서 울 의 대 잡 지 제17권 제2호
The Seoul Journal of Medicine
Vol. 17, No. 2, June, 1976

A New Colorimetric Method for the Determination of Guanine Aminohydrolase Activity

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INTRODUCTION

Through anabolic reactions, not only guanine(Lukens & Herrington, 1957), but also the xanthine (Moat & Friendman, 1960), which is formed by the action of guanine deaminase, as well as hypoxanthine (Lukens & Herrington, 1957), which is formed from xanthine by the reversible action of xanthine dehydrogenase, are known to be incorporated into nucleotides and nucleic acids. In addition, there is a salvage pathway of the free purine base to be utilized for incorporation into nucleic acids (Mandel et al, 1957).

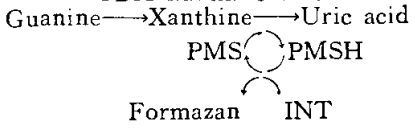
These guanine involved reactions, therefore, are well known for their competance with guanine deaminase (guanine aminohydrolase, EC 3.5.4.3) for the utilization of intracellular guanine; and thus it enables the statement that the deamination reaction may be expected to be subject to intracellular regulation, occupying a pivotal position in the metabolism of guanine. According to the earlier reports from this department, dealing with guanine deaminase revealed that it is localized abundantly and preferentially in the liver and brain tissues of rats and rabbits (Kimm et al, 1967; Lee, 1969; Talwar et al, 1961; Kumar et al, 1965). It was also suggested that the enzyme is characterized by separable two fractions (Kumar et al 1967; Kumar and Krishnan, 1970; Kumar et al: 1972) or several fractions (Kimm et al, 1967; Kumar et al, 1972; Panzica, 1968) when prepared from the above tissues and its significances as a diagnostic tool were reported as well (Mcleod, 1967; Prodanov and Astrug, 1971). Available informations on the enzyme, however, are at present very much obscure, although its importance has been stressed so much so far since Kalckar (Kalckar, 1947).

With regards to the isozymic and subunit nature, however, informations are so limited that an attempt in the author's laboratory is under progress with the use of phenazine methosulfate and INT to visualize the enzyme band on the electrophoretic gel bed based on the formation of formazan color. As a collateral study, a possibility of a new colorimetric assay method of guanine deaminase was studied adopting this system of staining the enzyme separated electrophoretically on the gel.

Before being more specific on the study to purify and characterize the enzyme, the present paper is planned to evaluate a new colorimetric assay system to apply it to succeeding studies in order to clear out some obscurity with regard to its properties. The colorimetric principle adopted in the present study is to utilize the red discoloration of INT (2-(p-iodophenyl)-3-(p-nitrophenyl)

<Received for Publication on June 8, 1976>

5-phenyl tetrazolium chloride) when reduced in the following reaction system, utilizing phenazine methosulfate (PMS) as an electron carrier. (Nachlas et al, 1960)



MATERIALS AND METHODS

The special reagents used in the studies, guanine, xanthine oxidase, phenazine methosulfate, and INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5 phenyl tetrazolium chloride) were procured from Sigma Chemical Co. (St. Louis, Mo.); and gelatin purchased from Difco (Detroit, Mich.), Tris-(hydroxymethyl) amino methane from Merck (Darmstadt, Germany).

The enzyme, guanine deaminase, utilized in this investigation was purchased from Mann Research Lab. The activity of guanine deaminase was 0.087 U/mg. protein of rabbit liver, and of xanthine oxidase 0.75 U/mg. protein of cow milk.

The assay system of guanine deaminase was principally consisted of coupling it with xanthine oxidase in order to convert guanine to uric acid via xanthine, during which phenazine methosulfate would be reduced and, in turn, the formazan discoloration could be detected colorimetrically at 492 nm.

The assay was carried out by incubating the 0.01 ml of guanine deaminase, with 1% gelatine, xanthine oxidase, INT, and phenazine methosulfate successively in 0.1 M Tris buffer, pH 8.0, starting the reaction by adding excess amount of the substrate, guanine, which was dissolved in hydrochloric acid to 3.0 mM as a working solution.

The standard and reference assay mixture per cuvette was consisted of 1.5 μ mole guanine, 5.0 mg. gelatine, 0.3 units xanthine oxidase, 1.2 mg. INT, 0.05 mg. PMS and known activity of guanine deaminase, dissolved in 0.3 mM Tris buffer, pH 8.0, in a total amount of 3.0 ml.; and the incubation was carried out at 30°C for various intervals of time, observing the changes in the optical densities at 492 nm at 5 minutes intervals.

The reference assay of the enzyme activity for control to compare with the present colorimetrical method coupled with xanthine oxidase was performed after the spectrophotometric estimation of Roush and Norris (Roush and Norris, 1950), measuring the decrease in the extinction of guanine during the incubation.

The activity of guanine deaminase was calculated, therefore, according to the following formula, where E stands for the extinction coefficient of INT at 492 nm and V for the total volume of assay system (3.0 ml)

$$\frac{\delta O.D.}{E_{492}} \times V \times 100 = \text{unit/ml. guanine deaminase}$$

RESULTS

1. Effect of the amounts of enzymes:

Since the reaction was GDA catalyzed reaction coupled with XO reaction producing uric acid from guanine, it is reasonable to use excess amount of XO, the indicator system catalyzer, as compared to the amount of GDA, the measuring system catalyzer. It was neceassary for this reason to establish an adequate amount of the former to be coupled with a given amount of the latter. With a given activity of GDA, 1.45 unit/ml., the effect of XO was observed as in Table 1.

As is apparent from the Table, all the figures were under the activity of the GDA added at the beginning of the incubation: that is, at the maximum the original activity of 1.45 u/

Table 1. The Effects of the amount of XO on GDA activity. The GDA activity in the assay system was 1.45 ut/ml. The incubation were carried out at 30°C. and pH of the buffer was 8.0.

(Unit/ml)

Incubation time / XO amount	5	10	15	20	25	30 min
0.015 unit	0.025	0.055	0.085	0.101	0.131	0.151
0.030 unit	0.096	0.148	0.193	0.225	0.257	0.271
0.150 unit	0.224	0.227	0.260	0.280	0.287	0.313
0.300 unit	0.328	0.322	0.321	0.322	0.320	0.325

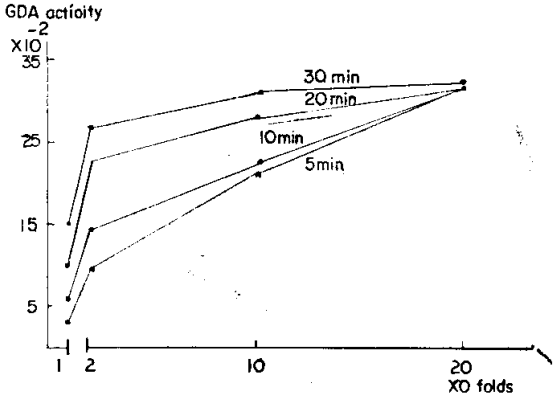


Fig. 1. See the legend of table 1. Figures denote mean values of several triple determination.

ml. fell as low as 0.325 u/ml. From the Figure 1, it is quite evident that the indicator enzyme, XO, should preferably as much as 20 fold activity against the measuring enzyme, GDA, because of the fact that GDA activity could be estimated maximum under the present condition of assay regardless the variations in the period of incubation.

When the activities of GDA were depicted as a function of time intervals of incubation, calculated on the base of O.D. observed during successive 5 minutes (Table 2, Fig. 2) it is also only with the system in which XO was added in excess, 20 folds of GDA, that showed not only the maximum activity but also linear activity profile against incubation period.

To 0.30 units of XO in the system various amounts of GDA were added to observe the effect if they were correctly reproducible. The results indicate as in Fig. 3 that the activities of GDA added were proportionately reproduc-

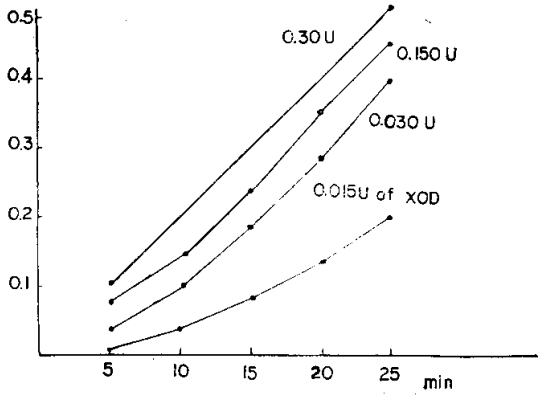


Fig. 2. See the legend of Table 2.

Table 2. The Effects of the amount of XO on O.D. increase. See the legend of table 1.

Incubation time / XO amount	5	10	15	20	25	30 min
0.015 unit	0.008	0.032	0.080	0.130	0.199	0.282
0.030 unit	0.030	0.092	0.180	0.279	0.395	0.505
0.150 unit	0.075	0.141	0.242	0.349	0.446	0.582
0.300 unit	0.102	0.200	0.299	0.400	0.498	0.594

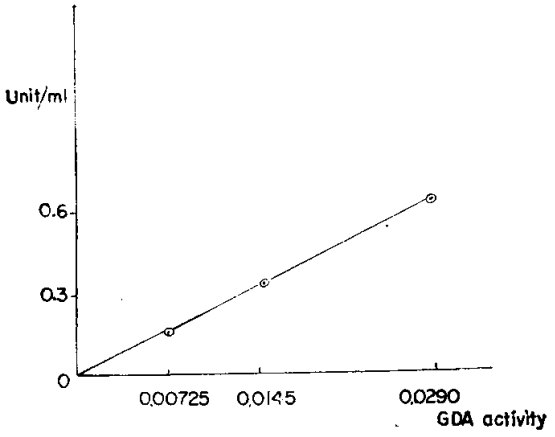
(O.D.)

ible when the ratio of XO to GDA ranges within 10 to 40 fold, provided that the incubation was carried out for 30 minutes at 30°C.

2. Effect of temperature and pH:

Keeping the ratio of XO to GDA 20 to 1, and incubating for 30 minutes, changes of GDA activity was followed below physiological temperature of 37°C (Fig. 4), which shows clearly that the activity of the enzyme could be maximally expected to appear at 37°C.

The influence of pH of the assay mixture was observed by the use of 0.1M Tris and 0.1 M phosphate buffers (Fig. 5) which displayed an activity profile characterized by the inactive effect on the acid side and the active effect on the alkaline side of pH, showing maximum activity of the enzyme at pH 8.0.



Fi :. The reproducibility of the various amount of GDA. The activity of XO was 0.30 Unit, and incubation was carried out at 30°C for 30 min. pH of the system was 8.0.

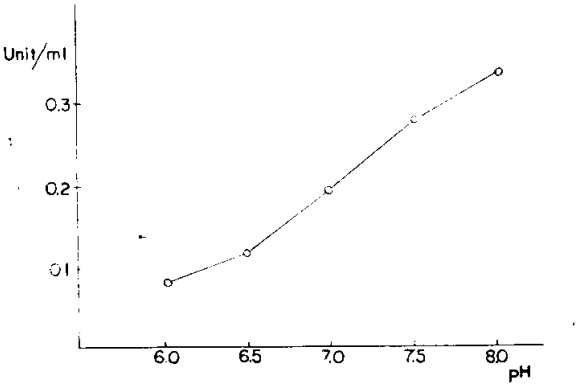


Fig. 5. Effects of pH on GDA activity. The activity of GDA was 1.45 Unit/ml, and of XO was 0.30 Unit. The incubation was carried at 30°C for 30 min.

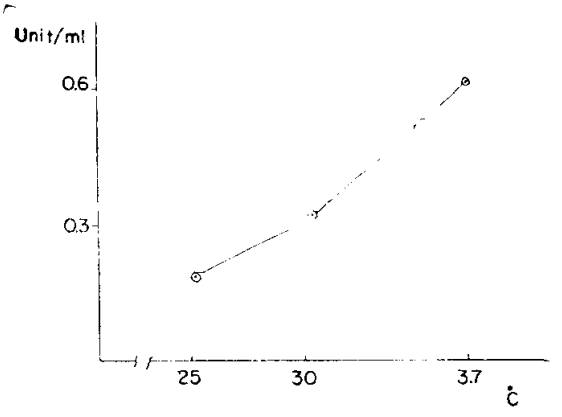


Fig. 4. Effects of temperature on GDA activity. The activity of GDA in assay system was 1.45 Unit/ml and of XO was 0.30 Unit. Incubation was carried out for 30 min. and pH of the system was 8.0.

3. Effects of PMS and INT:

Since XO contains flavin adenine dinucleotide in it, it can be reduced to $FADH_2$, which, in turn, can reduce INT to produce the formazan color. But as observed in the present study (Fig. 6) the formazan reaction is much more evident and stronger when small amount of PMS was added to the assay mixture as an electron carrier; 0.05 mg. of PMS per reaction mixture brought almost 25% more of the color intensity as compared to that of PMS depleted system. But the amount of PMS over 0.05 g. per present assay mixture caused, on the contrary, decrease in the color intensity to the extent comparable to that of PMS depleted system.

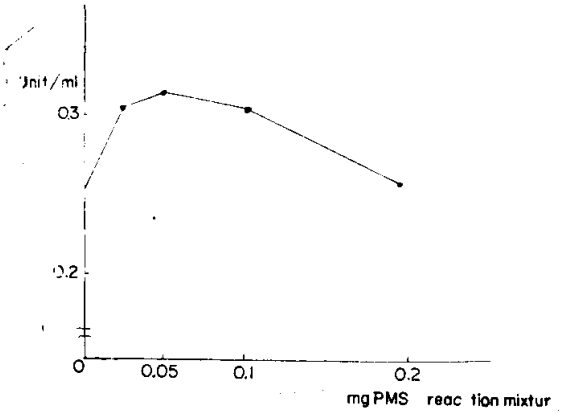


Fig. 6. Effects of the amounts of PMS on GDA activity. The activity of GDA was 1.45 Unit/ml, and XO was 0.30 Unit. The incubation was carried out at 30°C for 30min. and pH was 8.0.

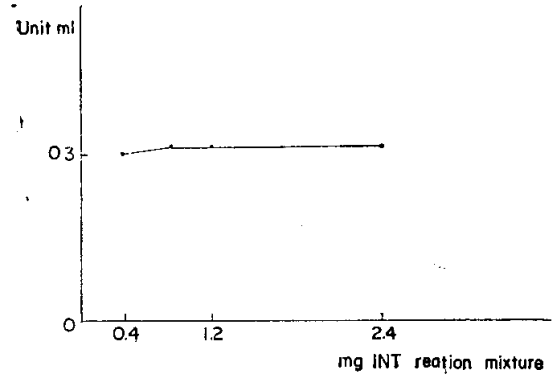


Fig. 7. Effects of the amont of INT on GDA activtiy. Incubation conditions and assay system were same as Fig. 6.

INT showed, by contrast to PMS, no appreciable variations in the final color production when added with the concentration below 2.4 mg. per reaction mixture(Fig. 7).

DISCUSSION

There are several reported methods for the estimation of GDA activity, notably Roush and Norris, Kalckar, Hue and Free (Hue and Free, 1965) and Knight (Knights et al, 1965) and radioactive assay method utilizing guanine-8-C^{14} (Aikhalidi et al, 1970). However, none of them has proved to be suitable in principle for application to visualize the GDA enzymogram on the electrophoretic gel bed, though they are very sensitive and reproducible with regard to the activity assay itself.

The present method is only dually purposeful; for one thing the basic principle involved can be applicable to the staining of GDA after electrophoretic separation and for another, applicable in estimation of its activity.

Although there was noticed that somewhat lowered activity of the enzyme was detected through this method, of which reason seems obscure presently and awaits further investigation, coupling of the GDA with XO with the use of PMS and INT in the present study was satisfactory to the demand of the author's laboratory. Firstly, adding small amount of PMS as electron carrier the magnitude of the reduction of INT was enhanced, unless otherwise FAD, contained in the molecule as a prosthetic group, alone could not be expected. Secondly, INT was preferred to NBT because of its high extinction coefficient, (Möllering et al, 1974) and the insoluble formazan produced were well dispersed by gelatin. Lastly, the data obtained by the present method showed much lowered values when compared to the results obtained by Roush and Norris method. It is, therefore, suggested to multiply with the factor shown below when one wants to convert the present data to Roush and Norris unit of activity.

$$\frac{\delta \text{ O.D.}}{\text{E } 492} \times V \times 100 \times F(4.5) = \text{unit/ml GDA}$$

The activity of GDA by the present method was not suitable actually at the pH aboe 8.0, because the PMS is known to be highly unst-

able at extreme alkaline pH (Nachlas et al, 1960). And it is well in accord with published report (Nachlas et al, 1960) that amount of INT has no serious effect in the formazan discoloration. But the system requires excess amount of indicator enzyme, XO, as compared to measuring enzyme, GDA, to the ratio of 20 to 1. It is surely because of the fact that Km of the indicator enzyme should exceed far above the concentration of intermediate substrate in a coupled system (Bergmeyer, 1974), xanthine in the present system, in order to aquire steady state between the two enzymes.

CONCLUSION

A new colorimetric assay method for guanine deaminase was reported, coupling the enzyme with xanthine oxidase and formazan reaction system adding phenazine methosulfate and INT (2-(p-iodophenyl)-3-(p-nitrophemyl)-5-phenyl tetrazolium chloride). The method was proved to be applicable both for the assay of GDA activity and for visualization of the enzymogram after electrophoresis.

The assay system consisted of GDA, XO as much as 20 fold of GDA, 0.16μmole of PMS, and 1.5μ mole of INT in a final volume of 3.0 ml. Tris buffer, pH 8.0. The reaction was started by the addition of 1.5μ mole guanine, followed by 30 minutes incubation at 30°C.

The present data was inconsistent with the reported data, but could be converted by a factor for the purpose of comparison. It appears to be a good merit to apply the present method in estimating the GDA activity after staining electrophorogram with the formazan reaction introduced in the present study directly utilizing extract of visual enzyme band.

≫國文抄錄≪

Guanine Aminohydrolase 活性의 新比色測定法

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朴鍊培 · 金昇元

Guanine deaminase (GDA)에 xanthine oxidase를 indicator enzyme으로 couple시키고, Phenazine methosulfate 및 INT(2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride)에 의한 formazan 生成반응을 利用하여 그 活性을 측정할 수 있는 새로운 比色測定法을 報告하였다.

이 方法은 GDA 活性측정 뿐만 아니라 電氣泳動後의 enzymogram을 發色 可能하게 함을 알았다.

Assay 液 組成은 GDA와 그 20배 이상의 活性에 該當하는 xanthine oxidase 그리고 PMS 0.016μmole, INT 0.15μmole을 pH 8.0의 Tris 緩衝液 0.3mmole에 配合하여 總量이 3.0 ml에 이르도록 하였으며, guanine 1.5μmole을 加해 反應을 시작하고 30°C에서 30分間 incubate하였다.

본 方法에 依하면 다른 方法에 依한 結果보다 낮은 GDA 活性을 보이나 實驗係數로써 活性을 補正할 수 있고 더욱이 電氣泳動後에 formazan에 의하여 染色된 electrophoregram에서 그 活性을 측정하는데는 좋은 方法이다.

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