Finally, it should be pointed out that these methods have successfully been applied to studies of the anthocyanins present in colour mutants of *Solanum phureja* and *Primula sinensis*, and in a variety of other plant material. Provisional structures for a number of newly discovered pigments have been advanced on the basis of spectral and chromatographic data, of identifying the sugars present (Harborne & Sherratt, 1957a) and of examining the simpler glycosides produced by partial acid hydrolysis. Further details of these results will appear in later papers.

SUMMARY

1. Spectral data for both the common and rare naturally occurring anthocyanidins are given.

2. Spectral measurements have been made of a large number of anthocyanins. It is shown that 3- and 3:5-glycosides have characteristically different absorption properties in the 400–450 mμ region.

3. Measurements of the absorption spectra in the ultraviolet region of anthocyanins acylated with hydroxy aromatic acids can be used to determine the hydroxy aromatic acid:pigment ratio in these complex pigments.

4. The value of these measurements for characterizing anthocyanins is discussed.

The author thanks Dr K. S. Dodds for his encouragement. He also thanks the following for gifts of anthocyanins: Mrs R. Meares (née Scott-Moncrieff), Miss V. C. Sturgess, Dr E. C. Bate-Smith, Dr T. Swain, Dr G. C. Forsyth and Dr E. Sondheimer.

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A Sensitive Method for Estimation of Oxaloacetate

BY G. KALNITSKY* AND D. F. TAPLEY†
Department of Biochemistry, University of Oxford

(Received 13 January 1958)

The coupling of acetoacetate with an excess of diazotized *p*-nitroaniline has been utilized by Walker (1954) to develop a sensitive colorimetric

* Travelling Fellow of the College of Medicine, State University of Iowa, Iowa City, Iowa.

† Fellow of the Jane Coffin Childs Memorial Fund, on leave of absence from the Department of Medicine, Columbia University College of Physicians and Surgeons, New York.

method for the estimation of acetoacetate. With defined conditions at pH 4.9–5.0, the reaction proceeds as in equation 1 ($R = p\text{-NO}_2\cdot\text{C}_6\text{H}_4$) with the formation of *NN'*-bis-(*p*-nitrophenyl)-*C*-acetylformazan.

In the present investigation, the method of Walker (1954) has been modified to measure very small quantities (0.003–0.03 μ mole) of oxaloacetic acid in the presence of moderate concentrations (0.1 μ mole) of acetoacetate. The method has been applied to the estimation of oxaloacetate in the livers of both fed and fasted rats.

EXPERIMENTAL AND RESULTS

Materials

p-Nitroaniline (0.05%, w/v) in 0.05N-hydrochloric acid. This solution was prepared by dissolving the *p*-nitroaniline (recrystallized from hot water) in boiling water and the required amount of HCl.

Diazo reagent. This was prepared immediately before use as described by Walker (1954). A volume (3 ml.) of NaNO₂ solution (0.5%, w/v; prepared daily) was added to 20 ml. of cold *p*-nitroaniline solution. The colourless solution was kept in an ice bath and 7 ml. of 0.2N-sodium acetate was added just before use.

Acetoacetic acid. This was provided through the generosity of Mr R. Kulka and had been prepared according to the method of Krebs & Eggleston (1945).

Oxaloacetic acid. This was provided through the generosity of Mr R. Hems and had been prepared according to the method of Wohl & Oesterlin (1901) and Wohl & Claussner (1907).

Tissue extracts. Tissue homogenates (20%) in a final concentration of 3% (w/v) trichloroacetic acid were prepared with livers of normal male rats [4 ml. of 5% (w/v) glucose was injected intraperitoneally 15 min. before death] and with livers of male animals fasted for either 24 or 69 hr. After decapitation of the rat, the liver was removed as rapidly as possible and plunged into liquid oxygen. The frozen liver was then weighed, crushed at -10° in a Hughes press (Hughes, 1951), rapidly transferred to a stainless-steel homogenizer containing 4 vol. of cold 3.75% (w/v) trichloroacetic acid and homogenized at 0–5 $^{\circ}$. A sample was taken for dry-weight determination (2 ml. contained approx. 100 mg.). The rest of the homogenate was centrifuged at approx. 900 g for 5 min. and a portion of the supernatant taken for analysis.

Standard method

The method finally adopted is a direct modification of that of Walker (1954). The modifications are based upon the findings that (1) the compound produced by the reaction of an excess of diazo reagent with oxaloacetate is unstable in acid (Walker, 1954), whereas that formed with acetoacetate is stable, and that (2) the addition of NaNO₂ at acid pH removes ascorbic acid which otherwise would interfere with the estimation of both oxaloacetate and acetoacetate. Measurements of the colour of solutions A, B and C treated as described below permit the simultaneous estimation of oxaloacetic and acetoacetic acids in biological materials.

To each of two glass-stoppered test tubes containing 2 ml. of supernatant from a 20% tissue homogenate (solutions A and B) is added 0.4 ml. of 0.25% NaNO₂ (to remove ascorbic acid). Exactly 30 sec. later 2 ml. of M-sodium acetate-acetic acid buffer, pH 5.2, is added, followed im-

mediately by 3 ml. of freshly prepared cold diazo reagent. After incubation for 20 min. at 25 $^{\circ}$, the tubes are placed in an ice-bath for 3 min. The colour due to reaction of the diazo reagent with both acetoacetate and oxaloacetate is determined with solution A by adding 6 ml. of ethyl acetate and then 6 ml. of 5N-HCl. The colour is immediately and quantitatively extracted into ethyl acetate by shaking the tubes 30–35 times. Not more than 7.5 sec. should elapse between the addition of acid and the extraction (see Fig. 5). With solution B the colour produced by oxaloacetate is destroyed by adding 6 ml. of 5N-HCl and placing the tube for exactly 8 min. in an ice-bath. The remaining colour is then extracted with 6 ml. of ethyl acetate. A reagent blank containing 2 ml. of 3% trichloroacetic acid instead of the tissue extract is subjected to each of the above procedures. For solution C both oxaloacetate and acetoacetate are destroyed by heating 2 ml. of the extract at 100 $^{\circ}$ for 5 min. The solution is then cooled rapidly and treated in the same way as solution B and read against the appropriate reagent blank.

The optical density of the ethyl acetate layer is read at 450 $m\mu$ in a 4 cm. cell (model SP. 500 Unicam spectrophotometer). The bottom of the cell is raised, and the cell carrier appropriately masked, so that readings can be made with a 5–6 ml. sample. The difference in optical density between solution A and solution B is taken to represent the colour produced by reaction of the diazo reagent with oxaloacetate, and between solutions B and C that with acetoacetate.

Sensitivity of the method

Oxaloacetate and acetoacetate couple with diazotized *p*-nitroaniline to give compounds which have almost identical absorption spectra, as shown in Fig. 1. Under standard conditions all readings were made at 450 $m\mu$. The time course of colour development with both compounds is given in Fig. 2. In order to minimize the colour contributed by acetoacetate, 20 min. was chosen as the reaction time in the standard method. The method can measure as little as 0.003 μ mole of oxaloacetate (Fig. 3).

Specificity of the method

A major problem in utilizing this reaction is that of estimating oxaloacetate in the presence of acetoacetate. Decarboxylation of oxaloacetate by multivalent cations (Krebs, 1942) before reaction with the diazo reagent has proved satisfactory only with pure solutions of these compounds. In the presence of tissue extracts, the addition of multivalent cations decreases the colour produced with acetoacetate to a varying extent. A satisfactory separation can be obtained when the products of the coupling reaction are kept in acid at 0 $^{\circ}$ for 8 min. As can be seen in Fig. 4, the coloured product obtained with acetoacetate is completely stable under these conditions, whereas that of oxaloacetate is nearly completely destroyed.

The addition of acid to the reaction mixture is essential to effect complete extraction of the resulting colour into the ethyl acetate layer. When

the extraction is performed within 7.5 sec. after the addition of acid (as with solution A) less than 5% of the colour obtained with oxaloacetate is lost; delaying the extraction for 20 sec. may result in a loss of as much as 20% (Fig. 5). Once extracted into ethyl acetate the colour yielded by both oxaloacetate and acetoacetate is stable for at least 6 hr.

A survey was made of compounds which might interfere with the estimation of oxaloacetate in tissues. The following 59 compounds (5 μ moles of each except where stated otherwise) failed to produce any significant colour with diazotized *p*-nitroaniline under the standard conditions: glycine, alanine, β -alanine, leucine, serine, threonine, cysteine, methionine, aspartic acid, asparagine, glutamic acid, lysine, arginine, histidine, phenylalanine, proline, hydroxyproline, glutathione, citrulline, glucose (50 μ moles), sucrose (50 μ moles), glucose 1-phosphate, fructose, sodium gluconate, carbamyl phosphate, 3-phosphoglyceric acid, α -glycerophosphate, glyoxal, oxalic, lactic, pyruvic, β -hydroxybutyric, citric, succinic, fumaric, malic, and laevulinic acid, sodium oleate, sodium crotonate, diphosphopyridine nucleotide, adenosine triphos-

phate, guanine, xanthine, thymine, uracil, acetoin, acetone, ethanol, urea, glycoxyamine, calcium pantothenate, inositol, folic acid, vitamin B₁₂, calciferol, α -tocopherol, choline, glycerol and cholesterol. A number of substances (Table 1) react with diazotized *p*-nitroaniline, but under the standard conditions are not decolorized in acid, and so do not interfere with the estimation of oxaloacetate.

Several compounds tested give with diazotized *p*-nitroaniline a colour which is partially labile in acid under standard conditions (Table 2). Of these, only the members of the vitamin B₆ group are

Fig. 1. Absorption spectra of the derivatives formed by coupling diazotized *p*-nitroaniline with oxaloacetate (O), and with acetoacetate (●). The products were formed by coupling diazotized *p*-nitroaniline with known samples of oxaloacetate and acetoacetate in 2 ml. of 3% trichloroacetic acid under standard conditions described for solution A in the text. The samples were read against ethyl acetate with the appropriate reagent blank (■) in a Carey recording spectrophotometer.

Fig. 2. Time curves for colour development with oxaloacetate (O) and with acetoacetate (●). Conditions were as described for solution A in the text.

Fig. 3. Calibration curves for the estimation of oxaloacetic acid (O) and of acetoacetic acid (●). Conditions as described for solution A in the text.

present in sufficient quantity in rat liver (Rabinowitz & Snell, 1948) to provide slight interference. Since the major portion of vitamin B₆ occurring in tissues is tightly bound to protein (Clark, 1953; Siegal, Melnick & Oser, 1943; Rabinowitz & Snell, 1947) its interference is negligible (0.003–0.005 optical-density units).

Fig. 4. Time curves for the stability in acid of the colours formed with oxaloacetate (O) and acetoacetate (●). Conditions were as for solution B in the text except for varying time in acid.

Table 1. Substances which react with diazotized *p*-nitroaniline to give a coloured product which is stable in acid under the standard conditions defined in the text for solution B

Substance	Amount (μmoles)	$E_{(450\text{ m}\mu)}$
Ribonucleic acid*	40†	0.025
Deoxyribonucleic acid*	40†	0.010
Glycogen*	500†	0.025
Vitamin A acetate*	5	0.150
Ascorbic acid	5	0.032
Riboflavin*	1	0.025
Thiamine*	5	0.045
Tyrosine	1.1	0.049
Tryptophan	1.8	1.155
Ethanolamine*	5	0.023
Creatinine*	5	0.045
Diacetyl*	5	0.083
Acetoacetate	0.025	0.325
<i>p</i> -Hydroxyphenylpyruvate*	0.5	0.415
α -Oxoglutarate	1.0	0.037
Dihydroxymaleic acid	0.91	0.027

* Conditions were as for solution B as defined in the text except that the tubes were in acid at room temp. for 10 min. instead of at 0° for 8 min.

† Mg.

Table 2. Substances which react with diazotized *p*-nitroaniline to give a coloured product which is labile in acid under the standard conditions defined in the text for solution B

Substance	Amount (μmole)	$E_{(450\text{ m}\mu)}$	
		Without acid	With acid
Oxaloacetate	0.03	0.530	0.000
α -Oxobutyrate	1.0	0.031	0.022
Malonic acid	1.0	1.315	1.275
Pyridoxine	0.1	0.410	0.317
Pyridoxamine	0.1	0.432	0.358
Pyridoxal	0.1	0.527	0.444

Fig. 5. Decrease in the colour of the oxaloacetate derivative during the first 20 sec. after the addition of acid. Conditions were as for solution A in the text except for the varying time at 0° before extraction.

Estimation of oxaloacetate in tissue preparations

As noted by Walker (1954), ascorbic acid interferes with colour development when acetoacetate is coupled with the diazo reagent. In fresh trichloroacetate extracts of normal liver there is sufficient ascorbate to interfere with the estimations of both acetoacetate and oxaloacetate (recoveries of added oxaloacetate and acetoacetate range from 60 to 80%). Walker (1954) removed ascorbate by the addition of cupric acetate to the extracts, but this treatment also destroys oxaloacetate. The difficulty was resolved by finding that ascorbic acid is completely removed by the addition of NaNO₂ for 30 sec. at acid pH (3% trichloroacetic acid),

whereas oxaloacetate under identical conditions is stable. This is illustrated in Fig. 6. With ascorbic acid thus eliminated, excellent recoveries of both acetoacetate and oxaloacetate are obtained (see Table 3).

Levels of oxaloacetate and acetoacetate in rat livers

Figs. 7 and 8 demonstrate that under the standard conditions adopted the colour produced is proportional to the amount of tissue analysed with the livers of both normal and fasted rats. Any significant variation in the technique may result in a non-linear relationship for solution B. This is particularly true for tissues from fasted animals.

Fig. 6. Effect of adding NaNO_2 to a solution of oxaloacetate ($0.03\text{ }\mu\text{mole}$; O) and of ascorbic acid ($0.05\text{ }\mu\text{mole}$; Δ) in 3% trichloroacetic acid for varying periods before the addition of the diazotized *p*-nitroaniline; conditions were otherwise as for solution A in the standard procedure.

Table 3. Recovery of oxaloacetate and acetoacetate added to extracts of liver from normal and starved rats

Extracts were prepared as described in the text and, after the additions indicated, were analysed by the standard method.

Addition to 2 ml. of extract (μmole)		Found (μmole)	
Oxaloacetate	Acetoacetate	Oxaloacetate	Acetoacetate
Normal rat liver			
—	—	0.003	0.004
0.02	—	0.022	—
—	0.047	0.004	0.051
0.02	0.047	0.021	0.051
Liver of rats starved 24 hr.			
—	—	0.004	0.034
0.02	—	0.024	—
—	0.017	0.002	0.051
0.02	0.017	0.022	0.053

Actual levels of oxaloacetate and of acetoacetate in the livers of normal and fasted rats are recorded in Table 4. It is of interest that the level of oxaloacetate in the livers of rats fasted 24 hr. is essentially the same as that in normal animals,

Fig. 7. Linearity of colour development with varying amounts of tissue from normal liver: □, solution A; ■, solution B; ○, oxaloacetate. Conditions were as for the standard method described in the text.

Fig. 8. Linearity of colour development with varying amounts of tissue from the livers of rats fasted for 27 hr.: □, solution A; ■, solution B; ○, oxaloacetate. Conditions were as for the standard method described in the text.

Table 4. *Estimation of oxaloacetate and acetoacetate in the livers of normal and fasted rats*

Estimations were made as described for the standard procedure in the text with 2 ml. of 20% trichloroacetic acid extract of normal livers and 1 ml. of 20% trichloroacetic acid extract of livers from fasted rats. Results are expressed as μ moles/100 g. dry wt. of tissue. Numbers in bold type are the means $\pm$ S.E.

Normal		Fasted, 24 hr.		Fasted, 69 hr.	
Oxaloacetate	Acetoacetate	Oxaloacetate	Acetoacetate	Oxaloacetate	Acetoacetate
6.3	3.6	6.0	91.4	<1	185.0
3.2	7.6	4.3	63.6	1.7	94.1
4.8	6.6	5.6	35.4	2.1	90.0
5.0	8.0	2.0	56.9	2.3	86.7
3.7	4.8	2.4	69.0	3.5	94.3
2.3	2.9	3.9	53.7	<1	112.3
3.0	7.8	5.3	70.8	<1	121.7
3.6	10.1	6.1	34.9	1.8	113.2
4.0	3.0	3.4	70.7	1.0	122.7
		4.8	62.1	3.9	84.0
		3.1	62.7	—	—
4.0 $\pm$ 0.9	6.0 $\pm$ 2.2	4.3 $\pm$ 1.2	61.0 $\pm$ 11.5	1.8 $\pm$ 0.9	110 $\pm$ 21

whereas in the same period acetoacetate has increased approximately tenfold. After fasting for 69 hr., there appears to be a decrease in the level of oxaloacetate. However, the colour due to acetoacetate in these livers is 20–50 times that contributed by oxaloacetate. Consequently, the results for oxaloacetate at 69 hr. may exceed the limit of reliability of the method.

DISCUSSION

The method described in this paper permits the accurate estimation of as little as 0.003 μ mole of oxaloacetate. This exceeds the sensitivity of the usual manometric method by more than 100-fold (Krebs & Eggleston, 1945). The method appears to be sufficiently sensitive and specific for estimating the steady-state tissue levels of this compound. Although an extensive survey of biological compounds has failed thus far to reveal any that seriously interfere with the estimation of oxaloacetate, such may be found in further studies.

The oxaloacetate values for normal rat-liver tissue reported here are lower than those found by Frohman, Orten & Smith (1951*a, b*). However, the determination of oxaloacetate by these authors involved certain technical difficulties which could not be completely resolved.

The major weakness of the present method is that oxaloacetate is determined in the presence of acetoacetate. In most experimental circumstances, the concentration of oxaloacetate may be expected to be very low in relation to that of acetoacetate. The difficulty in estimating oxaloacetate when more than 0.06 μ mole of acetoacetate is present in the sample will limit the general usefulness of the method. Attempts to separate the reaction pro-

ducts of oxaloacetate and acetoacetate by paper chromatography and a variety of solvent systems have been unsuccessful, owing to either inadequate separation or instability of the compounds.

The observation that the steady-state level of oxaloacetate is unchanged after fasting for 24 hr. is of interest. The two major paths for the disposition of acetyl-coenzyme A involve condensation either with acetyl-coenzyme A to form acetoacetate or with oxaloacetate to enter the tricarboxylic acid cycle. It has frequently been proposed that a diminished supply of oxaloacetate is one of the major factors contributing to the accumulation of acetoacetate in ketosis (cf. Lehninger, 1946; Krebs, 1948–9; Breusch, 1948; Weinhouse & Millington, 1951; Lynen, 1952–3; Chaikoff & Brown, 1954; Cantarow & Trumper, 1955; West & Todd, 1955). The present results indicate that significant ketosis can develop in the presence of normal levels of oxaloacetate in the liver. This appears to be in accord with the observation that the administration of succinate *in vivo* does not relieve ketosis in diabetic humans or rats (Lawrence, McCance & Archer, 1937; Deuel, Hallman & Murray, 1937; Terrell, 1938), and with the finding that the conversion of [14 C]acetate into 14 CO₂ proceeds at nearly identical rates in the livers of normal, fasted and alloxan-diabetic rats (Masoro, Chaikoff, Chernick & Felts, 1950; Chernick & Chaikoff, 1951; Lyon, Masri & Chaikoff, 1952; Medes, Thomas & Weinhouse, 1952; Medes, Spirtes & Weinhouse, 1953). However, it should be emphasized that the present observations show only that the level of oxaloacetate in liver may remain within normal limits early in ketosis. Information concerning the rate of turnover or the total quantity of oxaloacetate available for condensation with acetyl-coenzyme A is not provided by this study.

SUMMARY

1. The colorimetric method for estimation of acetoacetate by reaction with diazotized *p*-nitro-aniline has been adapted for measurements of oxaloacetate in amounts of 0.003–0.03 μ mole.

2. The steady-state level of oxaloacetate in the livers of normal and fasted rats was found to be about 4 μ moles/100 g. dry wt. of tissue. The level was essentially unchanged in starvation when the level of acetoacetate increased tenfold.

The authors wish to thank Professor Sir Hans Krebs, F.R.S., and the members of the Medical Research Council Unit for their help, advice and interest during the progress of this work, and Mr T. Gascoyne for skilful technical assistance during the last stages of this work. One of us (G.K.) gratefully acknowledges the support of the Traveling Fellowship of the College of Medicine of the State University of Iowa, the National Science Foundation and Office of Naval Research (U.S.A.).

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Oak-bark Tannins

By D. E. HATHWAY

The British Leather Manufacturers' Research Association, Milton Park, Egham, Surrey

(Received 14 January 1958)

The barks of the two common oaks, the pedunculate oak (*Quercus pedunculata* Ehrh.) and the sessile oak (*Quercus sessiliflora* Salisb.) have been the traditional tanning material of Britain and northern Germany since medieval times. The principal botanical difference between the species is that the pedunculate oak has acorns on a long peduncle and stalkless or nearly stalkless leaves, whereas the sessile oak has sessile or stalkless acorns and petiolated leaves, but intermediate or hybrid forms are common. For commercial purposes, the timber and bark of these trees are not differentiated. Our experience of this tanning material shows that best-quality oak bark contains

12–16% of tannin [cf. Howes (1953) who gives 12–14%].

Little information on oak-bark phlobatannins is available, but (+)-catechin and (+)-gallo catechin have recently been isolated from this source (Mayer, 1956; cf. Casparis & Reber, 1929). The nature of the partial-type formula for oak-bark phlobatannins has now been investigated. Phlobatannins have recently been isolated in high yield from the extractives of *Acacia catechu* heartwood and harvested *Uncaria gambir* leaves (Hathway & Seakins, 1957*a*), which have the same analytical properties and similar elementary analyses and absorption spectra to those of the polymers of