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A New Colorimetric Method for the Determination of Serum Isocitric Dehydrogenase Activity[1]

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A new colorimetric method is presented for the determination of serum isocitric dehydrogenase activity. This is based on the determination of 2-oxoglutaric acid formed in the enzyme reaction by means of the previously established selective color reaction of the acid. The method gives reliable results and is suitable to assay a large number of samples with a wide range of activity at the same time with 50 μl of sample.

Several photometric methods have been developed for the determination of NADP-dependent isocitric dehydrogenase[3] (ICD) activity in seurm and other biological fluids. Those methods are based either on ultraviolet light absorption of NADPH formed during the oxidative decarboxylation of isocitric acid to 2-oxoglutaric acid (2-OG)[4,5] or on coloration of 2,4-dinitrophenylhydrazone of 2-OG formed.[6,7] However, the former methods may be unsuited to the routine determination because they are time-consuming when applied to batch analyses and give sometimes incorrect values due to the deposition of manganese phosphate in the incubation mixture,[8] and the latter methods have rather narrow determinable range of activity. Furthermore, those methods require more than 0.2 ml of serum.

In the previous papers, we presented a selective microphotometric method for the determination of 2-OG on the basis of a color reaction with diazotized sulfanilic acid in sodium hydroxide solution in the presence of sodium sulfite and sodium hypophosphite,[9] and then applied to the determination of serum transaminase activities.[10] This paper extends an application of the method to the determination of ICD activity in serum, and presents a new method which permits the assay of a large number of samples at the same time with a minimum amount of sample, covering from normal values of activities to high ones.

Experimental[11]

Reagents[12] **Reagents for the Enzyme Reaction**——Sodium Chloride Solution: Dissolve 8.7 g of NaCl in 1000 ml of H_2O (0.15M).

Manganese Chloride Solution: Dissolve 0.80 g of $MnCl_2 \cdot 4H_2O$ in 1000 ml of NaCl solution (4 mM). This solution is stable at room temperature for at least 1 month.

1) This forms "Organic Analysis LXXXII." Part LXXXI: M. Kageura, Y. Ohkura and T. Momose, *Chem. Pharm. Bull.* (Tokyo), **19**, 2294 (1971).
2) Location: *Katakasu, Fukuoka.*
3) Ls-Isocitrate: NADP oxidoreductase (decarboxylating), EC. 1.1.1.42.
4) S.K. Wolfson, Jr. and H.G. Williams-Ashman, *Proc. Soc. Exptl. Biol. Med.*, **96**, 231 (1957).
5) G.N. Bowers, Jr., *Clin. Chem.*, **5**, 509 (1959).
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8) S. Ochoa, "Methods in Enzymology," Vol. I, ed. by S.P. Colowick and N.O. Kaplan, Academic Press, New York, N.Y., 1955, p. 699.
9) Ts. Momose, Y. Ohkura and Ta. Momose, *Talanta*, **16**, 1451 (1969).
10) Y. Ohkura, Ta. Momose and Ts. Momose, *Clin. Chem.*, in press.
11) The absorbance was measured by a Hitachi UV-Vis Spectrophotometer, model 139, and a Beckman DB Spectrophotometer with a cell of 10 mm optical length.
12) All reagents used were JIS Reagent Grade except noted.

Tris-(hydroxymethyl)-aminomethane(Tris) Buffer Solution: Dissolve 3.0 g of Tris in about 80 ml of H_2O, adjust the pH to 7.5 with diluted HCl and then diluted to 100 ml with H_2O (0.25M). This solution is usable for several months when stored at room temperature.

Tris-manganese Chloride Solution: Prepare by mixing Tris buffer solution and $MnCl_2$ solution in the ratio of 1:1. The solution is usable for at least 1 week when stored at room temperature.

NADP Solution: Dissolve 10 mg of NADP monosodium salt[13] in 10.0 ml of NaCl solution (1.1 mM) and keep frozen when not in use. The solution is stable for at least 2 days.

Isocitrate Solution: Dissolve 30 mg of trisodium *dl*-isocitrate[14] in 10.0 ml of NaCl solution (1.16M) and store in a refrigerator. This solution is usable for at least 1 week.

Reagents for the Color Development——Sulfanilic Acid Solution: Dissolve 7.0 g of sulfanilic acid in approixmate 600 ml of heated H_2O. After cooling, add 20 ml of concentrated HCl and dilute to 1000 ml with H_2O. This solution is stable for at least 6 months when stored in a refrigerator.

Sodium Nitrite Solution: Freshly prepare 3% aqueous solution.

Diazotized Sulfanilic Acid Solution: To 100 ml of sulfanilic acid solution, add 10 ml of $NaNO_2$ solution, both solutions being ice-cooled to about 5°, and shake the mixture occasionally during 10 min. The solution is stable for 4 hr at room temperature (about 25°), and remains usable for 2 days in a refrigerator (5°).

Alkaline Solution: Dissolve 80 g of NaOH in about 500 ml of H_2O. After cooling to room temperature, add a solution prepared by dissolving 10 g each of Na_2SO_3 and $NaH_2PO_2 \cdot H_2O$ in about 300 ml of H_2O and dilute to 1000 ml with H_2O. The solution is stable when stored in a tightly closed container.

2-Oxoglutaric Acid Standard Solutions——Prepare first 66 μg/ml solution by dissolving 132 mg of 2-OG in 2000 ml of H_2O. Using this solution, prepare 3.3, 6.6, 13.2, 19.8, 26.4 and 33 μg/ml solutions, which correspond to 15, 30, 60, 90, 120 and 150 units, respectively, in the procedure as described later. Add a drop of $CHCl_3$ per 100 ml of each solution as a preservative, tightly close and store in a refrigerator. The solutions are stable for at least 3 months.

Procedure——Pipet successively 0.4 ml of Tris-$MnCl_2$ solution, 0.3 ml of isocitrate solution and 50 μl of fresh serum[15] into a test tube and pre-heat in a water-bath at 37° for 5 min. At zero time, add 0.3 ml of NADP solution, mix well, cover the tube with Parafilm and incubate at 37° for exactly 30 min. Cool the tube in an ice-water bath to stop the enzyme reaction. Add successively 1.0 ml of diazotized sulfanilic acid solution and 8 ml of the alkaline solution and mix by inversion. Then warm at 37° for 45 min to develop the color and cool in running water (the volume of final reaction mixture: 10.05 ml). Prepare a serum blank in the same way as described above omitting the incubation.[16] Within 30 min after the end of the reaction period, read the absorbance (E) at 525 mμ against the serum blank.

If serum is expected to have a highly elevated activity, a shorter incubation time, 15 min, may be used.

Calibration Curve and Calculation of Units——Treat three 1.0 ml aliquots of each 2-OG standard solution and of H_2O for blanks to develop the color as described in the procedure (the volume of final reaction mixture: 10 ml), and read the absorbances against the pooled blank. The calibration curve thus drawn up is a straight line which passes through the origin. The absorbance for 33 μg/ml 2-OG standard solution was obtained as 0.570 ± 0.015.

In this method, the units of ICD activity are defined as the μ moles of 2-OG formed by the enzyme in 1000 ml of serum per min at 37°, similarly to the manner of representation of the international units. Therefore, the units are calculated by the following equation.

$$\text{Units} = \frac{10}{10.05} \times \frac{K}{146} \times \frac{1}{30} \times \frac{1000}{0.05} \times E$$

Where, K is the ratio of 2-OG concentration (μg/ml) to the absorbance given by this concentration of the acid in the preparation of calibration curve. For example, 57.9 was obtained in our study, and the units were calculated by $264 \times E$.

Result and Discussion

The absorption spectrum of the color developed in the procedure had a maximum at 525 mμ, and the shape was similar to that observed in the assay of transaminase activity in serum.[10]

13) Purchased from Worthington Biochemical Corporation, Freehold, New Jersey, USA. The content of NADP was 87% when determined by measuring the absorbance at 260 mμ: H.U. Bergmeyer (ed.), "Methods of Enzymatic Analysis," Academic Press, New York and London, 1963, P. 1029.

14) Purchased from British Drug Hauses Ltd., Polle, England.

15) The serum to be assayed should be without any trace hemolysis because the erythrocyte shows an extremely high ICD activity.

16) Isocitrate solution and NADP solution may be substituted by the saline solution as described later.

The each individual substance which was concerned with the enzyme reaction was first investigated as the effect on the color development of 2-OG under the conditions of procedure. *dl*-Isocitrate and Tris gave no absorbance and did not interfere with the color development. NADP showed only 0.012 of absorbance at the prescribed concentration. Manganese chloride caused turbidity in the color developing solution due to the formation of its hydroxide in the absence of Tris and serum. However, Tris and serum effectively removed this interference to show a complete recovery of 2-OG, 99%, under the prescribed conditions of the procedure. Therefore, EDTA was not necessary in the present method, though the reagent was required in the 2,4-dinitrophenylhydrazine methods.[6,7]

An almost constant absorbance of the serum blank, 0.098—0.122 (mean value, 0.110), was observed on 92 different sera. But, some sera showed higher values,[17] indicating that the serum blank could not be necessarily omitted in the procedure. *dl*-Isocitrate could be substituted by the saline solution in the preparation of serum blank for the reason already stated. The contribution of NADP to the absorbance of serum blank in the prescribed concentation was 0.028 ± 0.004 on 26 different sera, and then the saline solution might be substituted for the coenzyme, using this value of absorbance for the correction.

The conditions for the enzyme reaction were investigated so as to be zero order reaction. The ICD activity did not change over a concentration range of *dl*-isocitrate of 0.53—11.6 M with sera of fairly high activities, and the prescribed concentration, 1.16 M, was selected for a higher activity. The concentration of manganese chloride did not affect the determined activity in a range of 0.2—10 mM when examined with sera of high activities, and the prescribed concentration, 4 mM, was employed as the optimum quantity. The concentrations of NADP, 0.8, 1.1 and 2.2 mM, were observed to give the equal determined values of activity with 16 different sera of high activities, but a 0.55 mM solution gave a slightly lower value. Thus, 1.1 mM was selected as a sufficient concentration for fairly high activity.

When the prescribed concentrations of the substrates were used, a linear relationship between the incubation time and the μ moles of 2-OG formed was observed throughout at least 30. min incubation even on serum with a fairly elevated activity, 108 units, as shown in Fig. 1. This fact suggested that the present procedure might permit the determination of

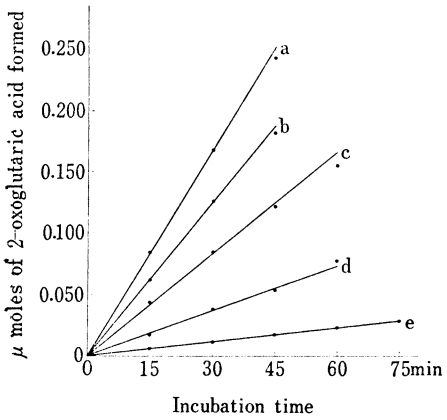

Fig. 1. Relationship between the Incubation Time and the Amount of 2-Oxoglutaric Acid Formed

a, 108; b, 83; c, 55; d, 26; e, 8 units serum. Each plot was the mean value of triplicate determinations.

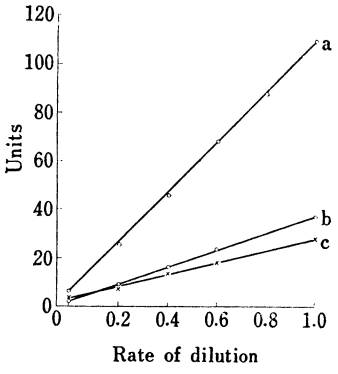

Fig. 2. Effect of the Dilution of Serum on the Measured ICD Activity

serum, a or b, was diluted with a low untis serum, c, with 5% bovine serum albumin solution. Each plot was the mean value of triplicate determinations.

17) The highest absorbance of serum blank, 0.241, was obtained.

activity up to 120 units. The range might be extended to a very high activity, about 240 units, yb employing a shorter incubation time, 15 min. The enzyme activity was proportional to the dilution of serum when diluted with serum of very low units or 5% bovine serum albumin (fraction V) solution as shown in Fig. 2. But, the activity slightly decreased by unknown factors when the serum was diluted with water or the saline solution.

Interfering substances were tested in regard to the color development, effects on the recovery of added 2-OG, and the enzyme activity. Bilirubin caused an increase of absorbance of 0.013 both in the test and serum blank at the concentration of 10 mg/100 ml in serum, and seemed to be a non-interfering substance in a lower concentration. Glucose in serum gave an absorbance of 0.017 at the concentration of 1000 mg/100 ml, which became negligible in a concentration occured in usual serum. Formaldehyde and *p*-aminosalicyclic acid gave no absorbance in concentration below 1 mg/100 ml. Urea and lactic acid did not influence the color development in concentrations below 100 and 50 mg/100 ml, respectively.

Other substances tested did not give the absorbance at 525 mμ even at a concentration of 25 mg/100 ml in serum. These were 20 different α-amino acids, pyruvic acid, oxalacetic acid, acetoacetic acid, 3-hydroxybutyric acid, ascorbic acid, glucuronolactone, citric acid, acetaldehyde, acetone, inositol, creatine, creatinine, uric acid, glutathione, *p*-aminobenzoic acid, salicylic acid and acetylsalicylic acid.

Furthermore those substances did not influence the recovery of 2-OG added in an amount of 0.15 μ moles and the assayed enzyme activities at the concentrations described above.

Parallel tests with the Bowers method[5] were carried out on 46 different sera with activities below 80 units. The results are shown in Fig. 3. Satisfactory coefficient of correlation for both methods, 0.96, was obtained. The units obtained by the present method (x) could be converted to the Bowers units (y, the mμ moles of 2-OG formed per ml of serum per hr at 25°) by the regression equation, $y=19x-25$. The same tendency was observed on sera with activities above 100 units, in which the 15 min incubation was employed (Fig. 3). The turbidity due to manganese phosphate was observed in two cases in the Bowers method.

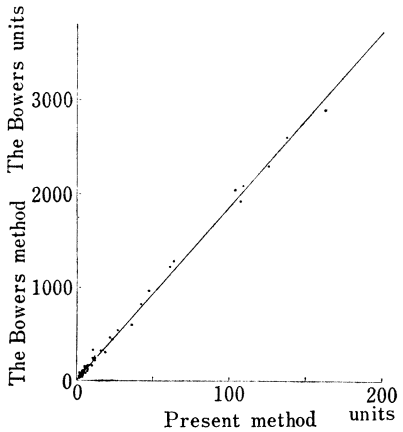

Fig. 3. Correlation between the Values obtained by the Present Method and the Bowers Method

×: Turbidity was observed in the Bowers method.

The precision of the present method was studied as to repeatability, which was obtained by performing separately 30 determinations on sera with the mean activities of 20 and 30 units. The coefficient of variation were 3.4 and 1.9 %, respectively.

Forty six determinations of the activity were carried out on sera of healthy individuals. The values obtained did not exceed 12 units, with a large incidence of 1—9 units, and the mean was 6 units.

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