

(Figures in brackets represent the difference between each result and that obtained by Method 7 on the same urine.)

Method	Riboflavin excreted in						
	Rat urine (μ g./rat/day)			Human urine (μ g./man/day)			
	A1	A2	B	C1	C2	C3	C3 after 1 month
1	1.7 (1.1)	1.7 (1.4)	24.4 (4.2)	530 (150)	1120 (200)	720 (180)	630 (160)
2	2.5 (1.9)	2.2 (1.9)	24.1 (3.9)	810 (430)	1100 (180)	750 (210)	770 (300)
3	1.9 (1.3)	2.5 (2.2)	22.0 (1.8)	800 (420)	1260 (340)	980 (440)	850 (380)
4	0.8 (0.2)	0.8 (0.5)	22.7 (2.5)	550 (170)	1050 (130)	700 (160)	620 (150)
7	0.6	0.3	20.2	380	920	540	470

In most cases, Methods 2 and 3 measure considerably more 'apparent riboflavin' in human urine than Method 1. This must be due to the production of 'apparent riboflavin' by the reaction of some precursor with the $\text{Na}_2\text{S}_2\text{O}_4$ and/or SnCl_2 in Method 2 and with the KMnO_4 in Method 3. Method 2 measured more and the other methods less 'apparent riboflavin' in urine C3 after storage for 1 month at room temperature as judged by comparison with figures obtained on the fresh urine.

The results obtained by Method 4 show that adsorption on Florisil does not completely separate 'apparent' from true riboflavin. The following experiment showed that 'apparent riboflavin' is actually formed during contact with Florisil in Method 4.

A volume of 20 ml. of urine was adjusted to pH 5.0 and diluted to 100 ml. A volume of 25 ml. was treated as in Method 1. Two further amounts of 25 ml. were pipetted into two 50 ml. centrifuge tubes, A and B. Florisil (0.5 g.) was added to each tube. Riboflavin solution (1 ml. of a solution containing $1.2 \mu\text{g./ml.}$) was added to tube B and 1 ml. H_2O to tube A. The contents of each tube were stirred mechanically for 5 min., centrifuged and the centrifugate poured into a 200 ml. standard flask. The residue was washed by stirring by hand for 2 min. with 20 ml. H_2O and again centrifuged, the washings being added to the previous centrifugate. The combined centrifugates were made up to 200 ml. The riboflavin was eluted from the Florisil as in Method 4 and the eluates were oxidized with 4 drops of 4% KMnO_4 , followed by 3% H_2O_2 and then made up to 200 ml.

The following results were obtained: riboflavin content of urine determined by Method 1, $0.271 \mu\text{g./ml.}$; of combined centrifugates and washings from adsorption in tube A, $0.222 \mu\text{g./ml.}$; of combined centrifugates and washings from adsorption in tube B, $0.225 \mu\text{g./ml.}$; of eluate from tube A, $0.165 \mu\text{g./ml.}$; and of eluate from tube B, $0.346 \mu\text{g./ml.}$ The small difference between the results on the centrifugates and washings from the two tubes shows that the adsorption on the Florisil must have been almost complete. The difference between eluates A

and B shows that only $0.181 \mu\text{g./ml.}$ of the $0.24 \mu\text{g./ml.}$ riboflavin added was recovered (i.e. 75.5%). This means that only 75.5% of the riboflavin was eluted from the Florisil. Assuming that the percentage of added riboflavin eluted is the same as that of the riboflavin adsorbed from the urine, the riboflavin content of the urine is

$$\frac{0.165}{0.181} \times 0.24 = 0.219 \mu\text{g./ml.}$$

Thus the total riboflavin adsorbed on the Florisil is $0.219 \mu\text{g./ml.}$ and the total riboflavin not adsorbed on the Florisil is $0.222 \mu\text{g./ml.}$, giving a total amount of $0.441 \mu\text{g./ml.}$ But the total riboflavin as measured by Method 1 is $0.271 \mu\text{g./ml.}$ which shows that at least $0.170 \mu\text{g./ml.}$ of 'apparent riboflavin' was produced during contact with the Florisil or during the oxidation of the eluates.

That at least some of this 'apparent riboflavin' is produced during the process of adsorption and washing was demonstrated in another experiment, in which the amount of riboflavin measured in the fraction of the urine not adsorbed on the Florisil exceeded that measured by Method 1 on the untreated urine:

Riboflavin content of untreated urine = $0.228 \mu\text{g./ml.}$

Riboflavin content of fraction not adsorbed on Florisil = $0.340 \mu\text{g./ml.}$

Thus at least $0.112 \mu\text{g./ml.}$ 'apparent riboflavin' has been formed during contact of the diluted urine with Florisil.

In Table 2, results obtained by Najjar's direct and indirect methods (Methods 5 and 6 respectively) are compared with those obtained by Method 7. The results show that both Methods 5 and 6 give low values for urines of high riboflavin concentration and high values for urines of low riboflavin concentration.

The following factors are responsible for these errors: (1) both methods are non-specific due to the presence of light-labile fluorescent compounds which are not destroyed by the small amount of KMnO_4 used; (2) the use of the external standard

Table 2. *Urinary excretion of riboflavin as measured by different methods*

(The figures in brackets represent the concentrations expressed as percentages of those given by Method 7.)

Method	Rat urine			Human urine			
	A1	A2	B	C1	C2	C3	C3 after 1 month
5	0.11 (216)	0.13 (670)	0.33 (68)	0.30 (85)	0.54 (47)	0.31 (77)	0.36 (96)
6	—	0.04 (205)	0.33 (69)	0.32 (91)	0.87 (76)	0.20 (50)	0.24 (64)
7	0.05 (100)	0.02 (100)	0.48 (100)	0.35 (100)	1.15 (100)	0.41 (100)	0.37 (100)

does not allow of a correction for quenching of the fluorescence; (3) no correction is made, in Method 6, for incomplete adsorption on and/or elution from the PbS. The first source of error leads to high figures while the other two cause low figures. The former becomes most important in the case of urines of low riboflavin content, while the latter are more important when urines high in riboflavin are assayed.

The importance of the errors caused in Method 5 by the use of the external standard is illustrated by the fact that widely differing results were obtained with different dilutions. The results in Table 3 were obtained with urine C2 diluted twice and fivefold respectively and both the internal and external standard procedures were followed.

Table 3. *Effect of degree of dilution on riboflavin content of urine as determined by Method 5, with internal and external standards*

Dilution	Riboflavin content of urine ($\mu\text{g./ml.}$ undiluted urine)		Quenching (%)
	Internal standard	External standard	
1 to 5	1.26	1.06	16
1 to 2	1.38	0.61	56

If the internal standard is used, the results on the more diluted urine are a little less than those on the more concentrated urine (possibly due to the greater excess of KMnO_4 in the former case). On the other hand, when the external standard is used, the results on the more concentrated urine are considerably the smaller, owing to the greater quenching.

Najjar (1941) does not specify the pH at which adsorption on PbS should be carried out. It was

Table 4. *Effect of pH on degree of adsorption of riboflavin on PbS*

pH	Fluorimeter reading (corrected for blank)
1.0	42.4
4.5	73.2
6.5	59.0

Table 5. *Residual blanks as measured by different methods*

(Equivalent to $\mu\text{g. riboflavin/day/animal.}$)

Method	Rat urine			Human urine			
	A1	A2	B	C1	C2	C3	C3 after month
1	2.2	4.8	7.6	580	420	480	930
2	3.1	5.1	13.2	670	450	470	750
3	1.3	1.6	5.0	270	260	360	530
4	0.4	0.7	2.4	120	100	150	170
5	1.7	2.6	7.6	400	310	180	570
6	—	0.3	1.5	130	100	40	15
7	0.9	2.0	3.7	240	280	300	—

found that the acidity had a considerable effect on the degree of adsorption as the results in Table 4 show. A pH of 5.0 (the same as that used for adsorption on Florisil) was adopted for our analyses, with the exception of urine C1 which was kept at its natural pH (6.5).

Residual blanks

In Table 5, the residual blanks, calculated as though the blank fluorescence measured in the fluorimeter was riboflavin, are compared. The residual blanks in Methods 1, 2, 3 and 5 increased considerably during 1 month's storage.

DISCUSSION

It has been shown that methods using the hydrosulphite blank are inaccurate since all fluorescent substances reduced by the hydrosulphite are measured as riboflavin. The 'apparent riboflavin' thus measured was especially high when procedures of purification which are commonly used were applied to the urines. Najjar's (1941) methods were also found unsatisfactory, giving high answers for urines of low riboflavin content and low answers for urines of high riboflavin content. Much of our knowledge of the metabolism of riboflavin is based on measurements of the urinary excretion of the vitamin. There may be a need to re-evaluate some of these experiments in the light of the findings regarding the specificity of the methods employed.

SUMMARY

1. Seven different methods of measuring riboflavin in rat and human urine have been compared.

2. Methods using the hydrosulphite blank, even those involving adsorption on Florisil, give high answers because they include 'apparent riboflavin', i.e. compounds other than riboflavin which fluoresce in the same spectral region and the fluorescence of which is destroyed by $\text{Na}_2\text{S}_2\text{O}_4$.

3. Urine contains not only 'apparent riboflavin', but also precursors of 'apparent riboflavin', which are changed to 'apparent riboflavin' by $\text{Na}_2\text{S}_2\text{O}_4$ - SnCl_2 reduction, by KMnO_4 oxidation or during the process of adsorption on Florisil.

4. The methods of Najjar (1941) were found not completely specific, giving high results for urines of low riboflavin concentration. They also give low figures for urines of high riboflavin content, due, in our opinion, to failure to allow for quenching of the fluorescence, or for incomplete adsorption on and/or elution from PbS.

5. It is pointed out that the lack of specificity of many fluorimetric methods hitherto used may necessitate a re-evaluation of some experiments on the metabolism of riboflavin, based on the urinary excretion of the vitamin.

REFERENCES

- Barton-Wright, E. C. & Booth, R. G. (1943). *Biochem. J.* **37**, 25.
Ferrebee, J. W. (1940). *J. clin. Invest.* **19**, 251.
Hodson, A. Z. & Norris, L. C. (1939). *J. biol. Chem.* **131**, 621.
Najjar, V. A. (1941). *J. biol. Chem.* **141**, 355.
Slater, E. C. & Morell, D. B. (1946a). *Biochem. J.* **40**, 644.
Slater, E. C. & Morell, D. B. (1946b). To be published.
Sure, B. (1944). *J. Nutrit.* **27**, 447.

Carotenoid Pigments of Badami Mango Fruit

By G. B. RAMASARMA, S. D. RAO AND D. N. HAKIM, *Department of Biochemistry, Indian Institute of Science, Bangalore, India*

(Received 7 June 1946)

The only attempt at the isolation of carotene from mango fruit seems to have been made by Yamamoto Osima & Goma (1932) who obtained 60 mg. of a crystalline preparation from 38 kg. of fresh fruit pulp. From a study of its optical activity and absorption spectrum they concluded that it was a mixture of α - and β -carotenes, which were, however, not separated. The inconclusive nature of their data has led us to re-investigate the carotenoid pigments of the mango fruit by the technique of chromatographic adsorption.

Earlier work from this laboratory (Ramasarma & Banerjee, 1938), as also elsewhere (Crawford & Perry, 1933; De, 1937), has shown that certain varieties of the mango fruit are very rich in carotene. The Badami mango fruit (almost the same as the famous Alfonso variety of Bombay) has been chosen for the present investigation, since it was found to be richer in carotene than any other variety so far studied.

A preliminary experiment of a qualitative nature showed that the mango xanthophylls are mostly in the form of esters and that nearly all of the epiphasic pigment is β -carotene (50 mg. per kg. of fresh pulp).

EXPERIMENTAL

Purification of solvents

Aldehyde-free ethanol was prepared by refluxing rectified spirit with 10 g./l. aluminium turnings and 10 g./l. potassium hydroxide, followed by fractional distillation.

Methanol was purified by the same method as the one used for ethanol. In order to obtain a 92% v/v methanol for the phase separation, 90 ml. of purified alcohol were mixed with 10 ml. of water.

As purified light petroleum was not available, ordinary car petrol was distilled and the fraction boiling between 60 and 75° was collected and purified according to the method of Castille & Henri (1924).

Ethyl ether was freed from peroxides by the procedure described by Brandt (1927). Only freshly distilled ether was used for the extraction of pigments.

Carbon disulphide used for spectrophotometry was purified according to Hammick & Howard (1932). The purified solvent was preserved in brown glass bottles and in the dark for periods not exceeding 1 week, after which it was purified again.

Extraction of pigments

Since the work could not be started immediately, the ripe fruit was preserved in the ice-room at 0° for a week, during which it became slightly soft but remained otherwise in good condition. The minced pulp from 50 Badami mangoes (7.7 kg.) was extracted by steeping overnight in 8 l. of absolute ethanol and the extract was pressed out in a tincture press. The residue was extracted similarly with 3 l. of absolute ethanol and the cake was set aside in cold storage for subsequent extraction. The combined alcoholic extract (17 l.) was concentrated under reduced pressure and in an atmosphere of carbon dioxide to about half the volume. After dilution with water, it was repeatedly extracted with freshly distilled ether till no more colour was taken up.

The dehydrated cake was broken up and thoroughly extracted with freshly distilled ether. The extract (4 l.) was concentrated to 500 ml. when most of the ether was distilled off and only alcohol remained. At this stage, small crystals of pigment appeared, and the solution was left in the refrigerator overnight in order to complete the crystallization. The glistening, dark red crystals were filtered off, washed with 25 ml. of absolute ethanol, dried *in vacuo* and preserved in an evacuated tube in the cold for subsequent work. The yield of crude pigment was 250 mg.

The deeply coloured mother liquor and the pigment solution obtained by the alcoholic extraction of the pulp were mixed and saponified by leaving overnight with 200 ml. of 10% alcoholic potash. The ether solution was next freed of alcohol, potash and soaps by washing it with water, and dried over anhydrous sodium sulphate. The ether was evaporated off under reduced pressure and the residue was dissolved in 200 ml. of light petroleum. On concentration to 50 ml. and cooling in the refrigerator, some colourless impurities came down and were removed by filtration. The filtrate was diluted to 300 ml. and extracted with an equal volume of 92% methanol. The epiphasic layer was further extracted four times with 100 ml. lots of 92% methanol in order to remove all the xanthophylls.