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hyperammonemia lymphoid cell
leukocyte ornithine transcarbamylase

Ornithine Transcarbamylase (OTC) in White Blood Cells

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Summary

A radiochemical assay method of ornithine transcarbamylase (OTC) was developed using labeled carbamyl phosphate as a substrate. The enzyme activities determined by this method in peripheral white blood cells from ten normal subjects were 1.32 ± 0.95 nmoles/mg/hr and the apparent K_m's, when assayed at pH 8.5, were 6.4 mM for ornithine and 0.6 mM for carbamyl phosphate. On the contrary, the apparent K_m's of human liver OTC were 0.6 mM for ornithine and 0.12 mM for carbamyl phosphate. The average OTC activity of granulocytes was 1.0 nmoles/mg/hr, whereas that of mononuclear cells was 0.4 nmoles/mg/hr.

Lymphoid cell lines were established from three normal subjects and an OTC-deficient infant. All these cell lines demonstrated no OTC activity. When arginine was removed from the medium and replaced by ornithine, the lymphoid cells were unable to grow in culture. On autoradiography, the lymphoid cells showed labeling at incubation in the presence of ^{14}C-citrulline, but not with ^{14}C-ornithine.

Speculation

The wide range of OTC activities observed in white blood cells from normal subjects might be due to a difference of the enzyme activities between granulocytes and mononuclear cells and in turn due to an individual difference of the two cell population.

OTC is either absent or inactivated in lymphoid cell lines, when grown in a culture medium with arginine. The enzyme of the peripheral white blood cells might be of a different genetic origin from that of the liver.

The diagnosis of inherited hyperammonemia has been made by measuring urea cycle enzymes in the liver (3, 4, 10) and other organs. Wolfe and Gatfield (17) recently demonstrated the presence of ornithine transcarbamylase [EC 2.1.3.3] (OTC) as well as other urea cycle enzymes in peripheral white blood cells from normal subjects. OTC deficiency was diagnosed by using peripheral blood white cells from the patients with OTC deficiency (7, 17). On the other hand, Snodgrass *et al.* (15) recently reported that OTC deficiency in the liver can not be inferred from the measurements of the enzyme's activity in peripheral white blood cells because the latter parameter was normal in their patients.

All these studies were carried out using a colorimetric method that is designed to measure the amount of citrulline produced in the assay system. The colorimetric method has several disadvantages: (1) the color complex formed is relatively light sensitive; (2) diacetylmonoxime reacts with carbamide compounds other than citrulline which give a color reaction; (3) the narrow range of optical density, which is from 0.01 to 0.03 according to Wolfe and Gatfield (17), is another problem in measuring OTC activity in white blood cells.

The use of radiochemical assay method as suggested by Wolfe and Gatfield (17), appears to be preferable in view of the fact that the OTC activity is low in the peripheral white blood cells and that the sensitivity of the radiochemical method is higher than that of the colorimetric method.

We would like to describe a radiochemical assay method using ^{14}C-carbamyl phosphate as a substrate and to discuss the presence of OTC activities in peripheral blood white cells and cultured lymphoid cells.

MATERIALS

L-Ornithine hydrochloride and carbamyl phosphate (dilithium salt) used were from Sigma Chemical Co., St. Louis, MO. ^{14}C-carbamyl phosphate (dilithium salt), ^{14}C-citrulline, and [^{14}C]-ornithine were purchased from New England Nuclear, Boston, MA. All the other chemicals used were of reagent quality. It was found in a pilot study that recrystallization of ^{14}C-carbamyl phosphate is unnecessary. OTC from bovine liver, partially purified by the method of Marshall and Cohen (9), was kindly supplied by Professor Tachibana.

METHOD

PREPARATION OF WHITE BLOOD CELL

White blood cells were prepared by the modified method of Wolfe and Gatfield (17). Five volumes of freshly drawn blood were quickly mixed with one volume of solution containing 5% dextran, 0.7% NaCl solution, and 0.05% heparin. After standing for 45 min at room temperature, the supernatant white blood cell-rich layer was removed and centrifuged at 600 × *g* at 4°C. The pellets were washed with ice-cold isotonic saline. Residual red cells were removed by gently mixing the pellets with 5 ml of distilled water for 60 sec. Five ml of 1.7% NaCl solution was added, and the pellets were recovered by centrifugation at 4000 × *g* at 3° C.

Separation of mononuclear cells from the granulocytes was accomplished by differential flotation using a modified method of Boÿum (1).

ESTABLISHMENT OF LYMPHOID CELL LINE

Long-term lymphoid cell lines were established from normal subjects (N = 3) and an OTC-deficient infant after incubation with Epstein-Barr (EB) virus as described previously (11). The infant with OTC deficiency was diagnosed by measuring the enzyme activity in biopsy specimen of the liver.

MEASUREMENT OF OTC ACTIVITY

Cell pellets were suspended in 0.1% cetylpyridinium chloride, frozen and thawed three times, and centrifuged at 12,000 × *g* for 25 min. The clear supernatants were used in radiochemical assay. The radiochemical assay method used in study was a modification of the method (14) of Goldstein *et al.* (3), which measured the

conversion rate of ^{14}C-carbamyl phosphate. One hundred μl of the supernatant ^{14}C-carbamyl phosphate (final concentration, 0.87 mM; specific activity, 1.31×10^3 cpm/nmole), and ornithine (final concentration, 5 mM) were mixed, and 0.05 M triethanolamine acetic acid buffer, pH 8.5, was added to make a total volume of 2 ml. The mixture was incubated at 37°C for 60 min unless otherwise stated. The reaction was then terminated by adding 100 μl of 3 N formic acid. After heating for 5 min in a boiling water bath, the excess $^{14}CO_2$ was removed by adding crushed dry ice. After centrifugation for 10 min at $2000 \times g$, the supernatants were transferred to counting vials and evaporated to dryness under air suction, and 0.3 ml of water and 5 ml of Aquasol-2 (New England Nuclear) were added. ^{14}C-Carbamyl phosphate incorporation was counted by Beckman liquid scintillation spectrometer. Boiled cell lysate was used as control blank material.

Apparent K_m's of OTC for ornithine and for carbamyl phosphate were obtained from Lineweaver-Burk plots.

Partially purified bovine liver OTC and human liver specimen obtained by surgical operation were used in a reference study. The enzyme activity was measured: (1) by a colorimetric method of Brown and Cohen (2); and (2) by a radiochemical assay. For the radiochemical assay, bovine OTC or crude homogenate of human liver (2) was diluted with distilled water and processed in the same manner as the cell lysates.

Protein was measured by the method of Lowry *et al.* (8).

GROWTH CURVE OF LYMPHOID CELLS

Lymphoid cells established from normal controls and the patient with OTC deficiency were cultured in two different media: (1) Roswell Park Memorial Institute medium 1640 (RPMI 1640) with 1 mM arginine (ornithine−, arginine+); and (2) RPMI 1640 with 1 or 5 mM ornithine substituted for arginine (ornithine+, arginine−). Total numbers of the cells were counted in a hemocytometer, and their viability was determined by trypan blue dye exclusion.

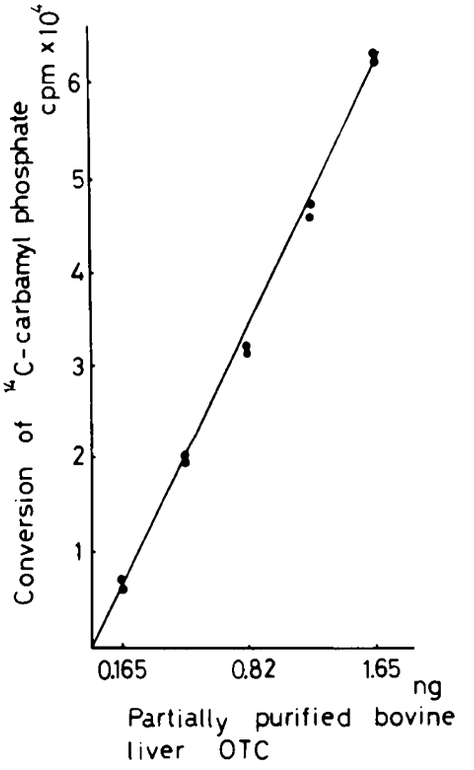

Fig. 1. Relationship of protein concentration of partially purified bovine liver OTC to the conversion of ^{14}C-labeled carbamyl phosphate. Reaction mixture was incubation for 10 min.

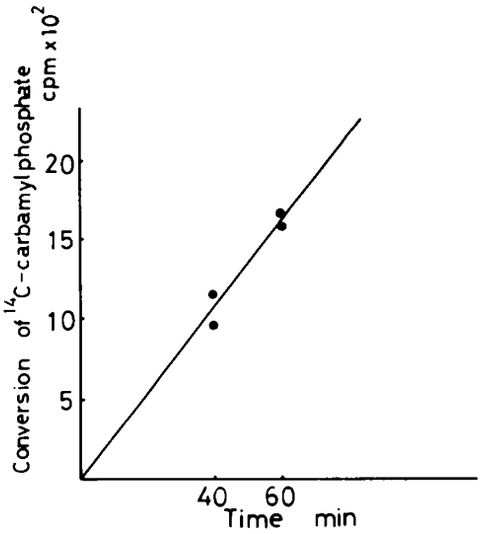

Fig. 2. Time course study of OTC activity in leukocyte lysate.

AUTORADIOGRAPHY

Logarithmically growing cells, each 5×10^5 in number from the OTC-deficient infant and control subjects were washed with arginine-free RPMI 1640 and incubated for 48 hr at 37°C in RPMI 1640, which contained ornithine [1 mM of cold ornithine and ^{14}C-ornithine (1 μCi/ml)] or citrulline [1 mM of cold citrulline and ^{14}C-citrulline (1 μCi/ml)] instead of arginine. After the incubation, cells were washed three times with Hanks' balanced salt solution and centrifuged at 1000 rpm. They were resuspended in fetal calf serum, placed on glass slides, and fixed with methanol. Slides were dipped in Kodak NTB3 emulsion, exposed for 5 days, developed in D-19 for 3 min, and stained with Giemsa.

RESULTS

TIME COURSE STUDY AND REACTION KINETICS OF PARTIALLY PURIFIED BOVINE LIVER OTC

Colorimetry. Linearity was found between the incubation time and citrulline production rates and also between citrulline production rates and enzyme protein levels. The range of detectable activity by this method was from 75 to 300 ng of enzyme protein.

Radiochemical Assay. Time course study was performed up to 40 min of incubation, and a positive linear relationship between the incubation time and the conversion rate of radioactive substrate was found. The relationship between the conversion rate of ^{14}C-carbamyl phosphate and enzyme protein, measured after 20 min incubation, was also linear. The range of detectable activity by this method was from 0.16 to 1.6 ng (Fig. 1). The mean enzyme activity obtained by the colorimetric method was approximately 13% higher than that obtained by the radiochemical assay.

APPARENT K_m OF HUMAN LIVER OTC

Radiochemical assay was used in measurement of human liver OTC and obtained results are listed in Table 2.

TIME COURSE STUDY AND REACTION KINETICS OF OTC OF HUMAN PERIPHERAL WHITE BLOOD CELLS

Significant radioactivity could not be obtained at 20 min incubation due to scanty content of the enzyme in white blood cells. OTC activity was, therefore, determined at 40 and 60 min incubation. Longer incubation was not feasible because carbamyl phosphate is unstable in solution. Using (unfortunately) only two time points and extrapolating to zero time, a positive linear relationship was found between incubation time and conversion of ^{14}C-carbamyl phosphate (Fig. 2). The relationship between the conversion rate of radioactive substrate and the amount of cell lysate, measured after 60 min incubation, was linear (Fig. 3).

Results obtained and apparent K_m of OTC for ornithine and carbamyl phosphate are shown in Tables 1 and 2. OTC activity of granulocytes appeared to be approximately two times higher than that of mononuclear cells.

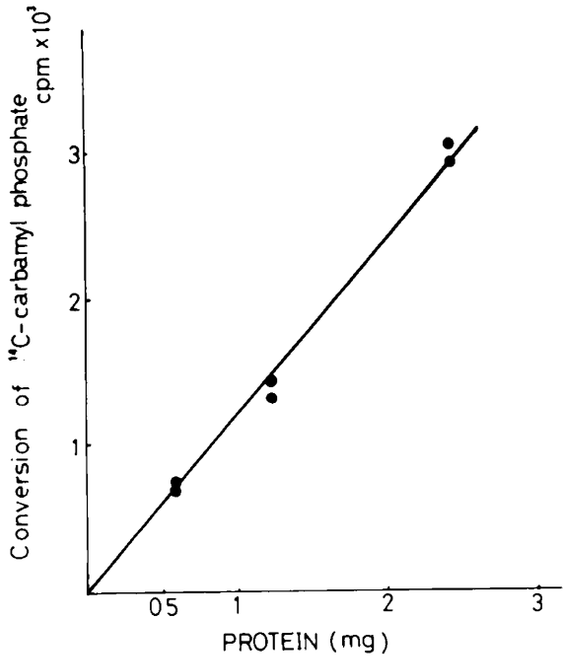

Fig. 3. Relationship of protein concentration of leukocyte lysate protein to OTC activity. Reaction mixture was incubated for 60 min.

Table 1. *Ornithine transcarbamylase in white blood cells and cultured lymphoid cells*

	Mean ± S.D. (nmoles/mg protein/hr)	Reference
White blood cells (N = 10)	1.32 ± 0.95	Present study
Granulocytes (N = 2)	1.0	
Mononuclear cells (N = 2)	0.4	
Cultured lymphoid cells (N = 4)[1]	0	
White blood cells (N = 7)	128 ± 67	Wolfe *et al.* (17)
White blood cells (N = 14)	63 ± 93 (2–345)	Snodgrass *et al.* (15)

[1] Including a sample derived from an OTC-deficient patient.

It was not possible to demonstrate by repeated measurements the enzyme activity in lymphoid cell lines derived from both the controls and the OTC-deficient patient.

GROWTH CURVES

Lymphoid cells from both the controls and the OTC-deficient infant showed normal logarithmic growths in the arginine (+) ornithine (−) medium, whereas the cells failed to grow in the arginine (−) ornithine (+) medium (Fig. 4), even when incubated for 40 days while changing medium every 3 days.

AUTORADIOGRAPHY

When incubated in the presence of ^{14}C-ornithine, few grains were found on cultured lymphoid cells from both the normal subjects and the OTC-deficient patient. On the other hand, when ^{14}C-citrulline was substituted for ^{14}C-ornithine, more than 80% of cultured lymphoid cells contained more than 20 grains per cell.

DISCUSSION

Although the results are in conflict with that by Rabier *et al.* (13) who could not find the activity of OTC in white blood cells, our study clearly demonstrated the presence of OTC in white blood cells, as found by Wolf and Gatfield (17) and Snodgrass *et al.* (15). The enzyme levels obtained by our study were lower than those in the other studies. The discrepancy might be attributable to the difference of the methods used or, alternatively, to the low substrate concentration in our assay system.

Apparent K_m's of white blood cells were measured at pH 8.5, which was reported to be optimum for the K_m measurement of OTC (4). At this pH, K_m's of OTC for carbamyl phosphate and for ornithine are approximately 5 and 10 times, respectively, higher in the white blood cells than those in liver. We could not afford to carry out a study to compare the OTC levels in the blood leukocytes and liver of the patient with OTC deficiency because the amount of blood sample available from the patient with OTC deficiency was limited. (For duplicate assay, at least 30 ml of whole blood is necessary, and the patient is below 2 years of age). Snodgrass *et al.* (15), based on their findings, suspected that OTC in the peripheral white blood cells is different from that in the liver. This hypothesis is supported by our finding that K_m's for the enzyme in the white blood cells are not identical with those in the liver.

Our study demonstrated that the OTC activity in the granulocytes is higher than that in the mononuclear cells. Wide variations of OTC activities in the white blood cells, observed both in our study (Fig. 1) and those by Wolfe and Gatfield (17) and Snodgrass *et al.* (15), may be related to individual differences in the ratio of the two cell population.

Cultured lymphoid cell lines have increasingly been used in the study of the inborn errors of metabolism. With this in mind, the lymphoid cells derived from both the normal subjects and from the OTC-deficient patient were studied for OTC activity. How-

Table 2. *K_m's of ornithine transcarbamylase in human white blood cells and human liver*

	K_m for ornithine (mM)	K_m for carbamyl phosphate (mM)	Reference
White blood cells *[1]1[2]	6.7, 6.2 (pH 8.5)***[3]	0.6 (ph 8.5)***	Present study
Liver *1	0.6 (pH 8.5)***	0.12 (ph 8.5)***	Present study
Liver *2	0.2 (pH 8.5)	0.2 (ph 8.5)	Heiden *et al.* (4)
Liver *2	0.8 (ph 8.0)	0.6 (ph 8.0)	Katsunuma *et al.* (6)
Liver **[4]2	0.2 (ph 8.0)	0.09 (ph 8.0)	Pierson *et al.* (12)

[1] *, crude homogenate;

[2] 1, radiochemical procedure; 2, colorimetric procedure.

[3] ***, specimens from two persons.

[4] **, purified sample.

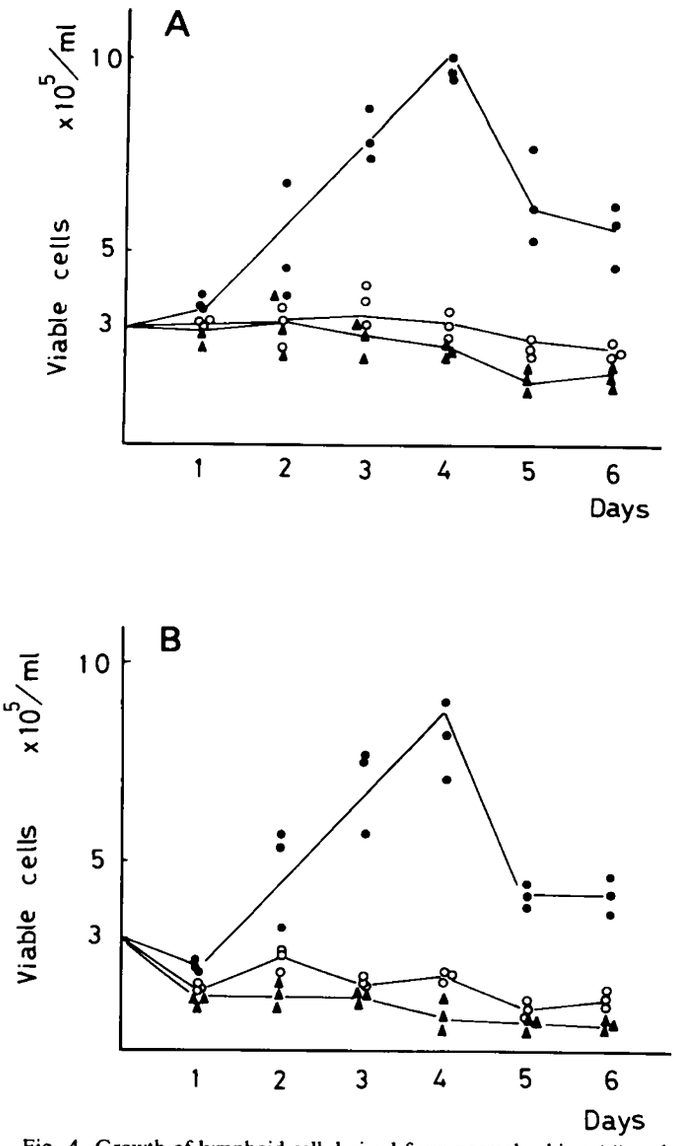

Fig. 4. Growth of lymphoid cell derived from normal subject (*A*) and an OTC-deficient infant (*B*) in RPMI 1640 (●—●) arginine (+), ornithine (−), or in medium with ornithine [1 mM (○) 5 mM (△)] substituted for the normally used arginine arginine (−), ornithine (+). Inoculations of 0.3×10^6 cells/ml were made into each of the three type of medium, and cells were incubated without medium changes.

ever, no detectable activity of the enzyme was noted in any of the lymphoid cell lines studied.

The lymphoid cells grew logarithmically in the ornithine (−) arginine (+) medium, but did not grow in the ornithine (+) arginine (−) medium. The finding was confirmed by autoradiography, which showed no grains after incubating the lymphoid cells in the arginine (−) medium with ^{14}C-ornithine, but massive grains after incubation in the arginine (−) medium with ^{14}C-citrulline.

The results suggest that conversion of ornithine to citrulline is impaired, whereas that of citrulline to arginine is normal (16). The observations are compatible with those of Jacoby (5) who demonstrated that cultured human lymphoid cells do not grow in the ornithine (+) citrulline (−) medium. To explain the phenomenon, two possibilities may be considered: (1) OTC is missing in the EB virus-reactive B-lymphocytes, but it is lost or inactivated during the process of establishing the lymphoid cell lines. In view of the known adaptive ability of human lymphoblasts in culture (5, 16), the latter possibility is more likely, whereas the mechanism involved remains to be elucidated.

Reinstitution of OTC activity in established lymphoid cell lines, if it becomes possible, may pave the way to genetic therapy of OTC deficiency.

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