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INCREASED ARGINASE ACTIVITY DURING LYMPHOCYTE MITOGENESIS*

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SUMMARY: A sensitive assay for arginase activity was developed using [*guanidino*-^{14}C]arginine as substrate and measuring the production of $^{14}CO_2$ from [^{14}C]urea in the presence of urease. Arginase activity was measured in bovine lymphocytes after activation by Concanavalin A. The specific enzymatic activity of arginase doubled in 6 hours and increased nearly 4-fold by 24 hours after stimulation. It is suggested that the role of arginase in these cells is to provide ornithine as substrate for the synthesis of putrescine, precursor of the polyamines spermidine and spermine.

The biosynthesis of the naturally occurring polyamines begins with the decarboxylation of ornithine to yield putrescine, with subsequent conversion of putrescine to spermidine and spermine (reviewed in reference 1). Polyamine biosynthesis is enhanced in many animal systems by a variety of stimuli usually associated with cell growth and proliferation. The rate of polyamine biosynthesis appears to be regulated in general by the level of ornithine decarboxylase and, in some systems, S-adenosylmethionine decarboxylase (reviewed in reference 2). The question we posed in this work was whether the level of arginase, one route of ornithine synthesis, was increased under conditions leading to stimulation of polyamine biosynthesis. Arginase increased in lymphocytes activated by Concanavalin A over a time period consistent with its participation in providing ornithine as substrate for polyamine production. A similar observation has been made in hormonally stimulated mammary explants (3).

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EXPERIMENTAL PROCEDURES:

Cell-free extracts. Bovine lymphocytes were obtained from suprapharangeal lymph glands and cultured in the presence of Concanavalin A (18 μg/ml) as previously described (4). Unless otherwise stated, all subsequent procedures were performed at 0-4°C. Cells were harvested by centrifugation at 200 x g for 10 min, washed by resuspension in 0.9% NaCl and recentrifuged. Cells were broken with a Dounce homogenizer in sufficient buffer [10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 5 mM dithiothreitol] to give a final protein concentration of approximately 3-7 mg/ml. The homogenates were stored at -20°C for assay at a later time. No loss of activity was noted during storage.

Enzyme assays. The stored extracts were thawed, rehomogenized and centrifuged for 15 min at 27,000 x g. The extracts were then brought to 15 mM $MnCl_2$ by addition of the solid salt and activated by incubation for 10 min at 55°C (5). This resulted in approximately a doubling of arginase activity.

For routine assays of arginase activity, the reaction mixtures contained the following components in a total volume of 0.3 ml: 85 mM [*guanidino*-^{14}C]L-arginine (20,000 cpm/μmole; purified as described under *Materials*), 50 mM glycine-NaOH (pH 9.75), 0.5 mM $MnCl_2$, 0.75 mg urease (treated as described under *Materials*) and an appropriate amount of enzyme extract. After incubation at 37°C for the desired time, the reaction was stopped by addition of trichloroacetic acid to a final concentration of 7% and the liberated $^{14}CO_2$ was trapped on a piece of filter paper and counted as previously described (6,7). One unit of enzyme activity is defined as that amount catalyzing the production of 1 nmole of urea per min.

For estimation of ornithine production, reaction mixtures were incubated as described above with uniformly labeled [^{14}C]arginine (2.5 x 10^5 cpm/μmole; purified as described under *Materials*). After trapping the liberated $^{14}CO_2$, the remaining reaction mixture was mixed with 0.7 ml 0.116 M sodium citrate (pH 5.3). The sample was then applied to a column (0.5 x 6.3 cm) of AG 50W-X8 (H^+, 200-400 mesh) and eluted with the citrate buffer (8). The ornithine-containing fractions (1 ml each) were counted in 10 ml of a scintillation fluid consisting of Triton X-100/xylene (1:3) containing 2,5-diphenyloxazole (3 g/l).

Protein concentration was determined by the method of Lowry *et al.* (9) with crystalline bovine serum albumin as a standard.

Materials. [*Guanidino*-^{14}C]L-arginine (23 mCi/mmole) was obtained from New England Nuclear). This material contained sufficient [^{14}C]urea to provide intolerable backgrounds in the arginase assay and was therefore purified by the following procedure. The radioactive arginine was applied to a column (0.8 x 10.5 cm) of AG 50W-X8 (H^+, 50-100 mesh). The urea contaminant was eluted with water and discarded. Arginine was removed from the column with 2 N HCl. After taking to dryness on a rotary evaporator, the arginine was redissolved in 0.1 N HCl and stored at -20°C. It was then diluted with unlabeled L-arginine to the appropriate specific radioactivity prior to use.

The uniformly labeled [^{14}C]L-arginine used for assessing ornithine production (New England Nuclear, 300 mCi/mole) was contaminated with both [^{14}C]urea and [^{14}C]ornithine. Urea was removed as described above for [*guanidino*-^{14}C]arginine. For removal of ornithine, the urea-free sample was evaporated to dryness and resuspended in 0.116 M sodium citrate (pH 5.3). The sample was then applied to a column of AG 50W-X8 and eluted as described above for the detection of ornithine synthesis. The fractions containing ornithine were discarded and continued elution with the citrate buffer removed the arginine. The arginine-containing samples were acidified by adding 0.01 volume of concentrated HCl. These samples were then desalted by applying to a column (0.8 x 10.5 cm) of AG 50W-X8 (H^+, 50-100 mesh) and eluting sequentially with 8 ml 0.1 N HCl, 16 ml 1.5 N HCl and 12 ml 6.0 N HCl. The arginine-containing

Table I

Stoichiometry of Arginase Assay

The incubation was carried out with uniformly labeled [^{14}C] arginine for 90 min as described in Experimental Procedures. The quantities of CO_2 and ornithine produced were estimated by assuming their specific radioactivities to be 1/6 and 5/6 that of arginine, respectively. The lymphocyte extract was from unstimulated cells.

	Additions to Reaction		Product Formed[a]	
	Extract	Urease	CO_2	Ornithine
1.	-	+	0.00	0.00
2.	+	+	0.55	0.59
3.	+	-	0.05	0.55

[a] µmoles

fraction which eluted with 6 N HCl was evaporated to dryness and redissolved in 0.01 N HCl. This purified arginine was stored at -20°C and diluted with unlabeled L-arginine before use.

We found that jack bean urease (2X crystallized, Worthington) contained sufficient arginase to interfere with the sensitive assay described here. This contaminating activity was inactivated, with no inhibition of urease activity, by dissolving the urease in 10^{-3} M EDTA (12.5 mg urease/ml) and heating at 55°C for 10 minutes.

RESULTS:

Because of the low levels of arginase activity encountered in lymphocyte extracts, we were unable to obtain reproducible results with the usual colorimetric assays for urea production (5,10). We therefore utilized a radiochemical assay with [*guanidino*-^{14}C]arginine as substrate. The arginase activity in the lymphocyte extracts was coupled with urease to produce $^{14}CO_2$ from the urea liberated in the assay. Similar assays have been used previously with yeast and *Neurospora* (11,12). The stoichiometry of this reaction is illustrated in Table I. When the cell-free extract was omitted from the reaction mixture *(Experiment 1)*, neither CO_2 nor ornithine were produced, whereas stoichiometric quantities were liberated in the complete reaction mixture *(Experiment 2)*. Omitting urease from the reaction mixture *(Experiment 3)* had no effect on the arginase activity as measured by ornithine production, but abolished CO_2

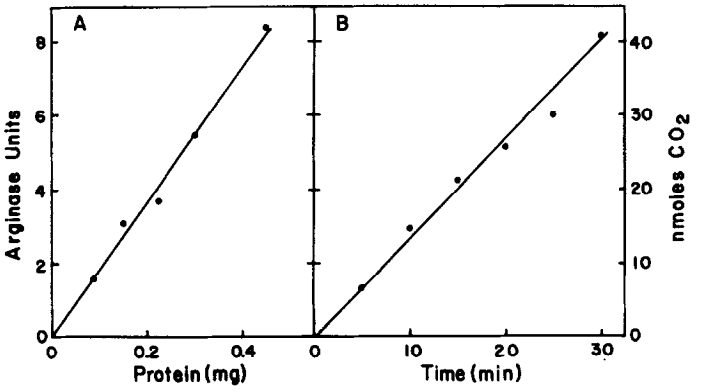

FIGURE 1. (A) Dependence of reaction rate on protein concentration. Arginase assays were performed as described in Experimental Procedures. The indicated amount of extract from cells exposed to Concanavalin A for 12 hrs was added to the 0.3-ml reaction mixtures. (B) Time course of the arginase assay. Reaction mixtures were incubated for the indicated times with an extract (1.1 mg per assay) of cells exposed for 48 hrs to Concanavalin A.

synthesis. Although this particular experiment was performed with unstimulated lymphocytes, similar results have been obtained with extracts from cells exposed to Concanavalin A for 24 hours. The reaction rate was linear with protein concentration to at least 0.45 mg added per 0.3 ml reaction mixture (Figure 1A). This type of a plot with at least 5 points was routinely performed to establish the specific enzymatic activity of a particular extract. The rate of $^{14}CO_2$ liberation in the reaction mixture was constant for at least 30 minutes (Figure 1B).

The arginase specific activity was estimated at various times after activation of lymphocytes by Concanavalin A (Table II). The activity rose nearly 4-fold in a 24 hour period, from the basal level of 10 units/mg in unstimulated lymphocytes. The activity had doubled by 6-12 hours after cellular activation.

DISCUSSION:

We have described here a sensitive arginase assay for use with those tissues containing extremely low levels of this enzyme. The arginase levels reported here are two orders of magnitude lower than in tissues specialized

Table II

Arginase Activity During Mitogenesis

Lymphocyte cultures were stimulated with Concanavalin A and assayed for arginase activity at various times as described in Experimental Procedures. At each time point the specific activity was determined by a plot of enzymatic activity versus protein as in Figure 1A.

Hours after Con A	Arginase Activity[a]
0	10
6	24
12	18
24	36
48	24

[a]units/mg protein

for urea synthesis. For example, a value of 4,000 units/mg protein (recalculated to our units) was reported for liver (13). On the other hand, these values for lymphocytes compare favorably with specific activities of 17-75 units/mg reported for mammary gland, a tissue which does not carry out the urea cycle (3). The level of arginase activity in lymphocytes is much higher than has been found for other enzymes involved in polyamine biosynthesis. Values in lymphocytes for ornithine decarboxylase and S-adenosylmethionine decarboxylase of 0.03-0.18 and 0.002-0.02 units/mg, respectively, have been reported (14-17). This might suggest that ornithine production would not be rate limiting for polyamine synthesis, although this extrapolation from optimal *in vitro* assay conditions to the *in vivo* situation is clearly tenuous.

Careful kinetic studies have been made of changes in the levels of polyamines and of their biosynthetic enzymes in lymphocytes undergoing mitogenesis (4,14-17). The levels of ornithine and S-adenosylmethionine decarboxylases began to increase about 3 hours after stimulation and were maximal at 10-20 hours. Putrescine began to accumulate by 8 hours after stimulation, followed by spermidine and spermine at 10-12 hours. Thus, the increase in arginase

occurs at the time of enhanced polyamine synthesis, consistent with a role for this enzyme in providing ornithine as a precursor of the polyamines. Oka and Perry have suggested a similar role for arginase during hormonal stimulation of the mammary gland (3).

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