67. A COLORIMETRIC METHOD FOR THE DETERMINATION OF CITRULLINE[1]

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IN the course of experiments on the mechanism of urea formation, we came to feel the need of a convenient method for the determination of small quantities of citrulline. The carbamido-diacetyl reaction described by Fearon [1939] appeared to offer a prospect in that direction. Fearon himself had utilized it in an approximate determination of the citrulline content of trypsin-digested casein, but in general his observations were qualitative. We are indebted to him for permitting, and indeed encouraging, us to explore independently the possibilities of his test as the basis of a quantitative method.

The reaction consists in the development of a red (in the case of urea a yellow) colour, when the reacting substance is heated with diacetyl monoxime in acid solution and the product subsequently oxidized with potassium persulphate. It is not specific for citrulline, being given apparently by all compounds of the general formula $R.NH.CO.NH_2$ and by some of the formula $R_1.NH.CO.NH.R_2$. To Fearon's list of substances tested and found to react positively we are able to add the α-carbamido derivatives of propionic, *n*-butyric, *n*-valeric, *iso*butylacetic, δ-guanidinovaleric acid and δ-aminovaleric acids, β-carbamidopropionic acid, ε-carbamidohexoic acid and 3-carbamido-2-piperidone [Hunter, 1938]. Among known tissue constituents, other than citrulline itself, the only ones that give the test are urea, allantoin, proteins and such higher protein derivatives as peptone and gelatin.

In adapting the reaction to the quantitative determination of citrulline we have employed the method of photoelectric colorimetry, using the instrument of Evelyn [1936]. This possesses the advantage over most similar forms of apparatus (of particular importance for the present application) that the reaction is carried out, and the colour developed, in the same special test tubes, which serve as the absorption cells of the colorimeter. The procedure recommended could doubtless be adapted to any other photoelectric colorimeter. For visual colorimetry it is hardly suitable.

Lacking facilities for a precise spectrophotometric study of the coloured reaction mixture, we had to be guided, in the choice of a suitable light filter, by an approximate procedure, in which the photocolorimeter itself is used as a spectrophotometer. A spectrogram of the red solution (for the preparation of which we are indebted to Dr Ireton and Dr Allin of the Department of Physics, University of Toronto) revealed an absorption band in the green and blue, extending roughly from 460 to 520 mμ. Following this indication, we applied the analytical procedure, presently to be described, to six 7 ml. samples of a 0·001 % citrulline solution, using with successive samples each in turn of the

[1] The method was described, and a preliminary account was given of some results already obtained with it, at a meeting of the Royal Society of Canada held at Kingston, Ontario, on 21 May 1941.

six Rubicon filters numbered (in correspondence with the dominant wave-length of the transmitted light) from 420 to 565 mμ. The six values for photometric density thus obtained were plotted against the filter numbers or dominant wave-lengths. The resulting curve suggested that the maximum of absorption occurs at or near 475 mμ. A filter ideal for that wave-length not being immediately procurable, No. 490 (transmission limits 465 to 530 mμ) was adopted as the best available compromise.

It may here be mentioned that we constructed similar rough absorption curves for urea, allantoin, methyl- and phenyl-urea, α- and β-carbamidopropionic acids, ε-carbamidohexoic acid and 3-carbamido-2-piperidone. For the four last of these the curves were to all appearance identical, both in form and position, with the curve for citrulline. From that pattern methyl- and phenyl-urea showed only minor deviations. The curve for urea, as was to be expected, was conspicuously different, its peak falling in the neighbourhood of 450 mμ. Allantoin yielded a curve differing little from that of urea.

In order to obtain consistent results in the quantitative application of Fearon's test it was found necessary to define very rigidly both the proportions of the reagents and the conditions under which the colour is developed. The technique we are about to describe, adopted after many exploratory experiments, not only ensures the reproducibility of the results, but enhances considerably the sensitivity of the reaction. It permits the estimation of citrulline in any concentration down to (for pure solutions) 0·2 or 0·3 mg./100 ml., with an error which, except at the very lowest concentrations, does not exceed 2% (see Table 2).

Description of the method

The following description takes for granted an acquaintance with the theory and the general operational technique of the Evelyn photoelectric colorimeter [Evelyn, 1936; Dann & Evelyn, 1938] and omits mention of details common to all methods in which that instrument is employed.

A constant-level water bath is provided with a cover and a perforated false bottom. The cover is pierced with a number of round holes, of a diameter (about 22 mm.) exactly fitting the absorption tubes of the colorimeter. The false bottom is fixed at such a level that the vertical distance between it and the cover is between 70 and 90 mm. The water level is adjusted so that the height of water above the false bottom is between 45 and 50 mm. The dimensions specified are such that during the period of heating only that part of the tube will be immersed which is charged with the reacting mixture, while half at least of its length will project above the cover. The objects sought are the standardization of the heating and the avoidance, as far as possible, of concentration through evaporation. The second of these objects requires that the projecting part of the tube should be kept as cool as possible. All the holes in the cover should therefore either be occupied by tubes or covered by small watch-glasses. It is of advantage also to have a number of holes in the wall of the bath below the cover, so that escaping steam will be directed away from the tubes.

While the water in the bath is kept gently, but steadily, boiling, a measured volume of the citrulline-containing solution is transferred to one of the special absorption test-tubes of the colorimeter. The volume should be such as to contain about 0·05–0·07 mg. of citrulline—in no case less than 0·02 or more than 0·12 mg. Water is added to make a total volume of exactly 7 ml. Alternatively the solution may first be diluted to a concentration of 0·7–1 mg./100 ml., 7 ml. being then measured into the absorption tube. If the concentration of citrulline is

quite unknown to begin with, one or more random trials will usually indicate the quantity or the dilution proper for a precise determination.

To the citrulline solution there are added (1) 4 ml. of conc. HCl (sp.gr. 1·18–1·19) and (2) 0·5 ml. of a 3 % solution of diacetyl monoxime. The reactants having been mixed, the tube is placed in the bath, and a 1 in. funnel is set in its mouth to act as a condenser. The tube is left in the boiling water for *exactly* 9 min. It is then removed, wiped dry and allowed to cool in the air for 6 min. This will reduce its temperature to about 65°. If citrulline is present, the mixture will already show a pink colour. To develop this more fully, there is now added one drop of 1 % potassium persulphate solution. The tube is thereupon placed in position in the colorimeter (already equipped with the appropriate filter, No. 490, and adjusted to the reagent-blank rest point), and the behaviour of the galvanometer spot is observed. As the colour deepens, the galvanometer reading, of course, falls. When it shows signs of becoming stationary, another drop of persulphate is added. If this produces a further fall, a third drop may be needed to make sure that the maximum attainable depth of colour and the minimum galvanometer reading have been reached. This is usually accomplished in 1–2 min. After a time the colour begins to fade, and the galvanometer reading to rise again. The lowest reading observed is the significant one. It is translated into terms of citrulline concentration by reference to a previously constructed calibration curve.

A calibration curve is necessary because, with the technique described, the logarithmic law of Lambert and Beer holds good only over a limited range of concentrations. The reason may be that the filter used is insufficiently selective, or that with the lower concentrations fading of the colour overtakes its full development. A curve for use with pure solutions may be constructed from the data recorded in Table 1. In practice only that part of the curve would ordinarily

Table 1. *Calibration curve for pure solutions of citrulline*

ml. of stock citrulline solution (5 mg./100 ml.) in a total volume of 7 ml.	Final citrulline concentration (mg./100 ml.)	Galvanometer reading (average of two to three trials)
0	0	100 (R.P. = 66)
0·3	0·214	88·75
0·6	0·429	73·7
0·9	0·643	59·9
1·2	0·857	48·0
1·5	1·071	38·0
1·8	1·285	29·4
2·1	1·50	23·7
2·4	1·715	19·3
2·7	1·93	16·25
3·0	2·14	13·3
3·6	2·57	10·0

be used, which lies between galvanometer readings of 80 and 20 (the respective citrulline concentrations being 0·35 and 1·7 mg./100 ml.). The concentration read from the curve is of course that existing in the volume of 7 ml. directly analysed. From this the concentration in the original solution is readily calculated.

The time occupied by a single determination (from the addition of the reagents to the final reading of the galvanometer) is 16–17 min., and so simple are the manipulations required, that a series of determinations can be carried along in orderly progression at regular intervals of 3 min.

To the foregoing description of the method we may add here the following comments. With a given amount of citrulline the depth of colour obtained depends upon the proportions of HCl and diacetyl, upon the intensity and duration of the heating and, in a smaller measure, upon the length of the subsequent cooling period. In the procedure prescribed the reagents are applied in what have been found to be the optimal concentrations with respect to colour development. The period of hearing, on the other hand, is a compromise. If it is shortened, less colour is produced, and the sensitivity of the method is correspondingly diminished. If it is lengthened, colour production and sensitivity are increased; but the method then becomes rather tedious. A change in either direction, while necessitating the construction of another calibration curve, is not only permissible, but might in special circumstances be of advantage. The essential point is not that the reaction mixture be heated for 9 min., but that both the time and the manner of heating be always exactly the same. The prescribed time of cooling need not be quite so rigidly adhered to.

Accuracy of the method with pure solutions

In the preparation of the calibration curve the agreement between duplicate or triplicate runs on any one concentration of citrulline was always within 2%. It was to be expected that in the analysis of pure solutions the results obtained would be equally good. This expectation is confirmed by the examples given in Table 2. Only occasionally does the error exceed the proportion named.

Table 2. *Analysis of pure solutions of citrulline of known concentration*

Concentration, mg./100 ml.	
Known	Found
0·236	0·22
0·59	0·58
0·67	0·67
0·75	0·73
0·75	0·75
0·826	0·83
1·00	1·00
1·00	0·99
1·00	1·02
1·46	1·46

Behaviour of reacting substances other than citrulline

In any practical application of the method, account has to be taken of certain difficulties, the first of which is the lack of specificity of the carbamido-diacetyl reaction. Among the substances which give this reaction there are at least three which may be expected to be present in practically every animal tissue or fluid: protein, urea and (except in human material) allantoin.

Protein and its higher derivatives occupy an exceptional position in that they yield, under the conditions prescribed, not only what appears to be a citrulline reaction, but also an additional violet colour (Liebermann reaction) partially or completely obscuring the other. Fearon discusses means of minimizing this effect in the qualitative test, but we have found no satisfactory way of preventing or compensating for it in the quantitative technique. Any solution to be analysed for free citrulline must therefore first be rendered protein-free. Among possible deproteinizing agents the most suitable is trichloroacetic acid. Uranyl acetate and tungstic acid may also be employed, although with the

latter care must be taken to avoid such an excess, as would precipitate on the addition of the hydrochloric acid.

In order to evaluate the disturbing effects of urea and allantoin we analysed solutions of each in routine fashion, just as if they were solutions of citrulline. As a matter of interest (although of less immediate practical importance), we also treated in the same way solutions of the other carbamido compounds listed in Table 3. Each substance was tested in at least two concentrations. These are stated in the third column of the table, while in the fourth there is shown for each that concentration of citrulline to which, in carbamido content, it is equivalent. The fifth column records the citrulline concentrations which the method appeared to indicate, while in the last the 'citrulline' thus found is expressed as a percentage of the citrulline equivalent. For citrulline itself the figure in the last column is of course 100.

Table 3. *Behaviour of various substituted ureas in the proposed method for citrulline*

No.	Substance	Concentration tested mg./100 ml.	Equivalent concentration of citrulline mg./100 ml.	Apparent citrulline concentration found	
				mg./100 ml.	% of citrulline equivalent
1	Urea	0·4	1·17	0·43	36·7
		2·0	5·84	1·56	26·7
		4·0	11·67	1·86	15·9
2	Allantoin	10·0	11·1	0·86	7·7
		20·0	22·2	1·33	6·0
3	Methylurea	0·212	0·50	0·55	110·0
		0·846	2·00	2·00	100·0
4	Phenylurea	0·39	0·50	0·38	76·0
		0·777	1·00	0·75	75·0
5	α-Carbamidopropionic acid	10·0	13·3	0·46	3·5
		20·0	26·5	1·04	3·9
6	α-Carbamido-*n*-butyric acid	10·0	12·0	0·24	2·0
		20·0	24·0	0·51	2·1
7	α-Carbamido-*n*-valeric acid	10·0	10·9	0·24	2·2
		20·0	21·9	0·57	2·6
8	α-Carbamido*iso*hexoic acid	10·0	10·0	0·17	1·7
		20·0	20·0	0·42	2·1
9	α-Carbamido-δ-guanidino-*n*-valeric acid	10·0	8·1	0·19	2·3
		20·0	16·1	0·38	2·4
10	α-Carbamido-δ-amino-*n*-valeric acid	10·0	10·0	0·18	1·8
		20·0	20·0	0·38	1·9
11	β-Carbamidopropionic acid	0·377	0·50	0·375	75·0
		0·754	1·00	0·775	77·5
12	δ-Carbamido-α-amino-*n*-valeric acid (citrulline)	—	—	—	100·0
13	ε-Carbamido-*n*-hexoic acid	0·50	0·50	0·56	112·0
		1·00	1·00	1·11	111·0
14	3-Carbamido-2-piperidone	10·0	11·2	0·75	6·7
		20·0	22·3	1·56	7·0

The reactivity of allantoin is seen at once to be of a very low order. It seems unlikely that in conventionally prepared blood or tissue filtrates one would ever encounter an allantoin concentration as great as 1 mg./100 ml. Table 3 shows that this would be equivalent to 1·11 mg. of citrulline, but would yield, over the spectral wave-band employed, only 7·7 % as much colour—would simulate, that is to say, a citrulline concentration of only 0·086 mg./100 ml. In the uses to

which we have hitherto put the method, this would be a negligible quantity. At the same time it must be recognized that substantial concentrations of allantoin, such for example as might occur in urine, would seriously complicate the application of the method.

The possible influence of urea is more important. A 1:10 protein-free filtrate of normal blood would contain about 3 mg./100 ml. of urea. From the data in Table 3 it may be estimated, by interpolation, that this would give about 20 % as much colour as the equivalent amount (8·76 mg.) of citrulline. In the complete absence, therefore, of citrulline itself, the filtrate would show an apparent citrulline content of as much as 1·75 mg./100 ml. Similarly it may be calculated that a saline tissue extract containing, when diluted for a citrulline determination, 1 mg./100 ml. each of citrulline and urea (a combination such as has frequently arisen in our experiments) would yield, by the prescribed method, a citrulline value (1·95) almost double the true one. Since the urea colour is different from the citrulline one, the difficulty could perhaps be met by making two measurements of photometric density, each with a different, appropriate and sufficiently selective filter; but it is simpler to destroy the urea with urease, and this expedient we have accordingly adopted as a necessary preliminary to the determination of citrulline in any biological fluid. The destruction of urea can of course be conducted as a step in its manometric determination [Krebs & Henseleit, 1932]. Afterwards the urease can be removed, along with pre-existing proteins, by any suitable reagent.

Urea and allantoin are the only substances in Table 3 which come under consideration as regular tissue constituents. The others (citrulline itself excepted) are of interest only from a theoretical point of view. Six of them (Nos. 3, 4, 5, 11, 13 and 14) have been shown to yield, in the diacetyl reaction, products with absorption spectra differing little, if at all, from that of the citrulline product. As far as visual observation goes, the colour given by the remaining five (Nos. 6–10) is of exactly the same reddish shade. For all of these therefore (but not for urea or allantoin) the figures in the last column of Table 3 indicate the relative intensity with which the carbamido group reacts in the various combinations represented.

Regarded from this point of view the data show that, as a test for substituted ureas, the carbamido-diacetyl reaction varies in sensitivity with the character of the substituting group. Thus methylurea gives about the same depth of colour as an equivalent quantity of citrulline, but phenylurea gives only three-fourths as much, and piperidonylurea only one-fifteenth. Of particular interest are the results within the special group of the carbamido-acids, to which citrulline itself belongs. Here the intensity of the reaction appears to depend directly upon the distance separating the urea residue from the carboxyl. With α-carbamido-acids the reaction is feeble, and is little, if at all, affected by either the length of the fatty acid chain or the presence of such other substituents as the amino- or guanidino-group. The β-acid gives about 25 times more colour than the average α-acid, but not so much as the δ-acid, citrulline. The reactivity of the latter is surpassed, in turn, by the ε-acid. As one special example of these relations, citrulline is seen to react more than 50 times as intensely as its isomer, α-carbamido-δ-amino-*n*-valeric acid (No. 10).

Application of the method in tissue analysis

The determination of citrulline in biological material (in blood, for example, or in tissue extracts) requires, as has been shown, the prior removal of urea and proteins; but, even with this accomplished, other complicating factors have still

to be dealt with. In the first place most urea- and protein-free filtrates, devoid though they may be of citrulline, still contain a trace of material giving the carbamido reaction. This trace may be too small to give a visible pink colour, yet large enough to simulate in the photocolorimeter a not entirely negligible content of citrulline. If this were all, the effect might be allowed for by deducting from all results with any particular material a constant 'blank', determined on similar material known to contain no citrulline. A more serious complication is, that tissue filtrates contain also something, which modifies in a peculiar way the development of the colour from any citrulline actually present. The effect is, that with low concentrations of citrulline colour development is greater, with high concentrations less, than in pure solutions. Under these circumstances the calibration curve already described might still give reasonably correct results for certain medium concentrations, but for all others it would be seriously misleading. It becomes necessary therefore to construct, for each practical application of the method, a special calibration curve.

The general procedure to be followed may be exemplified by describing in detail that application with which we have been most immediately concerned—the determination of citrulline, along with urea, in a saline medium containing the products derived from the metabolism of tissue slices. For the urea determination, to be carried out according to Krebs & Henseleit [1932], 3 ml. of the medium, it will be assumed, have been transferred to a suitable manometer vessel, and have then been treated with 0·3 ml. of acetate buffer (*p*H 5·0) and 0·3 ml. of urease solution. The manometer readings having been taken, the whole mixture is washed into a 15 ml. flask to a volume of about 12 ml. To this there is added 1 ml. of 50% trichloroacetic acid, whereupon the flask is heated for 2–3 min. in boiling water. After having been cooled, the flask is filled to the mark with water, its contents are mixed and it is kept for half an hour. The contents are then filtered, and of the filtrate 7 ml. (or some suitable smaller volume diluted to 7 ml.) are taken for analysis.

The analysis is carried out in the manner already described, except that galvanometer readings are referred to a special calibration curve. In the case under consideration the preparation of this special curve is simplified by the fact that the blank, and also the materials which disturb the development of the colour, are derived exclusively from the added urease. The data for the curve, which are given in Table 4, were accordingly obtained as follows. Into a series of 15 ml. volumetric flasks were measured the stated volumes of a solution containing in 100 ml. 14·6 mg. of citrulline. To each were added 0·3 ml. of acetate buffer and 0·3 ml. of the urease solution employed in the determination. The mixtures were then diluted to about 12 ml., deproteinized, brought to volume

Table 4. *Calibration curve for urease filtrates*

ml. of stock citrulline solution (14·6 mg./100 ml.) in a total volume of 15 ml.	Final citrulline concentration (mg./100 ml.)	Galvanometer reading
0	0	100 (R.P. = 75)
0·25	0·243	73·5
0·50	0·487	60·5
0·75	0·73	49·5
1·00	0·973	40·5
1·25	1·216	33
1·50	1·460	26·5
1·75	1·703	21·75
2·00	1·946	18

and filtered as already described. From the first filtrate, containing no citrulline and serving therefore as the blank control, 7 ml. were transferred to an absorption test tube, and the colour was developed in the regular way. When the photometric density had, upon careful addition of the oxidizing reagent, reached its maximum, the galvanometer was quickly set to 100. The tube was thereupon removed, and the 'rest point' noted. The colour was then developed in the remaining tubes, galvanometer readings being taken with adjustment each time to the rest point already determined. In this way the blank was automatically allowed for in each of the readings, while the effect of other interfering factors was reflected in the course of the curve relating the galvanometer readings to the known concentrations of citrulline.

The curve given by the data of Table 4 starts at a higher level than that drawn from Table 1, but, falling more steeply, crosses it at a citrulline concentration of about 1 mg./100 ml. It is applicable, but applicable only, to all analyses carried out on 7 ml. samples of filtrates prepared, in the way prescribed, from similar material treated with a urease solution of identical origin and concentration. An ideal requirement is that the rest-point obtaining during any series of determinations should be identical with that found during the preparation of the calibration curve. In practice a difference of several points is admissible.

For any other special application of the method another calibration curve, constructed on the same principles, would have to be prepared.

With respect to the urease it is of advantage, naturally, to employ a preparation which, in the quantity requisite, gives the lowest blank and the least interference. A crude jack-bean extract is, from this point of view, rather unsuitable. We have found it best to use a urease powder prepared from jack-bean meal by the method of Van Slyke & Cullen [1914] but further purified by a second extraction and a second precipitation with acetone. A 10% suspension of this powder is of ample potency, and yields (with 7 ml. of filtrate) a blank of only 0·2 mg./100 ml. It was such a preparation that was used in securing the data of Table 4.

To test the accuracy of the whole procedure we used it, exactly as described, in the analysis of known mixtures of urea and citrulline. The results, as shown in Table 5, were entirely satisfactory.

Table 5. *Analysis of citrulline-urea mixtures*

Mixtures analysed					Urea concentration (mg./100 ml.)		Citrulline concentration (mg./100 ml.)	
ml. of urea solution 27·3 mg./100 ml.	ml. of citrulline solution 16·5 mg./100 ml.	Water to ml.	ml. acetate buffer (*p*H 5·0)	ml. urease 10% solution	Present	Found	Present	Found
0·5	0·3	3	0·3	0·3	4·55	4·47	1·65	1·60
1·0	0·6	3	0·3	0·3	9·10	8·97	3·30	3·35
1·5	1·2	3	0·3	0·3	13·65	13·23	6·60	6·70
2·0	0·9	3	0·3	0·3	18·20	17·65	4·95	4·85

In conclusion it may be reported that the method has enabled us to demonstrate the production by liver slices of substantial quantities of citrulline from ornithine, and thus for the first time to establish citrulline as an actual product of tissue metabolism. A full account of the relevant observations is in process of preparation.

SUMMARY

The carbamido-diacetyl reaction of Fearon is made the basis of a method whereby, through the use of a photoelectric colorimeter, small quantities of citrulline may be estimated with an error not exceeding 2%. Procedures are described for overcoming certain difficulties that arise in the application of the method to tissue analysis. Incidentally a comparison is made of the reactivities to Fearon's test of various carbamido-acids and other urea derivatives.

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