The Direct Colorimetric Estimation of Reducing Sugars and other Reducing Substances with Tetrazolium Salts

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Since Kuhn & Jerchel (1941) showed that tetrazolium salts were reduced by bacteria, yeasts and germinating seeds, 2:3:5-triphenyltetrazolium chloride or bromide has come into prominence as a viability testing reagent (Cottrell, 1947) and for vital staining (Mattson, Jensen & Dutcher, 1947). Its specific advantages for this purpose and for the purposes which follow lie in its E_0 value of $-0{\cdot}08$ V. (Jerchel & Mohle, 1944) and, more especially, in the fact that it is colourless in the oxidized form and an intense cherry red colour in the reduced form. This latter peculiarity gives it a measure of uniqueness among organic compounds.

In oxidized form the compound is soluble in water; after reduction, which with sugars only takes place in alkaline solution, it becomes insoluble in water but soluble in many organic solvents. Recently Mattson & Jensen (1950) have described its use in a method for the estimation of reducing sugars. The advantage of such a method is obvious as the substitution of a direct colorimetric method for titration or for an indirect colorimetric or gravimetric method can lead to improved accuracies and a saving of time.

Apart from a general investigation of the properties and behaviour of the reagent, four possible specific applications were chosen—estimation of reducing sugars, of blood sugar, of cysteine and of ascorbic acid. Before any specific methods could be elaborated for the colorimetric measurement of reducing substances it was necessary to establish the general characteristics and stoicheiometrical relations of the reagent in both oxidized and reduced forms.

EXPERIMENTAL

Stability

2:3:5-Triphenyltetrazolium bromide (tetrazolium) in aqueous solution must be kept in the dark (an amber bottle is quite satisfactory), and it has been found advisable to make up solutions weekly. Tetrazolium is reduced to triphenyl formazan (formazan) which, in aqueous *iso*propanol, is unstable in alkaline solution, but becomes stable after neutralization. In neutral solution no appreciable fading of colour could be measured in a photoelectric colorimeter after standing for an hour in ordinary room lighting. Undissolved triphenyl formazan is decomposed rapidly at 100° and more slowly at room temperature when in contact with aqueous alkali.

Solubility

Formazan was found to be soluble in light petroleum, benzene, toluene, xylene, methanol, ethanol, *n*-propanol, *iso*propanol, *n*-butanol, *iso*butanol, amyl alcohol, glacial acetic acid, acetone, ethyl ether, chloroform, carbon tetrachloride and pyridine.

Optimum conditions of reaction

In vivo, reduction of tetrazolium may take place at a pH of about 5·7 (e.g. germinating cereal grains). *In vitro*, Kun & Abood (1949) found reduction to take place in the presence of succinic dehydrogenase at pH 7·4, but state that in the absence of succinate, tissue homogenates do not reduce tetrazolium except under strongly alkaline conditions. It was found that maximum *in vitro* reduction of tetrazolium by glucose, lactose or cysteine was only obtained in strongly alkaline solutions and at 100°. Ascorbic acid under the same conditions caused rapid *in vitro* reduction at room temperature. In view of the instability of formazan in alkali, the minimum strength of alkali which would give sufficiently rapid colour development was chosen. Because simultaneous production and destruction of formazan take place, it was necessary rigidly to control time, temperature and pH to ensure stopping the reaction when maximum formazan production had taken place. Moreover, the time taken to achieve optimal production of formazan varies for any given reducing agent according to its concentration, but it was found possible to choose a suitable range of concentrations such that the time factor was constant for maximum formazan production. In Fig. 1 are curves relating amount of formazan produced to reduction time, the variable being glucose concentration. Similar curves were prepared for the

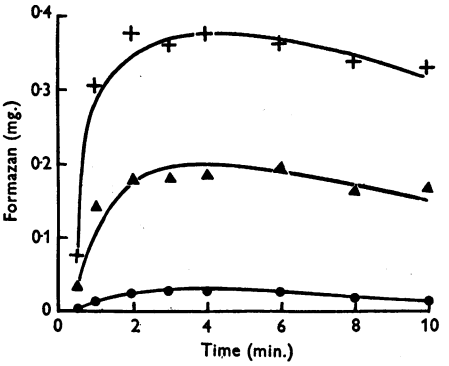

Fig. 1. Effect of reaction time on formazan produced. Glucose: ●—●, 0·2 mg.; ▲—▲, 1 mg.; +—+, 2 mg.

other reducing substances mentioned to establish optimum conditions for the reaction.

The amount of formazan produced in any specific reduction by sugar or other reducing agents bears no relationship to the quantity which would be expected from stoicheiometric considerations as alkaline destruction occurs concurrently with formation.

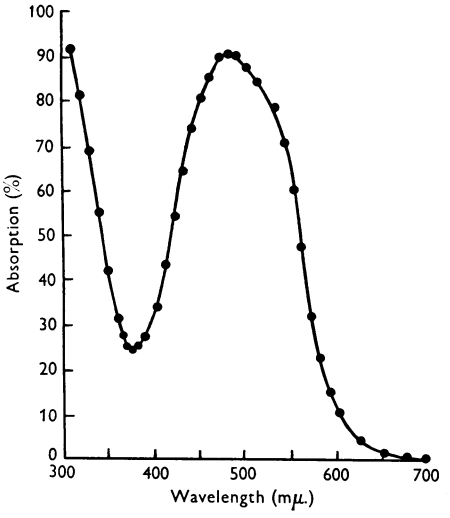

Fig. 2. Absorption curve of triphenyl formazan in *iso*propanol solution.

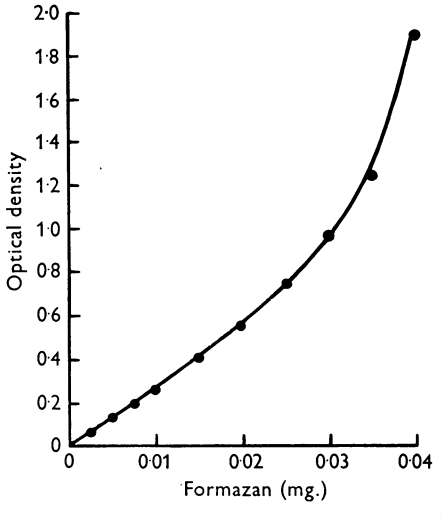

Fig. 3. Relation between absorption and concentration of formazan in *iso*propanol—Corning filter M515 in 5 mm. cell.

A sample of pure triphenyl formazan was prepared by the reduction of 1 g. of tetrazolium bromide by 1 g. of ascorbic acid dissolved in 25 ml. of distilled water + 5 ml. of 10% (w/v) NaOH at room temperature. After the reaction had proceeded for 20 min. the solution was neutralized, filtered and the triphenyl formazan washed on the filter paper and dried at 37°. It was used to determine the extinction coefficient and spectral absorption of formazan, using the Beckman spectrophotometer. Maximum absorption of triphenyl formazan in *iso*propanolic solution was found to be at 485 mμ.

The $E^{1\,cm.}_{0.01\%}$ 485 mμ. in *iso*propanol was found to be 5. The absorption curve is given in Fig. 2.

Formazan in solution obeys Beer's Law. Fig. 3 is a graph relating concentration to optical density using Corning filter M515 in the Lumetron colorimeter (5 mm. cell). Using Ilford filter 602 in the Spekker absorptiometer (1 cm. cell) the relationship between concentration and drum reading was found to be perfectly linear.

Method for glucose and lactose

Standard curve. Into clean dry 6 × 0·75 in. test tubes graduated at 20 ml. are pipetted dilutions of glucose or lactose to give a range of 0·2–2 mg. and the volume adjusted to 3 ml. with distilled water. In each series a blank is included containing 3 ml. distilled water. To each tube is added 1 ml. of 1% (w/v) solution of triphenyltetrazolium bromide ('Grodex', May and Baker) and 2 ml. N-NaOH. The tubes are heated in a boiling-water bath for exactly 3 min., and on removing from the bath, 2 ml. of approximately 1·1 N-acetic acid are added and the tubes immediately cooled in a cold-water bath. The volume is adjusted to 20 ml. with *iso*propanol and the mixture shaken to dissolve the formazan. Into another graduated test tube 2 ml. is pipetted, and the volume adjusted to 20 ml. with *iso*propanol. Colour measurement is made with a suitable colorimeter.

Standard concentration/extinction curves for glucose and lactose are similar to that shown for ascorbic acid in Fig. 5, and may be used for the determination of unknown concentrations of these sugars.

Method for determination of blood sugar

The method is suitable for the determination of glucose in blood, but because of the small quantities to be determined some modifications are required. Using the Lumetron colorimeter, tubes graduated at 20 ml. are required, whilst for the Spekker absorptiometer tubes graduated at 25 ml. are necessary. A suitable range of dilutions of standard is from 0·02 to 0·2 mg. glucose per 2 ml. The blank tube contains 2 ml. of distilled water.

To each tube add 1 ml. of 0·3% (w/v) triphenyltetrazolium bromide and 1 ml. 2 N-NaOH. Heat in boiling-water bath for exactly 3 min. On removal acidify with 1 ml. of 2·1 N-acetic acid. Cool in cold-water bath and adjust volume with *iso*propanol. A typical standard curve is shown in Fig. 4, together with a curve for lactose estimated under the same conditions.

Benzoic acid used to preserve standard solutions of glucose has no appreciable effect on the production of formazan. For the determination of blood glucose, a 0·2 ml. sample of blood is taken and the protein removed by the method of Somogyi (1945) as follows. The sample of blood is laked in 3 ml. distilled water and 0·4 ml. 0·3 N-$Ba(OH)_2$ added followed by 0·4 ml. 5% (w/v) $ZnSO_4.7H_2O$. The mixture is shaken, warmed and filtered. Accuracy of the concentration of the reagents is less important than the requirement that the

Table 1. *Blood sugar determinations by tetrazolium method and Hagedorn & Jensen method*

	Tetrazolium method		Method of Hagedorn & Jensen	
	Glucose (mg./100 ml.)	Glucose recovered (mg./100 ml.)	Glucose (mg./100 ml.)	Glucose recovered (mg./100 ml.)
Initial blood sugar	79		78	
Glucose added (mg./100 ml.):				
20	97	18	96	18
40	117	38	117	39
60	139	60	133	55
80	158	79	155	77
100	177	98	174	96

alkali must neutralize the $ZnSO_4$ precisely, volume for volume, using phenolphthalein as indicator. Filtrate (2 ml.) equivalent to 0·1 ml. of blood is pipetted into a graduated test tube, and 1 ml. of the tetrazolium solution and 1 ml. of 2 N-NaOH added. The tube is then treated in the same way as the standard tubes. The intensity of colour is measured as for glucose.

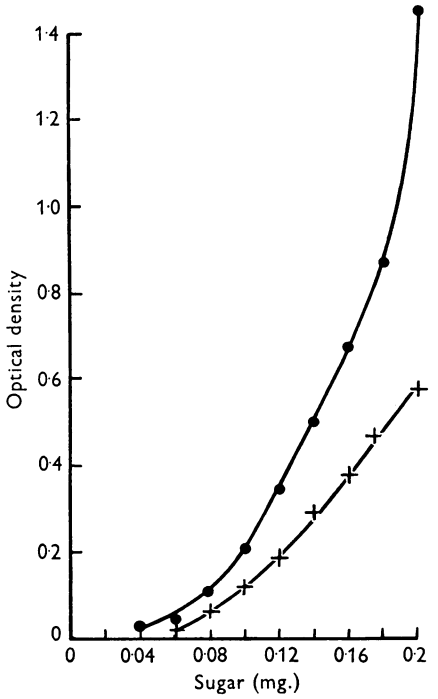

Fig. 4. Standard curves by blood-sugar method. ●—●, glucose; +—+, lactose.

Table 1, showing a comparison between the tetrazolium method and that of Hagedorn & Jensen in determining glucose added to whole blood, indicates that good agreement is obtained between the two methods. The results are the mean of duplicate determinations. In addition, the tetrazolium method proved much simpler and quicker in operation.

Method for ascorbic acid

Since the reaction between ascorbic acid and tetrazolium is extremely rapid at 100°, and difficulties due to the different rates of production of formazan at varying concentrations of ascorbic acid, and the destruction of formazan by alkali, arise, this reaction is best carried out close to room temperature and, to prevent reduction by light, in the dark.

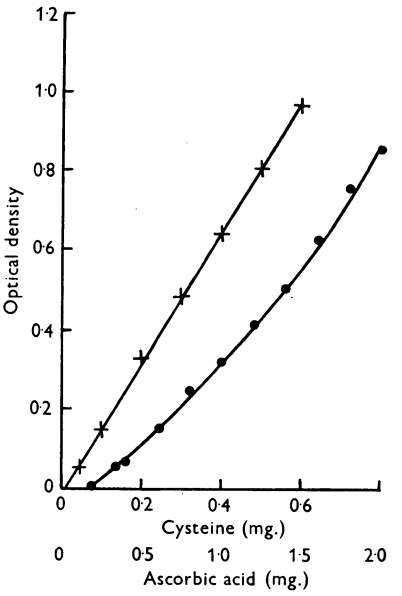

Fig. 5. Standard curves for ascorbic acid, ●—●, and cysteine, +—+.

Standard solutions of ascorbic acid are prepared in 3% (w/v) metaphosphoric acid and are used immediately after preparation. The method follows that of glucose with the difference that the time of reaction is 20 min. at room temperature, or preferably at 37° (incubator) and in the dark. To neutralize the HPO_3, 2 ml. of 2 N-NaOH are used instead of N-NaOH. As dilution with *iso*propanol causes precipitation of phosphates, it is necessary to add 5–6 ml. distilled water before adjusting the volume with *iso*propanol. Alternatively, the formazan may be extracted from the

reaction tubes with an immiscible solvent, the volume being adjusted in the usual way.

Fig. 5 relates a range of 0·2–2 mg. of ascorbic acid to optical densities. An attempt was made to determine the ascorbic acid content of fruit juices by this method, but owing to the presence of other strongly reducing substances no comparison with results determined by the 2:6-dichlorophenolindophenol method could be obtained.

Method for cysteine

Cysteine reacts with tetrazolium much more slowly than glucose or ascorbic acid and the optimum time of reaction under similar conditions is 20 min. at 100°. Moreover, prolonged heating of the formazan produces conglomerates which are difficult to dissolve. It was found advisable to use a more dilute solution of tetrazolium (0·2 %, w/v) which, with amounts of cysteine ranging from 0·05 to 0·6 mg., has an optimum reaction time of 25 min. The method is otherwise similar to that for glucose. After acidification the volume is adjusted to 20 ml. with *iso*propanol. No further dilution is necessary. A typical curve is shown in Fig. 5.

Analytical results

Results obtained by the various modifications of the basic method which have been described above are shown in Table 2. Each result is the mean of three

Table 2. *Results of colorimetric estimations using triphenyltetrazolium bromide*

	Concentration (mg./100 ml.)	
Substance	Found	Actual
D-Glucose	31·9	31·8
	41·7	39·4
	52·5	51·6
	60·4	59·7
Lactose	26·3	27·0
	40·1	40·6
	50·4	50·6
	59·4	60·9
Ascorbic acid	28·2	29·9
	40·2	41·0
	49·2	49·6
	59·9	60·4
Cysteine HCl	10·0	9·4
	19·5	20·0
	33·9	32·9
	46·8	48·1

single determinations made at widely spaced points on the 'standard' curves. Greater accuracy than is indicated here may be obtained, if necessary, by estimations carried out in duplicate or triplicate.

DISCUSSION

Tetrazolium is a new analytical reagent, which possesses several unique properties. In the first place it allows the replacement of volumetric methods by colorimetry, so replacing a subjective (titration) by an objective method (electrometric colorimetry). Secondly, by eliminating one stage from customary indirect methods it provides less latitude for error and gives increased speed. Thirdly, it could prove useful in reactions where it is necessary to extract the formazan from the reaction mixture with an immiscible solvent (chloroform, benzene) which would not extract any interfering water-soluble pigments which might be present.

The reagent lends itself to micro and ultra-micro estimations as the colour developed is very intense relatively to the material being estimated. If the alkaline destruction of the formazan could be prevented or reduced by the introduction of a stabilizer, e.g. cysteine, then this advantage could be even greater, but so far efforts in this direction have failed.

Though the method is quite empirical it is not different in this respect from many others which are in daily use. Whilst strict adherence to the standard conditions chosen is essential the conditions may be varied to suit particular cases. For example, the range of concentrations of reducing substances could be increased if suitable dilutions of the formazan solutions were made. However, it cannot be increased indefinitely under one set of standard conditions, as for each range of concentrations there is an optimum time of reaction, and if this time is exceeded the amount of formazan decomposed by the alkali increases. This is shown in the blood glucose curves where the conditions chosen are not the optimum for the whole range of concentrations necessary.

The method of Mattson & Jensen (1950) differs very markedly from the present method in several respects. The temperature used is $25 \pm 0{\cdot}1°$ which only permits the development of a small fraction of the colour which can be obtained, and hence lessens sensitivity. Furthermore, in this region, the temperature is very critical, and slight deviations can readily lead to errors of the order of 10 %. No such critical behaviour is found at 100° and the time of reaction is much shorter. Acetic acid is used to neutralize the sodium hydroxide as the buffering action of the acetate retards decomposition of the formazan if excess acid is added. Moreover, sodium acetate is sufficiently soluble in the miscible solvents to prevent precipitation, whereas, if sulphuric or hydrochloric acids are used, the sulphate or chloride formed tend to precipitate, making filtration necessary.

Acetone, methanol, ethanol or *iso*propanol may be used as solvents. *iso*Propanol was chosen because of its relatively low volatility and general convenience in use. It possesses several obvious advantages over pyridine, which was used by Mattson & Jensen (1950).

SUMMARY

1. A study has been made of the reaction between triphenyltetrazolium bromide and various reducing agents. Conditions for optimal production of formazan by this reaction were established.

2. Triphenylformazan has been prepared and its absorption and solubility characteristics determined.

3. Direct colorimetric methods for the estimation of blood sugar, glucose, lactose, cysteine and ascorbic acid based on the use of tetrazolium are described.

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The Nature and Mechanism of Action of the Enzymes Responsible for Tissue Respiration during Aseptic Autolysis

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It is known that tissues, isolated from organisms and maintained under aseptic conditions, undergo processes of autolysis, characterized by progressive hydrolysis of proteins brought about by the tissue cathepsins. The mechanism of catheptic action has been studied by several workers, and it has been shown that the activity of the cathepsins is stimulated by acids, thiols and cyanide. Less attention has been devoted to the action of other enzymes during autolysis. Edlbacher & Koller (1934) showed that necrotic tissue from Jensen's sarcoma is rich in arginase, and Bayerle (1936) extended the observation to necrotic non-cancerous tissues. Michelazzi (1947) studied the behaviour of the oxidative metabolism of tissues in conditions of aseptic autolysis, and obtained evidence that homogenates of guinea pig liver, kidney and brain can consume small amounts of oxygen even many days after the start of aseptic autolysis in the organs. Since boiling for 20 min. abolished the respiratory activity, Michelazzi concluded that this must be due to the persistence of certain oxidizing enzymes. He observed nevertheless that fumarate, lactate, aspartate, DL-alanine, phenylalanine and ethanol are not attacked by autolysing tissues and that cytochrome oxidase is completely inactive. The present work is also concerned with oxidative metabolism of the tissues in conditions of aseptic autolysis.

EXPERIMENTAL

Conditions of autolysis. Liver and kidney of guinea pigs, mice and rats were used. The organs were removed under sterile conditions from the bled animals and were placed in sterile vessels containing 30 ml. 0·067 M-acetate buffer (pH 4·5), which was then covered to a depth of 1 cm. with toluene. The vessels were closed with paraffined glass stoppers and were placed in a thermostat at 37·5° for periods of from 20 to 50 days. At the desired times the vessels were opened, the toluene removed, and the organs washed with 0·067 M-phosphate buffer (pH 7·4) blotted with filter paper, and weighed.

Homogenates were made by prolonged grinding in a mortar, the amounts of tissue used in each experiment being such that 0·5 g. of liver or 0·25 g. of kidney was contained in 3 ml. of the suspension. Controls for sterility were made by inoculation into broth, any contaminated specimens being discarded.

Measurement of respiration. Residual respiration was estimated by the Warburg technique over a period of 60 min. at 38° in 0·067 M-phosphate buffer (pH 7·4).

Succinoxidase activity was determined by the method of Schneider & Potter (1943), and lactate dehydrogenase by the method of Green & Brosteaux (1936): α-glycerophosphate dehydrogenase was studied by measuring the extra O_2 uptake in the presence of 0·2 ml. 0·2 M-sodium α-glycerophosphate (Hofmann-LaRoche). D- and L-Amino-acid oxidase activities were determined by the procedure of Rodney & Garner (1938), 0·2 ml. of DL- and L(+)-alanine (0·2 M) being used as substrates.