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FLUOROMETRIC DETERMINATION OF ORNITHINE DECARBOXYLASE ACTIVITY FROM ANIMAL TISSUES

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Ornithine decarboxylase (ODC) limits the rate of synthesis of polyamines, whose level in animal tissues determines the rate of cell proliferation [13], and of hyperplasia and hypertrophy [7]. Of all known enzymes it has the shortest half-life (10-20 min), and has high lability in response to the action of hormones, drugs, and physicochemical factors [12]. These unique regulatory properties of ODC are responsible for the widespread use of this enzyme as a research tool in biology [7] and medicine, and in particular, in cardiology [8], pharmacology [12], neurology, and psychiatry [11], and in the study of fine mechanisms of regulation of metabolism. However, these investiations are hampered by the low level of ODC activity in normal tissues of intact animals and by the absence of a sensitive, yet technically convenient method of determination of the activity of this enzyme.

The aim of this investigation was to develop a highly sensitive and relatively easy method of determining ODC activity.

EXPERIMENTAL METHOD

Experiments were carried out on noninbred male albino rats weighing 120-180 g and on male C3HA mice, not exceeding 18-20 g in weight. The experimental tumors used included transplantable hepatoma 48, induced primarily in C3HA mice with orthoaminoazotoluene [2], and hepatoma G-27, induced primarily in noninbred albino rats with nitrosodiethylamine (NDEA) [6]. The liver affected with malignant disease, and the regenerating and normal liver also were investigated. Hepatocarcinogenesis was induced by NDEA. The carcinogen was injected intraperitoneally once a week for 2 months in a dose of 100 mg/kg body weight [1]. The total dose of NDEA received by each animal was 154 mg. Partial hepatectomy was performed by the method in [9].

The animals were decapitated and the liver quickly removed, perfused on ice with ice-cold distilled water, dried with filter paper, thoroughly freed from connective tissue, and weighed. Tumor tissue was taken in the logarithmic phase of growth, mainly from peripheral zones, and the possibility of necrotic tissue being present in the sample was excluded.

ODC does not withstand freezing, and for that reason the supernatant of a 33% freshly prepared homogenate (weight: volume = 1:2), obtained by centrifugation at 15,000 rpm for 20 min, was used as the source of the enzyme. The tissue was homogenized in 0.05 mM phosphate

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buffer (pH 7.2), containing 0.1 mM dithiothreitol (Serva, West Germany) and 0.4 mM Pyridoxal phosphate (Reanal, Hungary). All manipulations with the tissue were carried out at 2-4°C.

ODC activity was determined by a micromethod devised by the writers. The incubation mixture contained (in 0.4 ml final volume): 4 mmoles ornithine (from Reakhim, USSR), 20 μmoles pyridoxal phosphate (Reanal, Hungary), 5 μmoles dithiothreitol (Serva, West Germany), 5 mmoles K,Na-phosphate buffer, and 9.5 ± 0.7 mg protein (the source of enzyme). The sample was incubated on a constant temperature waterbath at 37°C for 1 h, after which 0.1 ml of 0.2 M $HClO_4$ was added. The residue was separated by centrifugation at 6000 rpm for 5 min.

Dansylation was carried out by addition of 0.3 ml of a solution of dansyl chloride (Schuchardt, West Germany) in acetone and 6 mg of $Na_3CO_3 \cdot 10H_2O$ to 0.1 ml of the supernatant. The samples were incubated for 18-20 h in darkness at room temperature. The reaction products were extracted with 0.5 ml benzene. The organic layer was evaporated in a conical test tube overnight at room temperature in darkness. The dry residue was dissolved in 20 μl benzene and applied to Silufol UV-254 plates (Kavalier, Czechoslovakia). One-way ascending chromatography was carried out twice in a benzene–triethanolamine system (ratio 5:1) for 45 min. Spots of dansylpolyamines were found by their yellow fluorescence in UV light. The putrescine fraction was identified with the aid of a reference substance, namely a preparation of a hydrochloride derivative of putrescine (Serva). The intensity of fluorescence was measured by direct scanning of the spot on a PROTVA-S automatic microscope-analyzer. The intensity of emitted light was measured at 488 nm. The excitation wavelength was 365 nm. A DRSh-250-2 lamp was used as the source of light. The concentration of putrescine was calculated by means of a calibration graph.

The unit of ODC activity was taken to be 1 catal. Specific activity of the enzyme was expressed by the number of picocatals per milligram protein. The protein concentration was determined by Lowry's method [10] in the writers' modification [4].

The experimental results were subjected to statistical analysis by Montsevichyute-Éringene's method, using a Mondenhauer factor [3]. Differences between mean values (X) were considered statistically significant at the $P \leq 0.05$ level.

EXPERIMENTAL RESULTS

The spectrophotometric method of ODC determination developed previously [5] proved to be more sensitive than known methods of determining the activity of this enzyme on the basis of CO_2 production or by ion-exchange chromatography of putrescine, and electrophoresis of orni-

TABLE 1. Estimation of Significance and Reproducibility of Results of Determination of ODC Activity from Normal Liver of Intact Animals

Metrologic characteristics	ODC activity, picocatals/mg protein	
	A	B
Sample mean ($\bar{X}$)		
Standard deviation of mean result ($\pm S_{\bar{X}}$)	0,12±0,003	0,18±0,003
Absolute reproducibility		
mean deviation from average ($\bar{d}$)	0,008	0,007
standard deviation (s)	0,009	0,009
Relative reproducibility, %		
relative mean deviation ($E_{\bar{X}}$)	6,7	5,6
relative standard deviation (S_r)	7,5	5,0

Legend. Statistical parameters calculated on the basis of results of 10 parallel determinations of ODC activity in preparations from mouse (A) and rat (B) liver.

TABLE 2. ODC Activity in Hepatomas G-27 and 48 and in Normal and Regenerating Liver Tissue from Intact Animals and Animals Receiving NDEA ($\bar{X} \pm S_x$)

Experimental conditions	Number of animals	ODC activity, picocatals/mg protein
Normal		
mice	9	0,14±0,033
rats	28	0,19±0,028
Mock operation (control)	6	0,13±0,05
Hepatoma G-27	12	1,5±0,22†
Hepatoma 48	8	0,39±0,09*
Regeneration (24 h after operation)	10	2,2±0,2‡
NDEA (after 5 months)	8	1,1±0,36*

Legend. *p 0.05, †P < 0.001 compared with normal, ‡P < 0.001 compared with control.

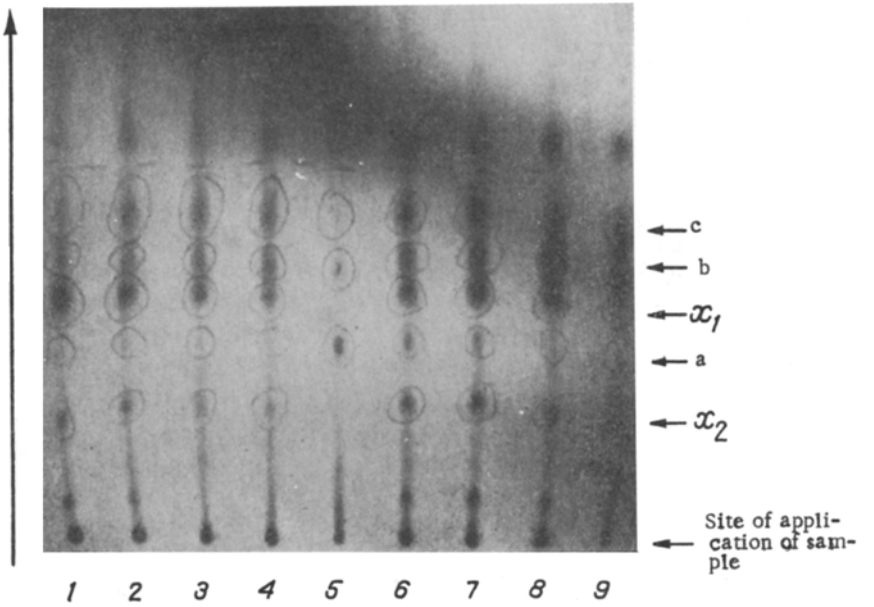

Fig. 1. Serial chromatogram of dansyl derivatives of polyamines and of putrescine. 1, 6) Incubation of ODC; 2, 7) control (ornithine added before dansylation reaction); 3, 4, 8, 9) samples controlling level of endogenous putrescine in tissue; 5) reference substances: a) putrescine, b) spermidine, c) spermine. x_1 and x_2) Fractions of unidentified amines. Vertical arrow shows direction of movement of solvent. Photographed in UV light at 360 nm.

thine-putrescine on paper. However, extraction of DNP-putr-scine was not sufficiently specific. The presence of DNP derivatives of other amino-containing compounds as impurities created a high background level of optical density of the samples. This reduced the sensitivity of the method. The suggested method is free from this disadvantage. The results given in Fig. 1 show that an individial pure fraction of dansyl-putrescine can be obtained by thin-layer chromatography. Dependence of the intensity of dansyl-putrescine on the quantity of putrescine in the sample was linear in character (Fig.1). The coefficient of instrumental sensitivity of the fluorometric method ($S_{f/c}$ = 3) was 1000 times higher than that of the spectrophotometric method ($S_{A/C}$ = 0.003). Calculation of the relative sensitivity of determination (C_{min}) showed that the suggested method (C_{min} = 0.3 nmole per sample) was 23 times more sensitive than the known method (C_{min} = 6.8 nmoles per sample). Since the error of determination is smaller if

the coefficient of instrumental sensitivity is greater, the accuracy of determination of ODC by the suggested method was higher than by the use of dinitrofluorobenzene [5].

The method also is distinguished by high reproducibility of the results of determination. Values of random errors of this method were estimated by calculating the basic metrologic characteristics d, w, s, E_x, and s_r from the results of 10 parallel determinations (Table 1). Specific activity of ODC from normal liver of an intact animal was close to that given in the literature [12]. The reliability, accuracy, and reproducibility of the results were all high. The standard error of the sample ($\pm S_x$) did not exceed 2.5%. The degree of scatter of the individual measurements around the mean was low, under 7.5%, i.e., this sample was homogeneous.

The method described above was tested in investigations of ODC activity in the liver of intact animals and in tissues with intensive cell proliferation. An increase in ODC activity was found in the liver in the presence of malignant disease, and also in the tissue of hepatoma G-27 and 48 (Table 2). These results are in good agreement with data in the literature showing a sharp increase in the velocity of ornithine decarboxylation in mouse leukemic tumor 1210 [14], in STAT-1 sarcoma, and in regenerating liver [15], obtained by a radiometric method. Data in the literature on increased ODC activity in response to the action of hormones and tissue growth stimulating factors [15], and also the analogous changes in ODC activity found in investigations of transplantable hepatomas, and of the malignant and regenerating liver, used as models of tissues with a raised mitotic index under normal and pathological conditions support the view that this enzyme participates in the complex process of regulation of cell proliferation.

The suggested method of determination of ODC activity in animal tissues is highly sensitive, reproducible, and reliable. It is relatively simple and available. It can therefore be recommended for widespread use in experimental practice in academic and clinical establishments.

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