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Optimization of Conditions for the Colorimetric Determination of Citrulline, Using Diacetyl Monoxime

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A method is described for colorimetric determination of citrulline following deproteinization, depending upon the reaction with diacetyl monoxime in the presence of sulfuric and phosphoric acids. Ferric chloride is included to sensitize the reaction and thiosemicarbazide to improve light stability of the chromogen. The method offers diminished heating time compared with existing procedures, combined with good sensitivity and photostability of the chromophore.

The colorimetric estimation of citrulline is most often required in the assay of ornithine transcarbamylase (OTC,[1] EC 2.1.3.3), which catalyzes the second step of urea synthesis in mammals (1,2), and is also by reason of its tissue-specific location a valuable indicator of liver damage (3–5). Depressed hepatic levels of OTC are found in genetic disorders causing hyperammonemia (6–10), in Reye's syndrome (11–13), cirrhosis (14), and acute fatty liver of pregnancy (15).

Several recent reports (16–18) describe methods for assay of serum OTC levels in which deproteinization is avoided. But such cannot be applied blindly to tissue samples or homogenates because of widely varying levels and because of the intramitochondrial location of the enzyme (19). There may be circumstances in which a method involving deproteinization should be used for serum samples also since a greater sensitivity is then obtainable. We have accordingly sought to optimize conditions for citrulline assay after removal of proteins and present a method which should prove widely applicable.

Determination of citrulline by the method of Archibald (20) is based on the Fearon reaction (21), in which carbamido compounds such as urea and citrulline form a color complex with diacetyl monoxime or diacetyl in acid solution. The original method (20) suffers from several disadvantages, namely, low sensitivity, nonlinearity of the calibration curve, and instability of the chromogen formed in the presence of light. Removal of protein prior to assay of citrulline has been found necessary in general (22), although several deproteinizing agents interfere in color development by producing opalescence (23).

Various modifications (24,25) of Archibald's method have proved to be tedious and unsatisfactory. The most widely used procedure (that of Guthöhrlein and Knappe (26)), though much more sensitive than Archibald's, requires a prolonged boiling time of 20 min for color development. Moreover, the standard curve for citrulline is nonlinear at low citrulline concentrations and the color complex although stable in the dark begins to fade after only 30 min in diffuse daylight.

Our method for citrulline is based on that of Favreau and Coulombe (27) for colorimetric determination of urea. The boiling time for color development has been cut to 5 min and addition of thiosemicarbazide has

[1] Abbreviations used: OTC, ornithine transcarbamylase; TCA, trichloroacetic acid; DAMO, diacetyl monoxime; TSC, thiosemicarbazide.

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led to stabilization of the color complex in light. The standard curve for citrulline is linear for quantities up to at least 0.1 μmol; and protein-precipitating agents like trichloroacetic acid (TCA) cause no inhibition in the development of color. The sensitivity of citrulline estimation is much greater than that in Guthöhrlein's procedure, which should enable OTC activity to be measured accurately in tissue homogenates containing depressed levels of the enzyme.

MATERIALS AND METHODS

Reagents

1. Acid–ferric solution. To 550 ml distilled water, add 250 ml concentrated sulfuric acid (95–98%, E. Merck, Darmstadt, W. Germany) and 200 ml concentrated phosphoric acid (85%, Mallinckrodt Chemical Co., St. Louis, Mo.). Cool to room temperature, and dissolve $FeCl_3$ (250 mg/liter) in the above solution. The solution when kept at room temperature is stable for more than 2 months. For the purposes of this paper, the total volume of this and similar solutions is taken to be 1 liter.

2. Diacetyl monoxime solution. To 100 ml of distilled water add 500 mg diacetyl monoxime (DAMO, 2,3-butanedione monoxime, Sigma Chemical Co., St. Louis, Mo.). Stored in a brown bottle, the solution is stable for more than a month.

3. Chromogenic reagent. Just before use add 5 mg thiosemicarbazide (TSC, Sigma Chemical Co.) to 50 ml of Reagent 2, followed by 100 ml of Reagent 1. This solution should be used no later than 1 h after preparation.

4. Citrulline standard solution (1 mmol/liter). Dissolve 17.52 mg DL-citrulline (Sigma Chemical Co.) in 100 ml distilled water. Kept frozen at −20°C, this can be stored for prolonged periods.

Procedure For Citrulline Estimation In Enzymatic Samples

Typically the sample is the reaction mixture following appropriate incubation for OTC assay and since urea also forms a color complex with diacetyl monoxime, urease should be included in the incubation mixture. (We generally used urease Type VII, Sigma Chemical Co.) The mixture may be deproteinized, and the enzyme reaction stopped, by adding TCA solution to a final concentration of at least 5%, then centrifuging and collecting the supernatant. (We occasionally experienced slight turbidity when using 50% TCA solution. This was overcome by using a larger volume of a more dilute solution and raising the final concentration to 7.5%.)

To 0.1 ml of supernatant add 3 ml of chromogenic solution (Reagent 3). Mix vigorously in a Vortex mixer (Whirlimixer, Fison's Scientific Apparatus, England), and boil at 100°C for 5 min. A covered water bath is preferable to minimize evaporation. Following boiling, cool the tubes to room temperature and measure absorbance at 530 nm, for example, in a Varian Series 634 double-beam spectrophotometer. Run a blank and a citrulline standard simultaneously with samples. Nanomoles of citrulline in the aliquot of supernatant can be read off from a standard curve, though the color development is so reproducible that we have habitually run only one confirmatory standard with each routine assay batch.

We used Pyrex glass tubes 12.5 cm long by 1.5 cm i.d., but have no evidence that tube size affects the results. Mixing was for 10–15 s so that this process occupied 5–10 min for a batch of 20–40 tubes: tubes already mixed but not yet boiled could be left on the bench in diffuse daylight for at least 30 min without affecting color development. Tubes were not stoppered during either mixing or boiling. Measurements were made in cuvettes with a 1-cm path length.

The sample volume may be increased to compensate for low citrulline levels (without changing reagent composition) up to a maximum of 1.0 ml. Preferably, a new standard curve should be constructed using the same volumes of aqueous citrulline standards as the selected sample volume, although in

terms of molar absorbance there is no detectable change in sensitivity as water content is increased. (Overall practical sensitivity—absorbance per micromoles—is diminished.)

EXPERIMENTAL RESULTS

Variation of Acids in Chromogenic Reagent (Fig. 1)

A strongly acid solution is necessary for color development. H_2SO_4 alone can increase the sensitivity of color development up to an optimum concentration of 250 ml per liter of acid solution, but the color formed is relatively light sensitive. A mixture of H_2SO_4 and H_3PO_4 is necessary to obtain both insensitivity to light and linearity of the standard curve. With H_3PO_4 in the absence of H_2SO_4, color production is negligible. Final concentrations chosen for the acid–ferric solution were 250 ml H_2SO_4 and 200 ml H_3PO_4 per liter because although color development under these conditions is not maximal, the chromogen is very much less sensitive to light.

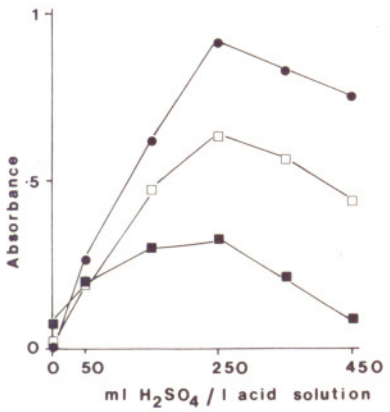

FIG. 1. Effect of acid variations on color development. 0.1 ml of citrulline solution (1 mmol/liter) was boiled for 5 min in the presence of 1 ml of DAMO–TSC solution (2 g DAMO and 100 mg TSC per liter and 2 ml of acid solution containing varying proportions (ml/liter) of H_2SO_4, (I) without H_3PO_4 (●), (II) with 200 ml H_3PO_4 per liter acid solution (□), and (III) with 450 ml H_3PO_4 per liter acid solution (■).

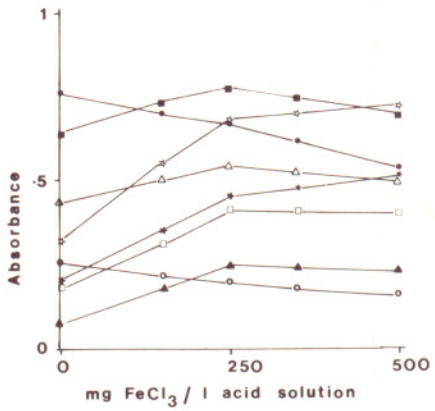

FIG. 2. Studies of sensitivity of citrulline measurement as a function of acid reagent compositions and concentrations of ferric chloride. Citrulline solution (0.1 ml, 1 mmol/liter) was boiled for 5 min in the presence of 1 ml of DAMO–TSC solution (2 g DAMO and 100 mg TSC per liter) and 2 ml of acid solution having the following compositions (per liter solution): (I) 50 ml H_2SO_4 (○), (II) 450 ml H_2SO_4 (●), (III) 50 ml H_2SO_4, 200 ml H_3PO_4 (□), (IV) 250 ml H_2SO_4, 200 ml H_3PO_4 (■), (V) 450 ml H_2SO_4, 200 ml H_3PO_4 (△), (VI) 450 ml H_3PO_4 (▲), (VII) 50 ml H_2SO_4, 450 ml H_3PO_4 (★), and (VIII) 250 ml H_2SO_4, 450 ml H_3PO_4 (☆). Ferric chloride in the requisite amount was added dissolved in the acid solution.

Following the method of urea estimation by Beale and Croft (28), we tried substituting mixtures of hydrochloric, nitric, and acetic acids in our recommended procedure. Replacing phosphoric acid with an equal concentration of hydrochloric or acetic acid diminished color development by 43 and 40%, respectively. Color development was inhibited by 8% when 50 ml HCl (per liter acid solution) was used in addition to H_2SO_4 and H_3PO_4 in the recommended procedure. Various combinations of sulfuric, phosphoric, hydrochloric, and nitric acids failed to improve color intensity.

Effect of $FeCl_3$ (Fig. 2)

Ferric salts are known to accelerate the carbamido–diacetyl reaction catalytically (29). We observed, however, that the effect of $FeCl_3$ varies with the composition of the acid reagent. For instance with sulfuric acid

only (no phosphoric acid), $FeCl_3$ inhibited color formation. With varying sulfuric acid concentrations in the presence of phosphoric acid (200 ml/liter acid reagent), progressively increasing sensitivities were obtained up to an optimum of 250 mg $FeCl_3$ per liter of acid solution (0.5 mg $FeCl_3$ per assay tube). With higher concentrations of phosphoric acid (450 ml/liter acid reagent), the sensitivity was found to increase up to a concentration of 500 mg $FeCl_3$ per liter of acid solution (1 mg $FeCl_3$ per assay tube).

The proportionate extent of improvement obtained by the use of $FeCl_3$ also varied with acid reagent composition. Not too surprisingly there was little improvement when using the optimum concentrations of H_2SO_4, H_3PO_4, DAMO, and TSC recommended above (+12%). Representing acid reagent compositions as milliliters per liter of H_2SO_4 and H_3PO_4 (H_2SO_4/H_3PO_4), we may cite some other results as follows (250 mg $FeCl_3$ per liter): —50/200, +119%; 50/450, +121%; 250/450, +113%.

Effect of Salts and Buffers

The influence of a number of cations upon the reaction rate of a carbamido compound (urea) was reported as previously by Ceriotti and Spandrio (29) and in our experiments we noticed a decrease of color if $FeCl_3$ is totally replaced by manganous salts. It was therefore of interest to study the effect of various salts on color formation. NaCl, KCl, KH_2PO_4, $MnCl_2$, $MnSO_4$, $CoSO_4$, $ZnSO_4$, and EDTA were added to the acid reagent in concentrations ranging from 5 to 100 mmol/liter. There was absolutely no effect on color formation with aqueous citrulline standards.

Many buffers have been advocated for assay of OTC (16,17,30), so it seemed worth checking whether there was any adverse effect. No alteration in color development could be detected when citrulline standards were made up in the following buffers instead of water: phosphate, 0.1–0.5 mol/ liter (pH 6.8–7.7); Tris/HCl 0.1–0.3 mol/ liter (pH 6.9–10.5); borate/sodium, 0.1–0.3 mol/ liter (pH 6.9–10.5); veronal acetate, 0.01–0.03 mol/liter (pH 6.9–10.5); triethanolamine/HCl, 0.05–0.3 mol/liter (pH 6.9–10.5).

Influence of Diacetyl Monoxime and Thiosemicarbazide (Fig. 3)

Various concentrations of DAMO from 0 to 10 g/liter (0 to 10 mg/assay tube) were tested to find the optimum concentration for color development. Color production decreased slightly when the DAMO concentration exceeded 5 g/liter. Thiosemicarbazide showed an optimum concentration at 100 mg/liter solution (0.1 mg per assay tube). Higher concentrations were found to be inhibitory as well as increasing the absorbancy of blanks.

Boiling Time

Maximum color development took place within 5 min of boiling in a water bath at 100°C. Further boiling diminished intensity of color (Fig. 4).

Stability of Color

In a well-illuminated room the color remains stable (after boiling and cooling) for 2 h. When kept in the dark, color is stable for at least 6 h.

Spectrum, Linearity

The absorbance spectrum of the citrulline reaction product has a broad peak with maximum absorbance (λ_{max}) at 530 nm. The standard curve was linear up to the maximum concentration of citrulline tested (0.1 μmol per assay tube).

Interferences

Equivalent molar concentrations of arginine, carbamyl aspartate, glycine, and urea produced, respectively, 2.4, 10, 2.1, and 35% of the color yielded by citrulline. Urea must be destroyed (as recommended) before at-

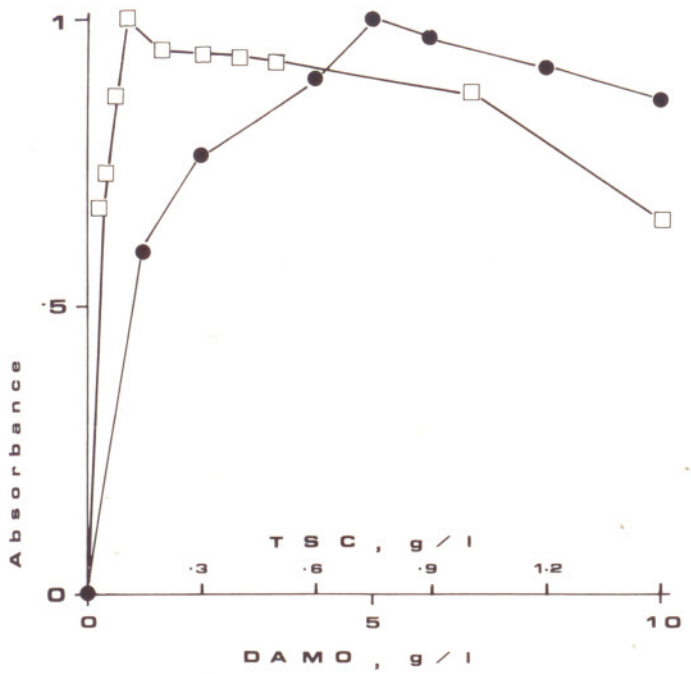

FIG. 3. Studies of sensitivity of citrulline measurement as a function of different concentrations of diacetyl monoxime and thiosemicarbazide. Citrulline solution (0.1 ml, 1 mmol/liter) was boiled for 5 min with 2 ml of acid–ferric solution (250 ml H_2SO_4, 200 ml H_3PO_4, 250 mg $FeCl_3$ per liter) and 1 ml of a solution containing either (a) 100 mg TSC per liter with appropriate amounts of DAMO (●), or (b) 5 g DAMO per liter with appropriate amounts of TSC (□).

tempting the assay of citrulline. The other interferences should not often prove serious.

Deproteinization by the Folin–Wu, Nelson–Somogyi, and $CdSO_4$–$Ba(OH)_2$ procedures did not affect color development. TCA up to 250 g/liter in an aqueous citrulline solu-

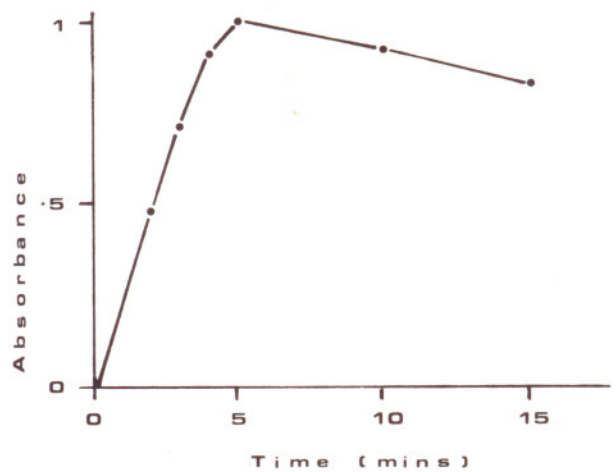

FIG. 4. Effect of boiling time of color development. Citrulline solution (0.1 ml, 1 mmol/liter) was boiled for various time periods with the recommended (optimum) chromogenic reagent.

TABLE 1

COMPARISON OF RECENT METHODS FOR CITRULLINE

Method (reference No.)	Constituents of chromogenic reagent		Boiling time for color development (min)	Total volume of mixture (ml)	λ_{max} of chromogen (nm)	Absorbance due to 0.1 μmol citrulline	Stability of color in light (h)
	Acids	Other components					
(16)	H_2SO_4, CH_3COOH	DAMO, antipyrine, $Fe_2(SO_4)_3$	15	3.075	460	0.841	1
(17)	H_3PO_4, H_2SO_4	DAMO, TSC, $FeCl_3$	15	6.70	515	0.207	3
(18)	H_2SO_4, CH_3COOH	DAMO, antipyrine	25	2.06	464	1.290	0.67
(23)	H_2SO_4, H_3PO_4, $HC10_4$	Dimethylglyoxime	15	3.00	495	0.020*	Several hours
(24)	H_2SO_4	Semidine/DAMO, $FeCl_3$	10	3.50	550	0.600*	1
(25)	H_2SO_4, H_3PO_4	DAMO, $MnSO_4$/ $FeCl_3$	30	3.75	490	0.240[a]	24
(26)	H_3PO_4, H_2SO_4	DAMO	20	3.00	490	0.450[a]	0.5
(30)	H_2SO_4, H_3PO_4	DAMO, NaCl, antipyrine, ferric ammonium sulfate	15	3.50	464	1.095	1
(33)	H_2SO_4, CH_3COOH	DAMO, antipyrine, $Fe_2(SO_4)_3$, ethylene glycol	30	4.50	460	0.711	Not mentioned
Present method	H_2SO_4, H_3PO_4	DAMO, TSC, $FeCl_3$	5	3.10	530	1.040	2

Note. Sensitivities are mostly as determined by us; exceptions are marked with an asterisk (as given in the original reference). (Statements in the literature about sensitivity are often not clearly phrased.) Light stability is as stated by the original authors. There are obviously great difficulties in evaluating this feature because of variations in room lighting: we refer to diffuse tropical daylight. For simplicity, the components of chromogenic reagents are classified here into acids and "other components." Thus the listing here does not directly reflect the practical grouping of reactants into stock solutions as recommended by the original authors.

[a] Calibration curve nonlinear.

tion did not affect color production by the recommended procedure: there was 4% inhibition at the highest concentration tested (450 g/liter).

Reproducibility

For aqueous citrulline standards (normally 0.1 μmol in 0.1 ml), tested by the recommended procedure, the coefficients of variation found were: within batch, 0.68%; between batches, 1.13% (mean of four tests each run over five batches, range 0.48–1.64).

DISCUSSION

Antipyrine (phenazone) and TSC are usually referred to as color intensifiers, although the absorbance maxima of the chromophores produced are quite different. This should be borne in mind when studying the comparison of various methods presented in Table 1. Antipyrine methods offer high sensitivity, but the chromophore photostability is generally not very good and there are other disadvantages including light sensitivity of the reagents as currently formu-

lated. When comparing sensitivities, it should also be borne in mind that the recommended procedure above is not the most sensitive we have studied, being preferred for other reasons.

The chromogenic reagent for citrulline measurement in the present method differs from that which we have found optimal for determining citrulline in the presence of serum proteins (manuscript in preparation). The presence of proteins causes the formation of brown pigments, possibly as a result of acid degradation and consequent liberation of tryptophan. On boiling in acid, carbohydrates present in serum would be converted to furfuraldehyde which might then give an aldehyde color reaction with tryptophan (Hopkin–Cole reaction) (31). With higher concentrations of acids in the reagent, proteins tend to precipitate; also $FeCl_3$ cannot be used because in its presence absorbances due to citrulline and to the protein blank are not additive. In the procedure without deproteinization, we therefore deliberately use low concentrations of acids and high concentrations of DAMO and TSC, but omit $FeCl_3$. This largely eliminates protein interference, at the cost of halving the sensitivity. A similar nondeproteinizing procedure (with more H_2SO_4) is very sensitive for the estimation of urea (32), whereas under the present conditions urea yields only about one-third the absorbance of citrulline.

The nature of the complex formed by citrulline and other carbamido compounds with diacetyl monoxime or diacetyl is apparently still unknown and a matter of controversy. Beale and Croft (28) postulated formation of triazines, while other authors have advocated involvement of glycolurils in the overall reaction scheme (34).

Besides OTC, citrulline is a product of arginine deiminase (EC 3.5.3.6) (and the above method could probably be used in assays of this enzyme) which, however, plays no part in mammalian metabolism. In mammals, apart from liver, OTC is also present in lesser amounts in other tissues like intestine and kidney (35–37), and it is in study of such tissues, and of conditions where tissue levels are much decreased, that the most sensitive possible method, free of interferences, may prove valuable. For example, elevated serum levels have been reported in intestinal disorders (38) and yet there are sharply conflicting reports even on normal tissue levels (35–37).

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