

## A New Photometric Assay Method of Serum Glutamate Dehydrogenase

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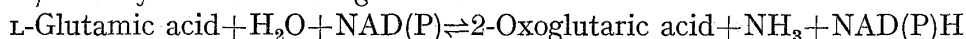
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A new photometric method is presented for the assay of serum glutamate dehydrogenase activity on the basis of the determination of 2-oxoglutaric acid produced in the enzyme reaction by means of the previously established method for selective determination of the acid with diazotized sulfanilic acid. The method gives reliable results and is suitable to assay a large number of samples at the same time with 0.1 ml of sample.

**Keywords**— diazotized sulfanilic acid; 2-oxoglutaric acid determination; color reaction; L-leucine and ADP as activators; oxalate as LDH inhibitor; small sample size

Nicotinamide adenine dinucleotide (phosphate) (NAD(P))-dependent glutamate dehydrogenase (GDH)<sup>2)</sup> catalyzes the following reaction.



Several photometric methods have been developed for the assay of GDH activity in serum and other biological fluids. The conventional methods are based on either ultraviolet light absorption of NADH consumed in the reverse reaction<sup>3)</sup> or that formed in the forward reaction<sup>4)</sup> (ultraviolet (UV) methods). Other methods so far proposed are based on coloration of a formazan produced from a tetrazolium salt by the reduction with reduced nicotinamide adenine dinucleotide (NADH) remained unreacted in the reverse reaction<sup>5)</sup> (tetrazolium method) or on coloration of 2,4-dinitrophenylhydrazone of 2-oxoglutaric acid (2-OG) remained in the same direction of the reaction<sup>6)</sup> (2,4-DNPZ method). However, the UV methods may be unsuited to the routine assay because they are time-consuming when applied to batch analyses and require more than 0.4 ml of serum. Both tetrazolium and 2,4-DNPZ methods also require more than 0.2 ml of serum and show very high absorbances of the blanks which cause sometimes incorrect values of the activity. And, in the 2,4-DNPZ method, an insufficient concentration of 2-OG for the enzyme reaction is employed to suppress the extremely high absorbance of the blank.

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 sulfite and hypophosphite,<sup>7,8)</sup> and applied to the assays of serum transaminase activities<sup>9)</sup> and serum NADP-dependent isocitrate dehydrogenase activity.<sup>10)</sup> This paper extends an application of the method to the assay of serum GDH activity and presents a new method which measures the amount of 2-OG formed in the forward enzyme reaction under its optimal conditions in the presence of L-leucine and adenosine

- 1) Location: *Maidashi, Higashi-ku, Fukuoka.*
- 2) L-glutamate: NAD(P) oxidoreductase (deaminating), EC 1.4.1.3.
- 3) E. Schmidt, "Methods of Enzymatic Analysis," ed. by H.-U. Bergmeyer, Academic Press, New York, 1963, p. 752.
- 4) Y. Kinoshita and Y. Hirazawa, "Kosokenkyuho," ed. by S. Akabori, Asakurashoten, 1959, p. 752.
- 5) A.M. Siegelman, A.S. Carlson, and T. Robertson, *Am. J. Clin. Pathol.*, **38**, 256 (1962).
- 6) J. King, "Methods of Enzymatic Analysis," Second edition, ed. by H.-U. Bergmeyer, Academic Press, New York, 1970, p. 650.
- 7) Ts. Momose, Y. Ohkura, and Ta. Momose, *Talanta*, **16**, 1451 (1969).
- 8) Y. Ohkura, M. Yamaguchi, and T. Momose, *Chem. Pharm. Bull.* (Tokyo), **22**, 1414 (1974).
- 9) Y. Ohkura, Ta. Momose, and Ts. Momose, *Enzymol. Biol. Clin.*, **10**, 343 (1969).
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diphosphate (ADP) as GDH activators and oxalate as lactate dehydrogenase (LDH) inhibitor. The method is precise enough to use practically and easily performed with a small amount of serum.

### Experimental<sup>11)</sup>

#### Reagents<sup>12)</sup>

**Reagents for the Enzyme Reaction**—Glutamic Acid Solution: Dissolve 3.86 g of L-glutamic acid and 1.0 g of tris(hydroxymethyl)aminomethane (Tris) in about 80 ml of 2% KOH, adjust the pH to 8.8 at 37° with diluted HCl and then diluted to 100 ml with H<sub>2</sub>O (the concentrations of L-glutamic acid and Tris are 250 and 83 mM, respectively). This solution is usable for a month when stored in a refrigerator.

Potassium Oxalate Solution: Dissolve 23.0 g of potassium oxalate monohydrate and 1.0 g of Tris in about 80 ml of 2% KOH, adjust the pH to 8.8 at 37° with diluted HCl and then diluted to 100 ml with H<sub>2</sub>O (the concentrations of the oxalate and Tris are 1250 and 83 mM, respectively). The solution is usable for at least a month when stored in a refrigerator.

Leucine Solution: Dissolve 0.6 g of L-leucine and 1.0 g of Tris in about 80 ml of H<sub>2</sub>O, adjust the pH to 8.8 at 37° with diluted HCl and then diluted to 100 ml with H<sub>2</sub>O (the concentrations of L-leucine and Tris are 50 and 83 mM, respectively). This solution is usable for a month when stored in a refrigerator.

NAD Solution: Dissolve 36 mg of NAD<sup>13)</sup> in 10.0 ml of H<sub>2</sub>O (5 mM) and keep frozen when not in use. The solution is usable for 2 days.

ADP·Leucine Solution: Freshly prepare by dissolving 24 mg of ADP<sup>14)</sup> in 10.0 ml of leucine solution (the concentrations of ADP, L-leucine and Tris are 5, 50, and 83 mM, respectively).

**Reagents for the Color Development**—Sulfanilic Acid Solution: Dissolve 7.0 g of sulfanilic acid in about 600 ml of heated H<sub>2</sub>O. After cooling, add 20 ml of concentrated HCl and dilute to 100 ml with H<sub>2</sub>O. 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<sub>2</sub> 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.

Alkaline Solution: Dissolve 80 g of NaOH in about 500 ml of H<sub>2</sub>O. After cooling to room temperature, add a solution prepared by dissolving 10 g each of Na<sub>2</sub>SO<sub>3</sub> and NaH<sub>2</sub>PO<sub>2</sub>·H<sub>2</sub>O in about 300 ml of H<sub>2</sub>O and dilute to 1000 ml with H<sub>2</sub>O. The solution is stable when stored in a tightly closed container.

**2-Oxoglutaric Acid Standard Solutions**—Prepare first 65.5 μg/ml solution by dissolving 132 mg of 2-OG in 2000 ml of H<sub>2</sub>O. Using this solution, prepare 3.27, 6.55, 9.82, 13.10, and 26.20 μg/ml solutions (corresponded to 0.022, 0.045, 0.067, 0.09, and 0.18 μmol/ml, respectively). Add a drop of CHCl<sub>3</sub> per 100 ml of each solution, tightly close and store in a refrigerator. The solutions are stable for at least 3 months.

**Procedure**—Pipet successively 0.2 ml of glutamic acid solution, 0.2 ml of oxalate solution, 0.2 ml of ADP·leucine solution and 0.1 ml of serum into a test tube and pre-incubate in a water bath at 37° for 5 min. At zero time, add 0.3 ml of NAD solution, mix well, cover the tube with Parafilm and incubate at 37° for exactly 45 min. Immediately after the incubation period, add 1.0 ml of diazotized sulfanilic acid solution under cooling in an ice-water bath and mix. Then, add 8.0 ml of the alkaline solution. Warm at 37° for 45 min to develop the color and cool in an ice-water bath. Prepare a serum blank in the same way as described above, but add 0.3 ml of NAD solution to the enzyme reaction mixture after the incubation. Within 30 min after the end of the reaction period, read the absorbance at 525 nm 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<sub>2</sub>O for blanks to develop the color as described in the procedure, and read the absorbances against the pooled blank. The calibration curve thus drawn up is a straight line which passes through the origin.

The units of GDH activity are defined as the μmol of 2-OG formed by the action of 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.

$$\text{units} = A \times \frac{K}{146.10} \times \frac{1000}{0.1} \times \frac{1}{45}$$

Where, *A*, 146.10, 0.1, and 45 are the absorbance obtained in the procedure, the molecular weight of 2-OG, the amount of serum (ml) and the incubation time (min), respectively. *K* is the ratio of 2-OG concentration (μg/

- 11) The absorbance and pH were measured by a Hitachi 124 Spectrophotometer with a cell of 10 mm optical path length and a Hitachi-Horiba M-7 pH meter, respectively.
- 12) All reagents used were Reagent Grade unless otherwise stated.
- 13) β-NAD (Kohjin) was used.
- 14) ADP (Seikagaku Kogyo) was used.

ml) to the value of A given by this concentration of the acid in the preparation of calibration curve. For example, 55.4 was obtained in the present experiment, and the units were calculated by  $84 \times A$ .

### Results and Discussion

The visible absorption spectrum of the color developed in the procedure had a maximum at 525 nm, and the shape was entirely identical to that observed in the determination of 2-OG.<sup>7)</sup>

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.

Potassium oxalate, Tris and L-leucine gave no absorbance and did not interfere with the color development at the prescribed concentrations in the enzyme reaction mixture. ADP, L-glutamic acid and NAD showed the absorbance of 0.05, 0.019 and 0.069, respectively, at the prescribed concentrations in the enzyme reaction mixture, but gave no interference with the color development of 2-OG to show a satisfactory recovery of 0.15  $\mu\text{mol}$  of 2-OG,  $100 \pm 2\%$ . Serum gave the absorbance of  $0.116 \pm 0.027$  (mean value  $\pm$  standard deviation) when examined with 15 sera.

An absorbance higher than the sum of the absorbances given by the individual components was observed when a solution containing all the components described above was treated to develop the color. This phenomenon was found to occur only when L-glutamic acid existed with NAD or serum, the value of absorbance depending on the concentration of L-glutamic acid and individual serum. The facts indicated that the color development for the preparation of serum blank should be carried out in the co-existence of all the components used in the enzyme reaction. The mixture of these components did not interfere with the color development of 2-OG to show satisfactory recoveries of the acid,  $93 \pm 1\%$ , in the concentrations of 0.05 and 0.1  $\mu\text{mol}$  (which corresponded to the enzyme activity of 11 and 22 units, respectively).

A slight increase of the absorbance of the final reaction mixture, 0.01, was observed on standing the mixture at 20° for 30 min, which was proved to be caused mainly by NAD remained unreacted. The increase of absorbance also occurred in the serum blank to the same extent, and therefore did not affect the absorbance measured in the procedure.

NADH formed in the enzyme reaction gave a small absorbance, 0.015, at the concentration of 0.1  $\mu\text{mol}$ , and did not affect the color development of 2-OG.

The enzyme reaction was investigated so as to be performed under the optimum conditions to avoid the factors unfavorably affected the amount of 2-OG formed by GDH.

Phosphate,<sup>6,15)</sup> triethanolamine<sup>8,16-18)</sup> and Tris<sup>19)</sup> buffers have been used for the assay of serum GDH activity. A 100 mM phosphate in the enzyme reaction mixture caused turbidity in the color developing solution by unknown factors though it could be removed by adding EDTA, and the phosphate buffer was unfavorable for the present purpose. Tris of 58.1 mM in the incubation mixture of pH 8.8 gave the same activity as that given by triethanolamine at 50 mM in the incubation mixture of pH 8.8. Tris was employed in the procedure for easiness of the preparation of the buffer. The enzyme activity increased with rising pH in a range of 7.0—8.5, and reached a constant value at 8.5—9.5. Thus, pH 8.8 was selected as the optimum.

The GDH activity increased with increasing concentration of NAD ranging from 1.7 to 5.0 mM with sera of high activities, reached the maximum at 5.0 mM and then became a practically constant in a range of 5.0—8.3 mM. Thus, 5.0 mM was selected for convenience.

L-Glutamic acid gave almost constant values of GDH activity with sera of both low and high activities in a concentration range of 250—500 mM, but lower values in a range of 100—225

15) J.A. Olson and C.B. Anfinsen, *J. Biol. Chem.*, **197**, 67 (1952).

16) K. Jung, *Clin. Chim. Acta*, **36**, 231 (1972).

17) J.-P. Persijn, W. Van Der Slik, C.J. Timmer, and A. Riethorst, *Clin. Chim. Acta*, **30**, 377 (1970).

18) G. Ellis and D.M. Goldberg, *Clin. Chem.*, **18**, 523 (1972).

19) A. Hershko and S.H. Kindler, *Biochem. J.*, **101**, 661 (1966).

mm with sera of high activities. Thus, 250 mm was selected as a sufficient concentration for fairly high activity.

The enzyme reaction rate was not so high enough in the absence of the activator for the sensitive assay of the activity that L-leucine and ADP were to be used as the activators<sup>16-20</sup> in the procedure. L-Leucine or ADP increased the activity by approximately 1.8 times that observed in the absence of them at a concentration of 50 or 5 mm, respectively, and a combination of them in these concentrations intensified the activation by approximately 2.2 times. The activation of GDH activity did not practically change over a L-leucine concentration range of 25—100 mm and an ADP concentration range of 3—5 mm. Therefore, the prescribed concentrations of L-leucine and ADP, 50 and 5 mm were selected, respectively, as their optimum concentrations.

The serum blank prepared with the incubation in the absence of NAD showed a slightly increased absorbance (0.01—0.02) in some sera when compared with that of a serum blank prepared without the incubation. This increase of absorbance was regarded to result from 2-OG produced extraneously from L-glutamic acid and small amounts of pyruvic and oxalacetic acids occurred in serum by the actions of glutamate pyruvate transaminase (GPT) and glutamate oxalacetate transaminase during the incubation. This assumption was proved by the fact that the increase of absorbance was more distinctly observed to occur when a small amount of sodium pyruvate (0.1  $\mu$ mol) was added to the incubation mixture and when serum with a high GPT activity (205 units<sup>21</sup>) was incubated. Therefore, the incubation was requisite for the preparation of serum blank in the procedure to cancel the interference from these enzymes.

An increased absorbance was also observed when serum was incubated with NAD solution under the conditions of procedure even in the absence of L-glutamic acid. This increase occurred in an absorbance range of 0.005—0.010 in normal sera, and was more evident in sera with relatively high LDH activity of 400—600 units<sup>22</sup>) and high lactic acid content of 40—60 mg/100 ml<sup>23</sup>) (the maximum increase of absorbance, 0.028, was observed), indicating that pyruvic acid was formed from lactic acid by the action of LDH during the incubation, which contributed to the increase of absorbance.<sup>24</sup>)

When glutamic acid and NAD solutions were incubated as in the procedure with normal serum which showed no GDH activity,<sup>25</sup>) an increased absorbance of 0.005—0.035 was observed. A remarkable increase of the absorbance, 0.180, was obtained when 7% albumin<sup>26</sup>) solution added with LDH, GPT<sup>27</sup>) and lactic acid<sup>28</sup>) so as to be 1200 units,<sup>22</sup>) 370 units<sup>21</sup>) and 50 mg/100 ml, respectively, was treated in the same way.

Those observations showed that pyruvic acid formed from lactic acid by the action of LDH was subsequently converted to 2-OG in the presence of glutamic acid and GPT, and these keto acids gave an extraneous absorbance which caused an error in the procedure. In order to eliminate the error, the inhibition of LDH, which also acted so as to suppress GPT

20) J. King, "Practical Clinical Enzymology," Van Nostrand, London, 1965, p. 66.

21) Assayed by the method: A. Karmen, *J. Clin. Invest.*, **34**, 131 (1955). GPT activity in normal serum was 0—30 Karmen units.

22) Assayed by the method: F. Wróblewski and J.S. La Due, *Proc. Soc. Exp. Biol. Med.*, **90**, 210 (1955). LDH activity in normal serum was 100—600 Wróblewski units.

23) Determined by the method: J.F. Neville, Jr. and R.L. Gelder, *Am. J. Clin. Path.*, **55**, 152 (1971). Lactic acid concentration in normal serum was 4—16 mg/100 ml: S. Tarui and K. Kuroda, *Nihonrinsho*, **31**, 1317 (1973).

24) The absorbance given by pyruvic acid was about 7% of that given by the equimolar concentration of 2-OG under the conditions of procedure.<sup>7)</sup>

25) Sera which showed no GDH activity when assayed by both the Schmidt method<sup>3)</sup> and the present method were used.

26) Bovine serum albumin (fraction V) with no GDH, LDH, and GPT activities was used.

27) LDH (pig heart, Sigma) and GPT (pig heart, Sigma) were used.

28) Lithium L-lactate monohydrate (Sigma) was used.

reaction by blocking the supply of pyruvic acid, was considered to be effective. Thus, oxalic acid as an inhibitor of LDH<sup>29)</sup> was employed as its potassium salt in the procedure.

The effect of the oxalate concentration on the inhibition was investigated on 7% albumin<sup>26)</sup> solution added with lactic acid and LDH<sup>27)</sup> so as to be 15 or 70 mg/100 ml and 2000 units, respectively, by monitoring the absorbance of NADH formed under the conditions of the present enzyme reaction in the procedure for 45 min. The examination was also carried out with pathological sera<sup>30)</sup> free from GDH activity<sup>25)</sup> containing lactic acid in the concentrations of 19–44 mg/100 ml, LDH activity in the range of 410–1030 units and GPT activity in the range of 16–195 units. As shown in Fig. 1, the formation of NADH was hindered with increasing concentration of the oxalate and reached the minimum and constant value at the concentrations of 1.25–1.75M. Thus, the prescribed concentration, 1.25M, was selected as a sufficient concentration. At this concentration of the oxalate, the NADH formation by the action of LDH was noted to diminish by more than 90% in low and high lactic acid concentrations, respectively.

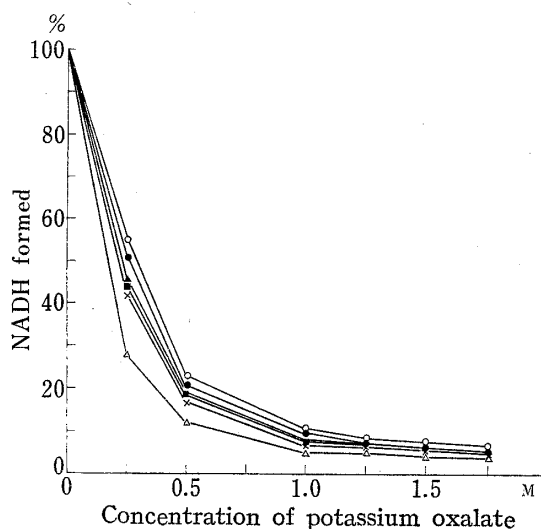


Fig. 1. Effect of Oxalate Concentration on NADH Formation

Albumin solution <sup>a)</sup> added with	Lactic acid (mg/100 ml)	LDH (Wróblewsky units)	GPT (Karmen units)
△—△	15	2000	—
○—○	70	2000	—
serum with			
■—■	19	410	34
▲—▲	30	670	36
●—●	41	700	195
×—×	44	1030	16

a) 7% bovine serum albumin (fraction V) solution

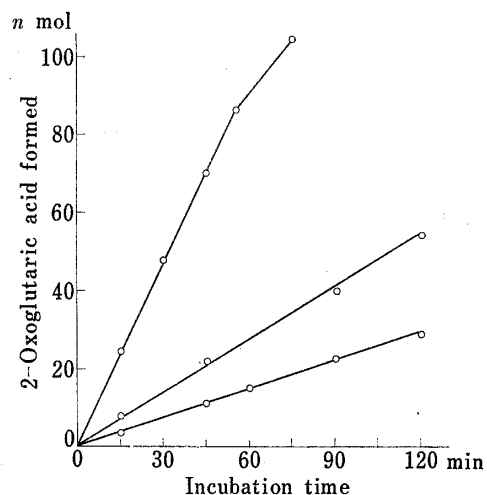


Fig. 2. Relationship between the Incubation Time and the Amount of 2-Oxoglutaric Acid Formed

a, 15.6; b, 5.1; c, 2.4 units serum. Each plot was the mean value of triplicate determinations and standard deviation for the each value was 1–2.5.

The effect of preincubation time on the suppression of NADH formation was examined for 5–15 min, and the prescribed time of 5 min was enough for the suppression.

29) J.B. Neilands, *J. Biol. Chem.*, **208**, 225 (1954).

30) The examination with serum of relatively high calcium content (more than 10 mg/100 ml) could not be successfully carried out because calcium caused turbidity due to the formation of its oxalate, which interfered with the absorbance measurement of NADH.

The interference of the oxalate with serum GDH was estimated on inactivated sera<sup>31)</sup> added with GDH preparation<sup>32)</sup> so as to be 1–18 units. The oxalate solution reduced the GDH activity only by less than 3%.

Oxamic acid as an inhibitor of LDH<sup>29)</sup> was also tested in the same way as described above and the maximum suppression of the NADH formation, 75%, was observed at the concentration of 3.7mM in the incubation mixture,<sup>17)</sup> showing that oxamic acid was inferior to the oxalate for the present purpose.

Under the prescribed conditions of the procedure, a practically linear relationship between the incubation time and the n mol of 2-OG formed was observed throughout at least 90 min incubation on sera with a slightly high activity, and also throughout 55 min and up to 84 n mol of 2-OG formed (which corresponded to 18.6 units on serum with a fairly elevated activity, as shown in Fig. 2. This fact suggested that the present procedure might permit the determination of GDH activity up to at least 18 units. The range might be extended to a very high activity, about 45 units, by employing a shorter incubation time, 15 min.

The enzyme activity was almost proportional to the dilution of serum when diluted with serum of low GDH activity, as shown in Fig. 3.

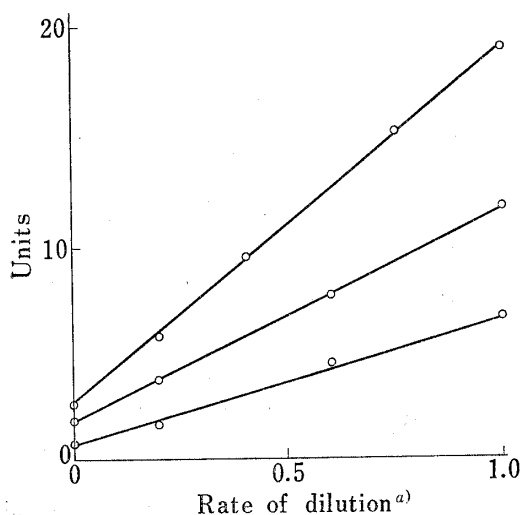


Fig. 3. Effect of the Dilution of Serum on the Measured GDH Activity

Sera were diluted with low units sera. Each plot was the mean value of triplicate determinations and standard deviation for the each value was 0.3–0.7.  
a) serum (ml)/serum(ml)+low units serum (ml)

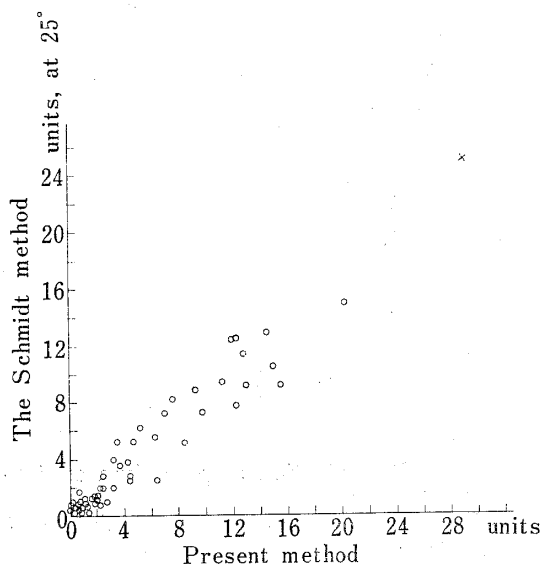


Fig. 4. Correlation between the Values obtained by the Present Method and the Schmidt Method

x: The incubation time of 15 min was used in the present method.

Parallel tests with the Schmidt method,<sup>3)</sup> most conventionally used method, were carried out on 65 different sera with activities below 20 units. The results are shown in Fig. 4. The coefficient of correlation for both methods, 0.95, was obtained. The units obtained by the present method ( $x$ ) could be converted to the Schmidt units ( $y$ , the  $\mu\text{mol}$  of NADH consumed per liter of serum per min at 25°) by the regression equation,  $y=0.8x+0.1$ .

The precision of the present method was studied as to repeatability, which was obtained by performing separately 36 and 20 determinations on sera with the mean activities of 14.9 and 2.8 units. The standard deviations were 0.7 and 0.3 (coefficient of variation, 4.4 and 12%), respectively.

31) Normal sera inactivated by heating at 60–70° for 30 min was used, which showed no LDH, GDH and GPT activities.

32) GDH (beef liver, Boehringer) was used.

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The values of GDH activity determined on sera of healthy 20 individuals did not exceed 2.0 units.

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