

A New Ultra-micro Microbioassay¹⁾

A method was developed which was capable of detecting minute amounts of carbohydrates as well as other biologically active substances. The principle of the method is to analyze an active substance by the turbidity or amounts of metabolites produced by a microorganism after two successive cultivations. We named this technique amplified cultivation.

A schematic representation of the method is shown in Fig. 1. The first cultivation is performed until a maximum difference is obtained in numbers of viable cells between a basal medium and that added with an aliquot of a substance (t_1). The second cultivation is started after adding complete medium to the cultures and stopped before the growth of microorganism in the blank reaches a certain detection limit (t_3), or at latest before it gains a maximum level (t_4).

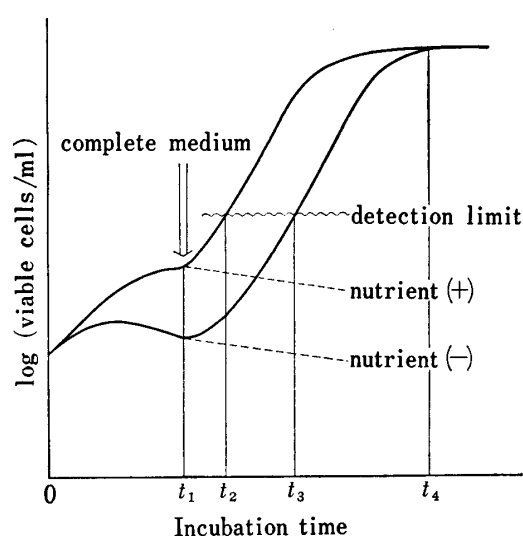


Fig. 1. Schematic Representation of Amplified Cultivation

At time t_1 , the bacterial growth is shifted up by addition of the complete medium to the culture. Analysis of a nutrient is carried out at a proper time between t_2 and t_4 . The dotted lines show the changes of viable cell numbers in the conventional microbioassay method.

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Microbioassay of Lactose and L-Cystine

The organism used was *Bifidobacterium bifidum* N₄²⁾ which was cultivated at 37° in an atmosphere of 90% N₂ and 10% CO₂ both in the first and the second cultivation. The inoculum was prepared by the previous manner.³⁾ For assay of these compounds, the medium used by György⁴⁾ was modified by replacing the enzymatic digestion of casein for casamino acids (acid-hydrolyzed casein), and calcium pantothenate for pantethine (17 mg/liter). The corresponding nutrient was omitted from the medium. Previously autoclaved (115°, 5 min) 6 mm diameter discs (Toyo Roshi

Kaisha Ltd., Tokyo), spotted either lactose or L-cystine in 10 μ l distilled water, were placed on the solidified seeded agar plate (1.5% Bacto-Agar, 4 mm thick) prepared from the basal medium. After performance of the first cultivation for 17–24 hr, the complete medium prepared by supplementing the respective basal medium with lactose or L-cystine was poured aseptically on the culture plate up to 4 mm depth over the agar, and then the second cultivation was carried out for 17–24 hr. Even 1 μ g of lactose or 10 ng of L-cystine was detected by the amplification technique as a growth zone, while the detection limits by the conventional methods are 1 mg of lactose and 10 μ g of L-cystine. The determination range of lactose was 50–1000 μ g per disc (Fig. 2a).

In the application of the present method to the test tube assay, 0.5 μ g of lactose was detected by titration method after 20 hr first and 36 hr second cultivation (100 μ g by the conventional test tube assay).

- 1) A part of this work was presented at the 89th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April, 1969.
- 2) M. Yoshioka, S. Yoshioka, Z. Tamura and K. Ohta, *Japan. J. Microbiol.*, **12**, 395 (1968).
- 3) H. Nakamura, K. Samejima and Z. Tamura, *Japan J. Microbiol.*, **14**, 9 (1970).
- 4) P. György, *Pediatrics*, **11**, 98 (1953).

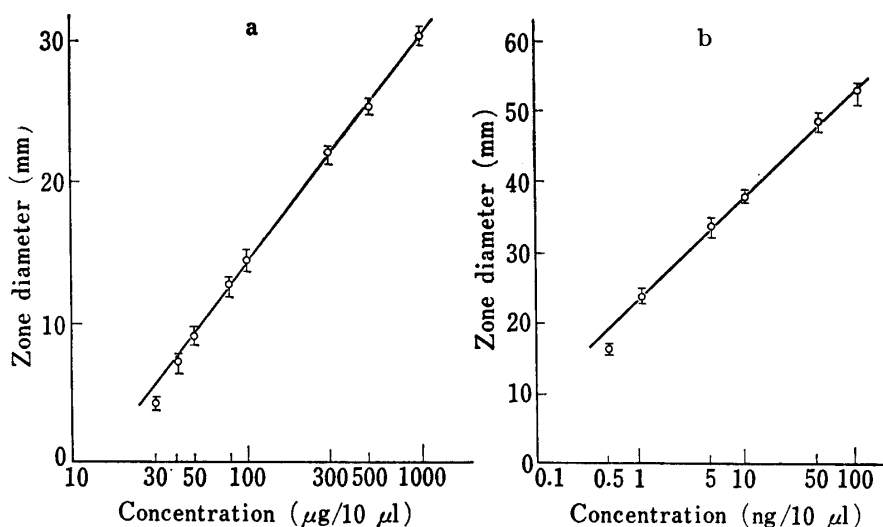


Fig. 2. Working Curves for Lactose (a) and Calcium D-Pantothenate (b) by the Amplified Cultivation

A series of concentrations of standard substance was incubated in a Petri dish (20 cm *i.d.*), and the range and the mean of values corresponding to each concentration, obtained from five plates are shown in the figure.

Microbioassay of D-Pantothenic Acid

Lactobacillus plantarum ATCC 8014 was used. The basal medium for assay was identical with the one described in U.S.P.XV (1955). Ten μl of 50% ethanolic solutions containing various amount of calcium D-pantothenate was applied to the pulp discs above mentioned. The discs were placed on the seeded agar (1%, 1.5 mm thick, 6×10^4 viable cells/ml) prepared from the basal medium mounted on the base agar (1%, 1.5 mm thick). After 21 hr of the first cultivation at 37°, the basal medium provided with calcium D-pantothenate (2 mg/liter) was poured on the agar up to 1.5 mm depth over the surface, and the second cultivation was carried out for 8 hr at 37°. If the bacterial growth was not enough for measuring the diameters of growth zones, additional incubation was carried out for 15 hr at 27°.

Lower detection limit of the vitamin was 0.1 ng (5 ng by the conventional method). Moreover, good linearity was achieved between amount of calcium D-pantothenate on the logarithmic scale and growth zone diameter on the linear scale ranging from 1 ng to 100 ng (Fig. 2b). Thus about 50 fold increase in sensitivity of detection and about 50 fold decrease in determination limit was successfully obtained relative to the other published standardized pulp disc method.⁵⁾

The principle of the present method would hold true in microbioassay of all the substances essentially required by microorganisms and also be useful for researching unknown active substances of trace amount.

Analyses of other bioactive substances, including biotin and nicotinic acid (*L. plantarum* ATCC 8014), fructose and nicotinamide (*L. fructosus* IFO 3516) and N-acetyl-D-glucosamine (*L. bifidus* var. *Pennsylvanicus*) *etc.* by this method is now in progress and the detailed paper will be presented in the near future.

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5) H. Kojima, Y. Matsuya, H. Ozawa and T. Uemura, *Nippon Nogeikagaku Kaishi*, **32**, 33 (1958).

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Occurrence of Insect-Moulting Substances Ecdysterone and Inokosterone in Callus Tissues of *Achyranthes*

Achyranthes fauriei LÉVEILLÉ et VANIOT (Amaranthaceae) is one of the plant sources which were first demonstrated to contain ecdysterols.¹⁾ Later the ecdysterols ecdysterone and inokosterone were shown to occur also in the other *Achyranthes* spp.²⁾ While ecdysterols are quite interesting substances in the respects that they disclose not only the dramatic effects in the moulting and metamorphosis of arthropods but also the unique physiological activities in higher animals.

In the hope that plant tissue cultures may provide an effective tool in studying the production and the metabolic pathway of ecdysterols, we have started the present work. Thus, induction and growth of seedling callus tissues from the *Achyranthes* plants were examined on the surface of basal media containing some of coconut milk, yeast extract, casein hydrolysate, and plant growth regulators (2,4-dichlorophenoxyacetic acid (2,4-D), α -naphthaleneacetic acid, 3-indolacetic acid, kinetin, and gibberellin). As the results, it was shown that callus tissues are best induced when the White's basal medium was supplemented with 10% of coconut milk and 1 ppm of 2,4-D for *A. fauriei*, *A. japonica*, *A. japonica* var. *hachijoensis*, *A. obtusifolia*, and *A. rubrofusca*, and with 10% of coconut milk and 4 ppm 2,4-D for *A. longifolia*. On the other hand, it was found that the growth of the callus tissues is best effected when the Murashige-Skoog's basal medium was supplemented with 10% of coconut milk, 1 ppm of 2,4-D, and 1 ppm of kinetin for *A. fauriei*, *A. japonica*, *A. japonica* var. *hachijoensis*, *A. obtusifolia*, and *A. rubrofusca*, and with 10% of coconut milk, 4 ppm of 2,4-D, and 1 ppm of kinetin for *A. longifolia*. Extracts of the callus tissues grown under various conditions were shown to exhibit intense insect moulting hormone activity in the *Sarcophaga* test, indicating the presence of ecdysterols. Identification of the ecdysterols as ecdysterone and inokosterone in the extracts was carried out by thin-layer chromatography of the extracts and their acetylation products. Certain extracts were further subjected to the liquid chromatography using an Amberlite XAD-2 column³⁾ to corroborate the identity. However, the contents of the ecdysterols in the callus tissues were very small (<0.002%) as compared with those in the normal plants. Increase of the ecdysterols contents in callus tissues by modification of the medium components is the future problem.

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- 1) T. Takemoto, S. Ogawa, and N. Nishimoto, *Yakugaku Zasshi*, **87**, 325, 1469, 1474 (1967).
- 2) T. Takemoto, S. Ogawa, N. Nishimoto, and S. Taniguchi, *Yakugaku Zasshi*, **87**, 1478 (1967); T. Takemoto, S. Ogawa, N. Nishimoto, K.-Y. Yen, K. Abe, T. Sato, K. Osawa, and M. Takahashi, *ibid.*, **87**, 1521 (1967); T. Takemoto, S. Ogawa, N. Nishimoto, H. Hirayama, and S. Taniguchi, *ibid.*, **88**, 1293 (1968).
- 3) M. Hori, *Steroids*, **14**, 33 (1969).