A Simplified Method for Determination of Amino Acid Amidases in Tissue Cultures.* (24196)

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Amino acid amidases (AAAases) catalyze enzymic hydrolysis of amino acid amides to corresponding amino acids with concomitant liberation of ammonia(1). For quantitative assay of AAAase activity in tissues either amino acid or ammonia concentration may be measured(1). Development of rapid microcolorimetric technics for determination of ammonia has resulted in numerous procedures which combine microdiffusion with sensitive colorimetric procedures(2,3). The present study was undertaken to incorporate in a compartmented flask the enzymic assay of AAAase from tissues grown *in vitro*, eliminating deproteinization and the transfer of an aliquot prior to microdiffusion. The enzymic-diffusion reactor (EDR) flask is a modification of Seligson and Hirahara's(2) diffusion bottle for measurement of ammonia in blood, erythrocytes, and plasma.

Materials and methods. The cortices of bovine kidneys from 14 to 20 inch embryos and swine kidneys from 4-week-old pigs were minced and trypsinized according to the method of Young *et al.*(4). Trypsin-dispersed cells were seeded in T-60 flasks and overlaid with nutrient media containing 20% homologous serum as reported previously(5). After incubation at 37°C in stationary position from 7 to 9 days, the cellular monolayers were harvested and washed twice with Hanks' balanced salt solution. The cells were homogenized in TenBroeck homogenizer at 4°C in 0.02 M phosphate buffer, pH 7.8, containing 0.001 M disodium ethylenediamine tetraacetate to give a final concentration of 5% tissue. This homogenate was stored at −70°C and used within a 5-day period for the assay of AAAase. AAAase activity was measured in compartmented flask shown in Fig. 1. Routinely to a 35 ml EDR flask a mixture of $KHCO_3$ (1 g) and $K_2CO_3 \cdot 1\text{-}\frac{1}{2}\ H_2O$ (0.5 g) was placed in one compartment. Buffer, activating ion, substrate, and homogenate in total volume of 1.2 ml were added to the other compartment. The cell homogenates were diluted so that not more than 0.5 μM of NH_3 was liberated during the incubation period. Control flasks contained similar mixtures with deionized water in place of homogenate or substrate. (Specific quantities are noted in the legends to Table I and Fig. 2.) The ground surface of receiving rods were dipped into 1 N H_2SO_4 to the indentation, inserted into the EDR flasks which were incubated at 38°C for 30 or 60 minutes. After incubation the flasks were rotated at RT on a wheel (25 rpm) for 30 minutes. The diffused NH_3 trapped by the acid film was washed from the ground surface with 2.5 ml of deionized water. Stone's(6) colorimetric procedure was used for measurement of ammonia. Protein was determined by the method of Lowry *et al.*(7). Specific activity is expressed as μM of NH_3 formed per mg protein per hour. The L-amino acid amides (AAAs) tested were ob-

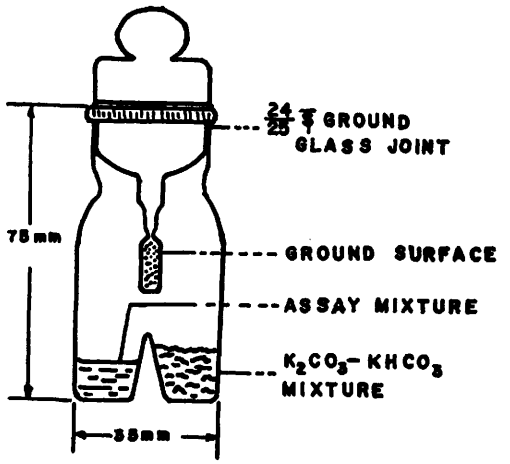

FIG. 1. Enzymic diffusion reactor flask for determination of amino acid amidases.

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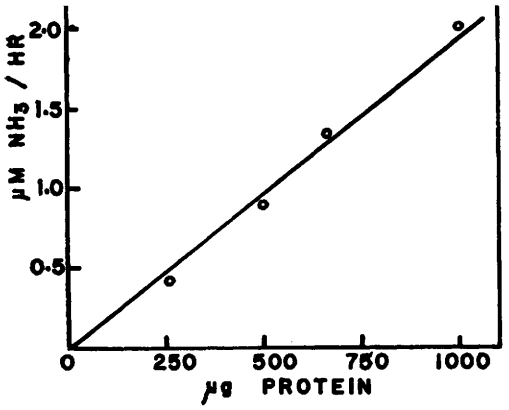

FIG. 2. Effect of enzyme conc. on leucinamidase activity of normal swine kidney cells grown *in vitro*. Assay system contained: L-leucinamide HCl, 100 μM; $MnCl_2$, 30 μM; aliquot of homogenate of the desired protein conc.; and tris buffer, 0.1 M, pH 8.3 to a final vol of 1.2 ml.

tained from the Mann Research Laboratories.

Results. Crude cell homogenates of bovine and swine kidney tissue cultures were capable of deamidating L-asparagine, L-glutamine, glycinamide · HCl, L-leucinamide HCl, and L-tyrosine amide. The AAAases from swine kidney tissue had about the same activity as the AAAases from bovine kidney tissue. Asparaginase was found to be the least active and leucinamidase the most active in deamidations of the AAAs tested. Typical data are summarized in Table I.

The activity of leucinamidase as a function of enzyme concentration is shown in Fig. 2. The results show that liberation of NH_3 from L-leucinamide HCl was related linearly to the enzyme concentration.

Discussion. The results indicate that modification of Seligson and Hiarahara's(2) method provides a rapid, accurate means of measuring enzyme reactions in which NH_3 is liberated. Since reproducible values were obtained with crude homogenates the method should be appropriate with purified preparations.

It is recognized by the author that leucine amidase and leucine aminopeptidase could be the same enzyme. Other enzymes may be present in the crude homogenate such as aminopeptidase which could release NH_3 if endogenous substrates were present. But, Spackman *et al.*(8) reported that crude enzyme preparations of swine kidney (leucine aminopeptidase) which hydrolyzed L-leucinamide required a prior incubation of 2 to 3 hours with Mn^{++} ions. The author observed almost an immediate effect with Mn^{++} which would tend to indicate the presence of leucinamidase. In this case the estimated AAAase activity, for example leucinamidase, would be large in comparison to the relative rates of the side reactions. The endogenous values were found to be very low and considered beneath the reliable range of the colorimetric procedure. The inclusion of a flask containing the AAA with homogenate omitted, showed negligible non-enzymatic hydrolysis in the assay. In Fig. 2 the rate of NH_3 release appears to be proportional to the enzyme concentration over the entire range of concentrations tested. The proportionality suggests that the substrate must be at a sufficiently high concentration to saturate the enzyme throughout the incubation period.

Brown *et al.*(3) have reported that deproteinization of human plasma and rat tissues prior to alkalinization and ammonia diffusion results in a varying degree of amide hydrolysis. Since similar situation has been encountered by the author with cell homogenates the enzyme assay and microdiffusion were combined in a single flask with the omission of a deproteinization procedure. Thus the EDR flask may be used to great advantage in studying enzymic reactions which are dependent upon ammonia determinations.

TABLE I. Deamidation of Amino Acid Amides by Crude Cell Homogenates of Bovine and Swine Kidney Tissue Cultures.

Substrate	μM NH_3/mg protein/hr	
	Bovine	Swine
*L-asparagine	.07	.05
Glycinamide HCl	.10	.11
*L-glutamine	.42	.52
L-tyrosine amide	.82	1.08
L-leucinamide HCl	1.76	2.06

Assay system for AAAs: L-amino acid, 100 μM; $MnCl_2$, 30 μM; suitable aliquot of homogenate; and tris(hydroxymethyl) aminomethane buffer, 0.1 M, pH 8.3 to final vol of 1.2 ml. The control flasks were similar without L-amino acid amide or homogenate. Incubation period was 30 or 60 min. at 38°C.

Values = Complete System (CS) − [(CS + Substrate) + (CS + Homogenate)].

* $MnCl_2$ was omitted from the assay.

Summary. A simple enzymic-diffusion reactor flask is described for the enzymic assay of amino acid amidases from tissues grown *in vitro*. The procedure is rapid, precise, and applicable for measuring enzymic reactions which involve the determination of ammonia.

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Technics for Separation of Plasma Cholesterol Esters for Determination of Iodine Value, and of Cholesterol.* (24197)

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To investigate the effects of ingestion of such preparations as ethyl linoleate and ethyl oleate upon fatty acid composition of the plasma cholesterol esters, new methodology was required. Such methods are here reported, as well as a modification of the Schoenheimer-Sperry technic for plasma cholesterol determination which substitutes relatively stable colorimetry for the Liebermann-Burchard reaction.

I. *Separation of cholesterol esters from other plasma lipids.* A number of adsorbents were tried in conjunction with various solvents or mixtures of solvents. Activated and non-activated silicic acid was used during the phase of standardization. In our hands, equal parts by weight of celite and "non-activated" silicic acid have produced the best separation. Many investigators using silicic acid for separation of the different lipid components advise certain mesh size and also "activation" of the silicic acid by heat or by using dehydrating solvents. Such procedures increase the efficiency of the column considerably where maximum efficiency is desired, but these additional steps are unnecessary and time-consuming when one is separating small quantities of lipids. Silicic acid, as received from the manufacturer,† contains about 22% water. The column consists of a 25 ml burette with self-lubricating teflon plug valve assembly,‡ and a 125 ml flask fused to the top of the burette as a reservoir. A glass wool plug is introduced, and the celite-silicic acid mixture is added, with tamping, to a height of 10 cm. An aliquot of the plasma filtrate (in acetone alcohol) containing approximately 20 to 30 mg of total lipid is evaporated just to dryness under partial vacuum at approximately 50°C, and the lipids dissolved immediately in a small amount of petroleum ether (5 ml). This is poured upon the *dry* column. (In a recent report, Hirsch also recommends addition of the dissolved lipids to the column in the dry state)(1). The test tube containing the lipids is washed 3 times with 2 ml portions of petroleum ether. These are added to the column and allowed to flow in without pressure until the liquid has been completely absorbed. The cholesterol esters are eluted with a total volume of 100 ml of petroleum ether under slight positive pressure to maintain a flow rate of approximately 1 ml per

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† J. T. Baker Chemical Co., Phillipsburg, N. J.

‡ "Ultramax", Fischer and Porter Co.