ENZYMES OF THE ORNITHINE-UREA CYCLE IN LYMPHOCYTES IN LONG-TERM CULTURE

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This is to certify that the

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Enzymes of the Ornithine - Urea Cycle in Lymphocytes in Long-Term Culture

presented by

Lou Betty Bood

has been accepted towards fulfillment
of the requirements for

PhD degree in Zoology

James Higgins
Major professor

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ABSTRACT

ENZYMES OF THE ORNITHINE-UREA CYCLE IN LYMPHOCYTES IN LONG-TERM CULTURE

By

Lou Betty Rood

The present study was undertaken to determine which of the enzymes of the ornithine-urea cycle occur in lymphocytes in long-term culture. Lymphocytes in logarithmic growth phase were used for the enzymatic determinations. Enzyme activity levels were established for ornithine carbamoyl transferase, argininosuccinic acid lyase and arginase. Specific activity for ornithine carbamoyl transferase was .41 μmoles citrulline per milligram protein per hour. Specific activity for argininosuccinic acid lyase was 5.25 mμ moles urea per milligram protein per hour. Arginase specific activity was 23 mμ moles urea per milligram protein per hour. Michaelis-Menten constants were established for the substrates of ornithine carbamoyl transferase and arginase. For ornithine carbamoyl transferase the ornithine Km was between 15 and 17.5 mM; the

carbamoyl phosphate Km was 1.5 to 2.2 mM; the Km for arginase was between 2.0 and 2.2 mM.

The kinetic studies of ornithine carbamoyl transferase, using ornithine as substrate, and arginase suggest that lymphocytes contain distinct isozymes when compared with rat liver. Lymphocytes in long-term cultures are initiated by Epstein-Barr virus. The possibility is discussed that the isozymes described are products of viral rather than lymphocytic DNA.

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Lou Betty Rood

A DISSERTATION

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INTRODUCTION

A new technique has been established for obtaining apparently permanent cell lines of lymphocytes (Choi and Bloom, 1971). This new development is important as a tool for studying metabolic errors in human beings. This investigation was undertaken to determine which of the enzymes of the urea cycle could be demonstrated in cultured lymphocytes. Argininosuccinic acid synthetase and argininosuccinic acid lyase are widely found in cultured tissue (Eagle, 1959; Schimke, 1963). From analogy, Spector and Bloom (1973) have shown indirectly that the two enzymes known to be in cultured fibroblasts are present in cultured lymphocytes. Using labelled citrulline, an amino acid not used in protein synthesis, as a substitute for arginine in culture medium, they showed that lymphocytes of normal people, but not those of a patient with citrullinemia (argininosuccinic acid synthetase deficiency), could incorporate the label into the trichloroacetic acid precipitable fraction. They also reported that Kennaway, using

radio-chemical methods, has shown activity of that enzyme in lymphocytes from normal people, but not in those from the citrullinemic patient. Arginase activity had been demonstrated in HeLa strains and in a mouse fibroblast strain (Schimke, 1963) as well as in red blood cells (Tomlinson and Westall, 1964) and freshly drawn "leukocytes" (Reynolds et al., 1957). This last activity was highly variable, from 0-270 mg urea nitrogen liberated per 10^{10} leukocytes.

Ornithine transcarbamylase activity has been reported in significant amounts only in liver and small intestine (Reichard, 1960). It does not occur in cultured fibroblasts (Schimke, 1963). It is often measured in serum, where it is directly proportional to the amount of liver degeneration occurring, and it is used as an estimate of the extent of liver damage.

This study attempts to show that arginase and ornithine transcarbamylase activity are present in lymphocytes in long-term culture.

LITERATURE REVIEW

Ammonia Disposal

Of the three major caloric components of animal diets, fats and carbohydrates can be completely oxidized to carbon dioxide and water, but proteins provide an additional metabolite, ammonia, which may require additional enzymes for its disposal. Many water dwellers can excrete ammonia through the gills or skin directly into the environment, but the development of impermeable skins and exploitation of water-poor environments has been made possible by two alternate methods of ammonia disposal. Urea, which is nontoxic and quite water soluble, is made by mammals and some reptiles. Uric acid, which is very insoluble, and therefore nontoxic even at saturating concentrations, is formed by birds and by many reptiles to dispose of ammonia. Some reptiles make use of both of these methods (Mora et al., 1965).

As the reptilian ancestors of mammals and birds were probably both ureotelic and uricotelic, it is not

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surprising that some of the enzymes from each cycle are active in most animals. In mammals, for example, purine and pyrimidine production is dependent upon some of the same enzymes as those found in the uric acid pathway, although other enzymes of the pathway are missing. It has also been reported that the first four enzymes active in urea biosynthesis (but not arginase) increase and decrease concordantly on a specific basis, perhaps suggesting a common control mechanism for these enzymes (Mora et al., 1965).

In humans the urea cycle normally protects individuals from ammonia intoxication. Because of the occurrence of inborn errors of metabolism of the urea cycle, the enzyme activities of the cycle are of interest in human genetics.

Urea Synthesis

Urea synthesis (Figure 1) involves the attachment of two ammonia groups to a carbon atom through a series of five steps (Krebs and Henseleit, 1932; Ratner and Pappas, 1949). Both ammonia molecules are derived from ammonia

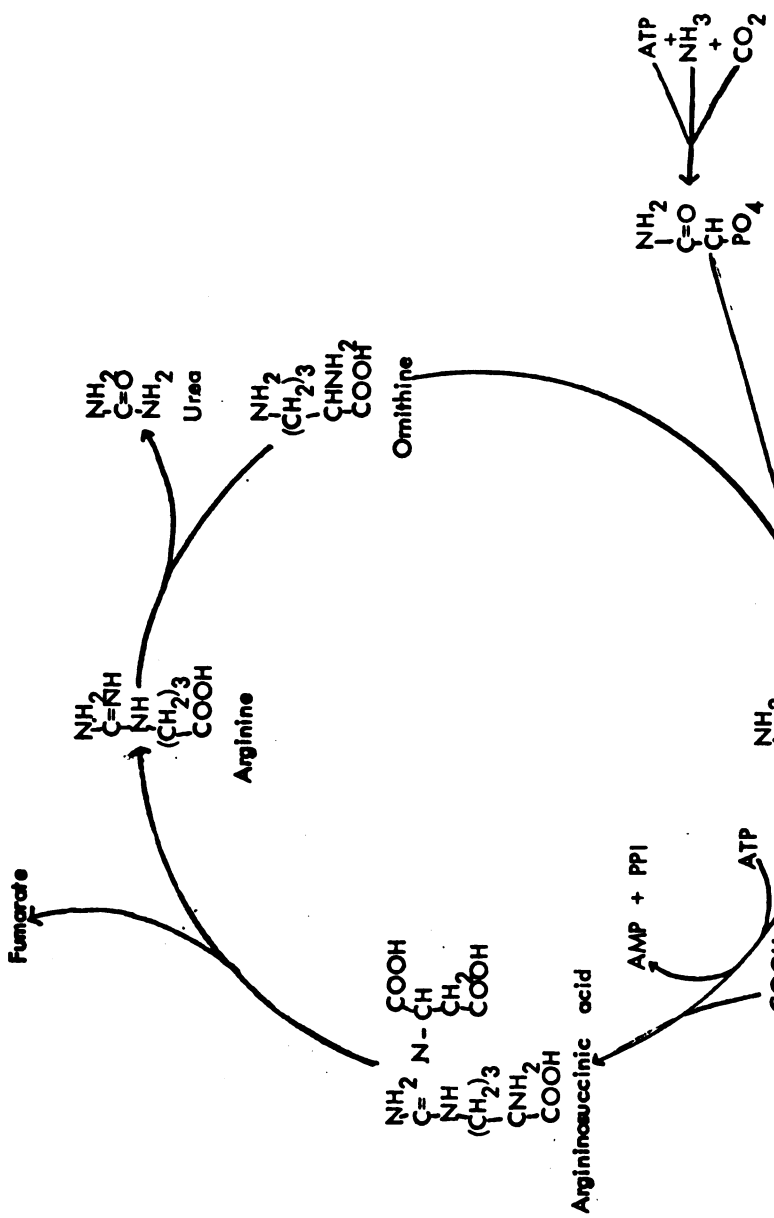


Figure 1. The Krebs-Henseleit ornithine-urea cycle.

residues of amino acids. One is attached to the carbon atom of CO_2 upon incorporation into carbamoyl phosphate, while the other is derived from the α-amino group of aspartic acid during a two-step process (Ratner and Pappas, 1949). The manufacture of carbamoyl phosphate from free NH_3, ATP, and CO_2 is considered to be the first step in urea synthesis. The next four steps form a cycle, using an ornithine moiety as a backbone to which are attached various other moieties that are subsequently altered and split off, finally resulting in the release of urea and the return of ornithine to its original state.

The first stage of the cyclic part of urea synthesis is the condensation of carbamoyl phosphate with ornithine to produce citrulline and Pi. Next aspartic acid condenses with the citrulline to form argininosuccinic acid with the cleavage of ATP to AMP and PPi providing the energy. Argininosuccinic acid (ASA) is then cleaved, producing arginine and fumarate, which is a metabolite of the Krebs carboxylic acid cycle. Arginine may be cleaved to form ornithine and urea or may be used as a building block for protein synthesis.

Because of interconnections with other cycles and pathways in the cell, especially the Krebs TCA cycle, the fumarate released by the cleavage enzyme may be reconstituted as either ornithine or aspartic acid (Figure 2).

Urea Cycle Errors

It is conceivable that errors in any one of the several pathways that connect with the urea cycle may have effects upon an organism's ability to regulate its ammonia disposal.

The inborn errors of metabolism that are apparently NOT connected with the urea cycle but that are associated with hyperammonemia, are hyperlysinemia (Colombo et al., 1967), low absorption of basic amino acids (Perkeentupa and Visakorpi, 1965), hyperornithinemia and homocitrullinuria (Shih et al., 1969), methylmalonic acidemia (Shih and Efron, 1972), and hyperglycinemia associated with an isoleucine metabolic defect (Keating et al., 1972). At present there are no known interrelationships among these diseases except for the associated hyperammonemia.

Abbreviations used

α-KG = α-ketoglutarate
ARG = arginine
ASA = argininosuccinate
ASP = aspartate
CIT = citrulline
CP= carbamoyl phosphate
CO_2= carbon dioxide
ATP = adenosine triphosphate
GLU = glutamate
GSA = glutamic semialdehyde
FUM = fumarate
MAL = malate
NH_2 = ammonia
ORN = ornithine
OAA = oxaloacetate
SUC = succinate

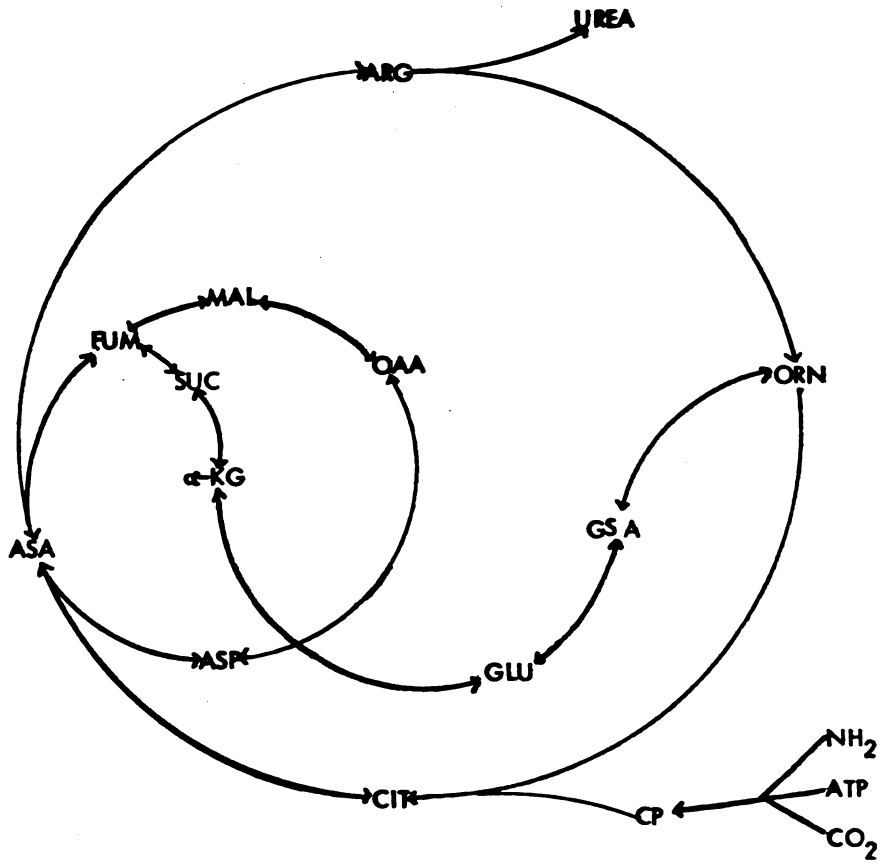


Fig. 2. Interrelationship of the ornithine-urea and the tricarboxylic acid cycles.

There are many other conditions that affect blood ammonia levels, liver failure being the major clinical cause of hyperammonemia. Among the less commonly seen conditions are several rare metabolic disorders which result in hyperammonemia. Five of these conditions would be directly predictable from the urea cycle as it is now understood (Figure 1).

In the following review, the established international nomenclature and numerical designation are used to identify each enzyme, followed by the few-letter abbreviation that shall be used (Levin, 1971).

Carbamate kinase EC 2.7.2.2 (CPS)

The formation of carbamoyl phosphate from ammonia, bicarbonate, and ATP is the first step in urea synthesis. Carbamoyl phosphate synthetase (carbamate kinase) is the mediating enzyme. A defect in its function causes the predictable hyperammonemia, and also is related to unusual amino acid levels in blood or urine. In one case of partial CPS deficiency, there was increased excretion of ornithine and proline (Kirkman and Kiesel, 1969). In

another case, in which the primary error was very low OCT activity accompanied by 20% CPS activity, the excretion of glutamine was greatly elevated (Levin and Russell, 1967). CPS deficiency is very rare, only two primary cases having been reported in addition to the one immediately above (Freeman et al., 1964; Hommes et al., 1969; Kirkman and Kiesel, 1969).

Ornithine carbamoyl transferase EC 2.1.3.3 (OCT)

Ornithine transcarbamylase mediated condensation of carbamoyl phosphate with ornithine makes citrulline. Its deficiency is the second most commonly reported defect for the urea cycle, some twenty cases having been reported to date (Russell et al., 1962; Levin and Russell, 1967; Corbeel et al., 1968; Hopkins et al., 1969; Levin et al., 1969a; Schneider et al., 1970; Campbell et al., 1971; Matusuda et al., 1971; Sunshine et al., 1972; Campbell et al., 1973; Short et al., 1973). Of the few males described, only two, with unusually mild forms of the disease, have survived early infancy (Levin et al., 1969b;

MacLeod et al., 1972). The age of onset and the severity of symptoms varies widely among females, ranging from habitual protein avoidance, to severe episodes of lethargy, seizures, retarded mental and physical development, and early death.

Argininosuccinic acid synthetase EC 6.3.4.5 (ASAS)

ASAS is the enzyme responsible for the condensation of aspartate with citrulline to make argininosuccinic acid, releasing pyrophosphate. Lack of the enzyme leads to citrullinemia, as well as to the expected hyperammonemia with high protein dietary stress (Scott-Emuakpor et al., 1972; McMurray, 1963). Two of three surviving cases are associated with severe mental retardation. The one reported adult is mildly retarded. Three of the four cases of this disorder had normal blood urea nitrogen and normal urea output. One case, which was fatal in infancy, showed no activity of the enzyme in the liver but some low level activity of ASAS in the kidney (Vidailhet et al., 1971). One could hypothesize either an isozymal system or

organ-specific control mechanisms to account for this observation. Morrow et al. (1967) reported a patient in whom urea production was low; Tedesco and Mellman (1967) showed that the citrulline Km of the enzyme in fibroblasts from that patient was many times normal, meaning that the affinity of the enzyme for citrulline was very low. One possible case of citrullinemia was reported to be associated with cystinuria, but the patient died before further studies could be initiated (Visakorpi, 1962).

Argininosuccinate lyase EC 4.3.2.1 (ASase)

Immediately upon the manufacture of argininosuccinate, the splitting enzyme, ASase, catalyses the reaction of ASA to arginine + fumarate. The enzyme catalyzing this reversible step, hypothesized by Ratner and Pappas in 1949, was proven to be correct by the first description of a person affected with ASase deficiency in 1958 (Allan et al., 1958). The patient showed massive excretion of ASA in the urine and elevations of ASA in serum. This is the most commonly reported error of the urea cycle, with twenty-three

known cases as of 1972 (Shih and Efron, 1972). The effects of the disease are highly variable. Some of the children are severely retarded; some have close to normal intelligence. The symptoms often include friable, patchy hair. The severity of effect may be unrelated to amount of deficiency. One child, diagnosed in early infancy, completely lacked ASase activity in cultured fibroblasts, but was apparently normal mentally after two years on a low protein diet (Shih, 1972). Another patient with very low, but measurable, activity died at six days of age (Kint and Carton, 1968).

Arginase EC 3.5.3.1

The final enzyme in the series, arginase, has been reported to be deficient in only two families (Peralta-Serrano, 1965; Terheggen et al., 1969). Terheggen's family was the product of a consanguineous mating, and included two female patients with very low or absent levels of arginase activity in their red blood cells, the patients' two sisters and the parents with low levels of arginase, and one sister with normal levels.

Inheritance

The genes for ASase deficiency and arginase deficiency would seem to be autosomal recessive because of sibling involvement and reduced enzyme activity in both parents when studied. There are too few cases to judge CPS deficiency inheritance. Although there are no reported sibships involved in ASAS deficiency, the fact that both males and females have been reported with this disease would lead one to speculate that this disease is caused by an autosomal recessive gene. OCT deficiency may be caused by a sex-linked gene (Short et al., 1973). Several families were described with this defect. In one of the families, a partial defect was transmitted from females to females with apparently no affected males. In another family, three male infants of a woman with a partial defect died shortly after birth with a total deficiency of OCT.

Lyon (1961) has proposed that in females, one or the other of the X chromosomes in each cell becomes inactive in early embryogenesis. All descendants of each cell will have the same active X and the same inactive one as the original cell. The descendants of an embryonic liver cell in which the X bearing a defective gene for

OCT was active would form a clone of cells deficient for OCT. As the original inactivation process is random and early in development, one would expect a wide range of effect on the carriers of one normal and one defective gene for OCT. The first reported family with this defect included a set of identical female twins and their female cousin. The mothers of the patients were sisters. The mothers appeared unaffected. One twin died in childhood, the other had no symptoms until the age of nine (Russell et al., 1962; Levin and Russell, 1967). Lyonization offers an explanation for the wide variability.

Study Methods

Among the methods of studying the genetic and metabolic bases for variation in humans are: 1) observing anomalous findings in the body fluids, and speculating from the biochemical literature the metabolic pathways which might be involved; 2) enzyme analysis of biopsy and autopsy materials; 3) stressing the supposedly defective pathway by loading the patient with a substrate of the suspect enzyme; 4) administering substrates labelled with

radioisotopes to patients with metabolic errors to check for rates and products of degradation; and 5) performing the enzyme analysis, stressing the suspect pathways, and using radioisotopes on human tissues grown in culture.

The most common type of tissue culture is that of fibroblasts; however, the usefulness in errors of the urea cycle is limited by the fact that one of the enzymes is missing in the fibroblast. ASAS and ASase are present in these cells, as is arginase, but OCT is not.

Recently, Choi and Bloom (1970) described a technique for maintaining human lymphocytes in apparently permanent culture. At the time of the initiation of the present research the enzymes of the urea cycle had not been thoroughly studied in lymphocytes.

Many genetic diseases may be diagnosed by enzyme analysis on freshly drawn lymphocytes (Hsia, 1972), and others on lymphocytes that have been induced to divide in temporary culture (Nadler and Egan, 1970; Hirschhorn et al., 1969). The technique of Choi and Bloom (1970) has provided a way to gain a population of white blood cells with an apparently infinite life span in culture. As fibroblasts have a finite number of divisions in culture,

lymphocytes potentially overcome the limitations of time that hamper the analysis of fibroblast cultures.

Enzymes of the Urea Cycle in Lymphocytes

Spector and Bloom (1973) have shown indirectly that ASAS and ASase are probably present in normal lymphocytoblastoid cells in culture. They found that normal cells would grow with citrulline as an arginine source while those from a patient with citrullinemia would not substitute citrulline for arginine.

Arginase has been reported to be present in freshly drawn white blood cells, but the activity varied so extensively that it has not been utilized as a technique for measuring arginase activities (0-270 mg urea nitrogen liberated per 10^{10} leukocytes) (Reynolds et al., 1957).

Ornithine carbamoyl transferase has not been reported to be present in white cells. Its presence in serum is related to the degeneration of liver cells, and serum OCT levels are used in testing for liver failure. Up to the present time, all studies on people heterozygous for OCT deficiency have been done by liver biopsy.

Tests for urea cycle enzymes would be useful in establishing whether the enzymes are active in cultured lymphocytes, and would provide an alternate system with which to study inborn errors of the urea cycle.

The discovery of useful colorimetric tests for such assays would put diagnostic procedures within the reach of most laboratories.

MATERIALS AND METHODS

Liver Controls

Adult Sprague-Dawley rats were maintained on a standard laboratory diet. They were guillotined and their livers were chilled and divided into small sections, weighed, wrapped individually in aluminum foil and stored at -80°C. The liver was used to set activity standards for assays, to establish lower limits of detectable activities, and to check Km's of the crude liver homogenate against those of the cell lysates.

Cell Lines

Maintenance

The lymphocytic cell lines UM 43 and 61, from males; and 54 and 56, from females, were supplied by Dr. Arthur Bloom's laboratory at the University of Michigan. They had been established by the method of Choi

and Bloom (1970), in which a lysate from a previously established line was used to stimulate growth of a new line. The donor was of sex opposite to that of the established line. They were maintained in RPMI 1640 (Grand Island Biological Company), enriched with 20% heat-inactivated fetal calf serum, and contained 60,000 units penicillin G and 60 mg streptomycin per liter of RPMI 1640. They were incubated in a 5% CO_2 humidified atmosphere at 37°C. The flasks used were disposable plastic 25 ml Falcon tissue culture flasks containing 10 ml of cell suspension and medium or 260 cc Greiner tissue culture flasks with 30 ml of cell suspension and medium. The lymphocytes grew clumped together in semi-suspension, settling to the bottom of the flasks, but easily suspended by shaking the flask gently.

The medium was changed when its pH dropped below 7 or when it was desired to have the cells in logarithmic growth phase within two or three days. To change the medium, nine-tenths of the culture medium was drawn off and the cells were suspended in the remainder. Fresh medium at 37°C was added under sterile conditions. No pipetting by mouth was done, as the cells were presumably

infected with an Epstein-Barr virus (Gerber, 1973), and were treated as pathogenic.

Harvesting

Harvesting was done with the cells in logarithmic growth phase, one or two days after changing medium. Two-thirds of the visible colonies were drawn off with a sterile pipette from the bottom of the flask. The cells and accompanying medium were put into screw-top test tubes and centrifuged at 1,000 RPM for 10 minutes. All cells harvested from a single line were vigorously resuspended in 10 ml of medium.

Two-tenths ml of the resuspended cells were added to 0.3 ml of 1% Trypan blue and mixed vigorously. The suspension was then sampled and counted on a hemocytometer. Total cells harvested were calculated by standard methods. Viability was determined by dye exclusion.

The suspension was recentrifuged, the medium discarded into a beaker, and the cells were washed in an isotonic salt solution followed by centrifugation. The

salt solution was also discarded into the beaker, and the button was stored dry in the test tube at -80°C until use.

All materials and medium that came in contact with the cells were autoclaved before cleaning or discarding as a precaution against infection.

Preparation of Cell Lysate and Liver Homogenate

At the time of assay, the cells were placed in suspensions of 2×10^7 cells per ml, so that differences in activities between lines and between cell lines and liver could be compared.

Preparation of the lysates was accomplished by freezing and thawing ten times in alcohol and dry ice alternating with a 37°C water bath. Schimke (1963) has shown that freezing and thawing is an acceptable method for preparing lysates from HeLa and fibroblast cultures for determinations of arginase, argininosuccinic acid synthetase and argininosuccinic acid lyase. The freezing and thawing were done in the test tube used for storage, which maximized the amount of recoverable lysate and minimized the possible exposure to the E-B virus.

Liver was prepared by homogenizing in ice cold water with a Potter glass homogenizer. The final dilution was 1 g of liver in 200 ml of homogenate.

The Colorimetric Assays

The four enzymes assayed were those involving the ornithine moeity. Citrulline determinations were done for ornithine carbamoyl transferase and argininosuccinic acid synthetase by the method of Archibald (1944) as modified by Ratner (1955).

One half ml of deproteininzed reaction mixture was mixed with 0.2 ml of .75% diacetyl monoxime and one ml of an acid mixture. The acid was composed of commercial grade H_2SO_4, 85% H_3PO_4, and water in proportions of 1:3:6. After boiling in the dark for 15 minutes and cooling in the dark for 15 minutes, the samples were read at 290 nm on a Hitachi spectrophotometer or a Bausch and Lomb colorimeter.

Urea determinations were done for ASase and arginase assays. One-tenth ml of 1.6% α-isonitroso propriophenone in 100% Ethanol was added to 0.5 ml of deproteinized reaction mixture. One ml of H_2SO_4, H_3PO_4, and water (1:3:5)

was added, the mixture was boiled in the dark for one hour, cooled for 15 minutes. Optical densities were read at 540 nm.

Standard curves were established each time an assay was run. Activities were expressed in μMoles mg liver hours.

The Enzyme Assays

The enzymes ornithine carbamoyl transferase, argininosuccinic acid synthetase, argininosuccinic acid lyase, and arginase were tested for activity. Each assay was performed in triplicate and repeated at least once.

Ornithine Carbamoyl Transferase

The reaction produces citrulline from ornithine and carbamoyl phosphate, and is measured as the rate of appearance of citrulline. The assay medium contained 20 mM dilithium carbamoyl phosphate and 15 mM ornithine in a 50 mM glycylglycine buffer at pH 8.3. To 0.2 ml of

30 mM ornithine, pH 8.3, was added 50 µl of liver homogenate or 50 µl of cell lysate. The ornithine with the liver or cell preparation was brought to the incubation temperature of 37°C. Carbamoyl phosphate was added to 0.1 M glycylglycine buffer (pH 8.3, 37°C), mixed quickly and .2 ml was added to the ornithine mixture to start the reaction. The incubation period was 15 minutes at 37°C. The reaction was stopped with 0.4 ml of 15% perchloric acid and centrifuged to remove the protein. One half ml was taken for color development.

Controls for this assay were particularly important as the reaction proceeds nonenzymatically. Therefore, each time period and/or substrate level was checked for development of background color with no enzyme. In addition, carbamoyl phosphate reacts with the glycylglycine buffer to produce background color in the reaction. Because of the fact that the non-enzymatic reaction is not stopped by the perchloric acid treatment, no delays between completion of the reaction and determination of product were allowable.

Argininosuccinic Acid Synthetase

The production of argininosuccinic acid from citrulline, aspartate and ATP is measured by detection of a depletion of the amount of citrulline in the reaction mixture. As urea produces color development in the citrulline assay, urea interfered with the determination of citrulline depletion. Therefore urease was added to the reaction mixture to eliminate the urea (Wixom et al., 1971). The assay medium was made up in 5 X final strength: 0.5 M Tris buffer, pH 7.5, 25 mM aspartate, 50 mM $MgSO_4$, 5 mM citrulline, and 25 mM ATP. Ten µl ASase, 1 mg arginase and 0.5 mg grade II urease (Sigma) were added per ml final volume. To 50 µl of the assay medium was added 200 µl of the liver homogenate or the cell lysate, and the mixture was incubated for one hour at 37°C. The cell lysate was prepared in 0.01 M Tris buffer, pH 7.5, as was the liver homogenate.

The reaction was stopped in a boiling water bath for five minutes. Protein was removed by centrifugation for 10 minutes at 2,000 RPM. Fifty µl of supernatant was taken for color development.

Argininosuccinic Acid Lyase

Argininosuccinic acid is cleaved by argininosuccinic acid lyase to produce arginine and fumarate. The reaction is measured as the production of urea in the presence of excess arginase. The method is that of Schimke (1962) modified for our conditions. One half ml of 10 mM barium argininosuccinic acid (Sigma) in 50 mM potassium phosphate buffer, pH 8.3, was added to 50 µl of cell lysate of liver homogenate. Lysate in this case was about 4×10^8 cells per ml, prepared by suspending the button in a minimal amount of medium. Liver homogenate was 1 g: 20 ml water. One half ml of 30% perchloric acid containing .1 M Na_2SO_4 was added to stop the reactions after incubation and to precipitate the barium ion before color development. The zero time control was pre-treated with the acid before adding the reaction mixture. Protein was removed by centrifugation at 2,000 RPM for 15 minutes. For color development 0.5 ml of the supernatant was taken.

Arginase

The cleavage of arginine to ornithine and urea is measured by the rate of appearance of urea. The assay medium was .250 M arginine in .001 M $MnSO_4$, pH 9.7. To one half ml of the assay medium was added 50 µl of pretreated lysate or liver homogenate. The pretreatment was incubation in .05 M $MnSO_4$ for five minutes at 55°C to activate the enzyme. The final concentration of cells in the lysate was 2 X 10^7 cells/ml. Liver was 1g:800 ml H_2O. Incubation was for one hour at 37°C. The reaction was stopped by 1/2 ml of 15% perchloric acid. After centrifugation to remove protein, 0.5 ml was taken for color development.

RESULTS

Lymphocytes

The enzyme assays required constant supplies of fresh lymphocytes, which were maintained on RPMI 1640 medium (Appendix I). These were cell lines derived from normal people (U.M. lines 43, 54, 56, and 61).

Growth Rates

The lines could be harvested once or twice a week, depending upon the rate of growth of the lymphocytes or the density of the cultures remaining after harvesting. Table 1 shows that over an eight-week period, the amount of harvested cells per flask varied with the line of cells. Line 43 had the lowest production rate, less than 10^6 lymphocytes per flask per week; while line 61 had the highest, or nearly 10^7 cells per flask per week. Therefore, line 43 was expanded to seven flasks in order to supply comparable numbers of cells from each line.

TABLE 1.--Lymphocytes harvested from four lines during one eight-week period.

Line	Bottle-weeks	Total cells harvested X 10^6	Cells per week per flask
43	50	45	.91
54	32	83.6	2.6
56	32	64.3	2.1
61	32	280	8.76

Viability

Viability checks were done at each harvest. The viabilities ranged from 65 to 93%. When viability was below 75%, the cells from that harvest were not used in the experiments. Table 2 shows the viabilities for the harvests during a representative eight-week period for each line. Ten per cent of all harvests were below the level of acceptability; 7.5 per cent were above 90% viability.

Protein Content

A protein determination (Lowry et al., 1951) was done to establish the amount of protein for a given number of cells. Line 61, the rapidly growing lymphocyte line, was chosen for this determination (see Table 1). There were 1.3 mg protein per 10^6 lymphocytes. This estimate was used throughout to establish specific enzyme activities.

TABLE 2.--Viabilities for four lines of lymphocytes harvested during an eight-week period.

Line	Date	% Viability
43	6/29	81
	7/4	85
	7/9	87
	7/17	76
	7/27	75
	7/30	73
	8/6	82
	8/19	78
54	6/29	82
	7/14	82
	7/17	86
	8/3	65
	8/19	85
56	7/14	85
	7/17	86
	8/3	69
	8/14	82
	8/19	89
61	6/29	76
	7/4	88
	7/5	77
	7/9	84
	7/11	78
	7/14	90
	7/17	93
	8/3	87
	8/14	83
	8/19	88

Growth on Arginine-Free Medium

In order to determine if the entire cycle was present in the lymphocytes, ornithine was substituted for arginine in the growth medium. It was decided to use arginine-free D' medium with ornithine added in place of arginine. D' medium (see Appendix II) enriched with 5% fetal calf serum was available in both arginine-free and arginine-containing forms, and was selected to be used for this experiment. Lymphocytes from line 43 were centrifuged at 50 X G, the RPMI 1640 medium was removed, the lymphocytes were washed with arginine-free D' medium, resuspended in 10 ml of D' medium, and distributed in 2.5 ml aliquots to four culture flasks, two with arginine, two with ornithine. No increase in numbers of cells was observed in either medium, and after four weeks, there were no observable clusters of cells on the bottom of the flasks. The lymphocytes did not seem to be able to adapt to the D' medium.

Urea and Citrulline Determinations

Urea was determined by the method of Archibald (1944). Citrulline was determined by the method of Archibald as modified by Ratner (1955). Optical density vs µmoles of citrulline and/or urea were established for the appropriate substances at the time of each experiment. Typical curves are shown in Figure 3a for urea, and Figure 3b for citrulline. Both of the assays followed Beer's law between .02 µMoles and 1.0 µMoles.

The Enzymatic Reactions

Three of the four enzymatic reactions of the ornithine-urea cycle were demonstrated colorimetrically in the lymphocytes. They were: OCT, Asase, and arginase, but not ASAS.

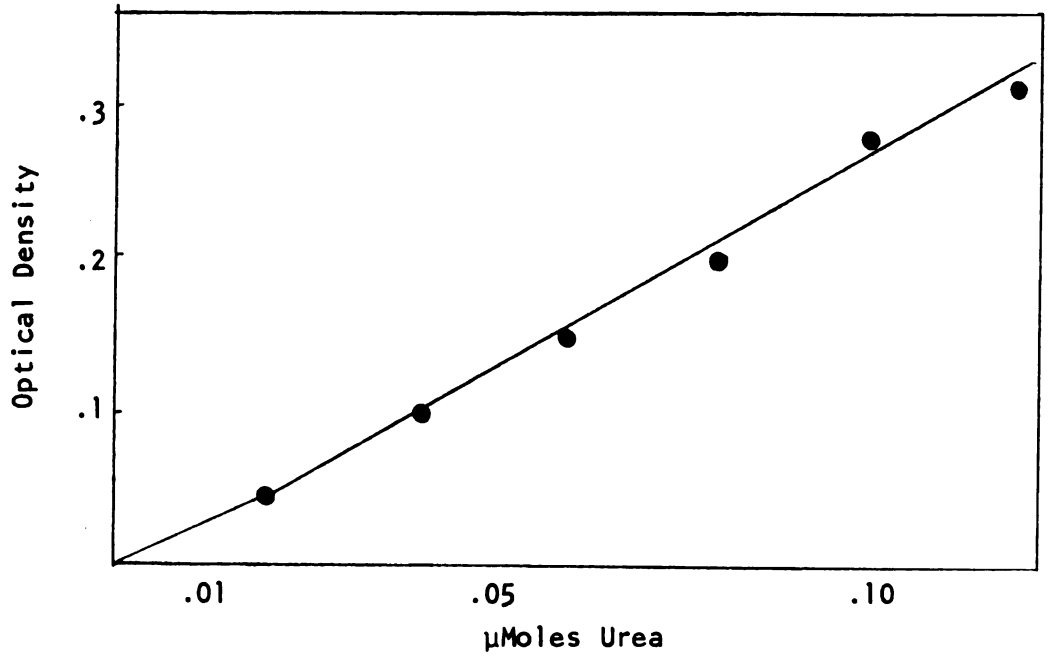


Fig. 3a.--Optical density vs µMoles urea.

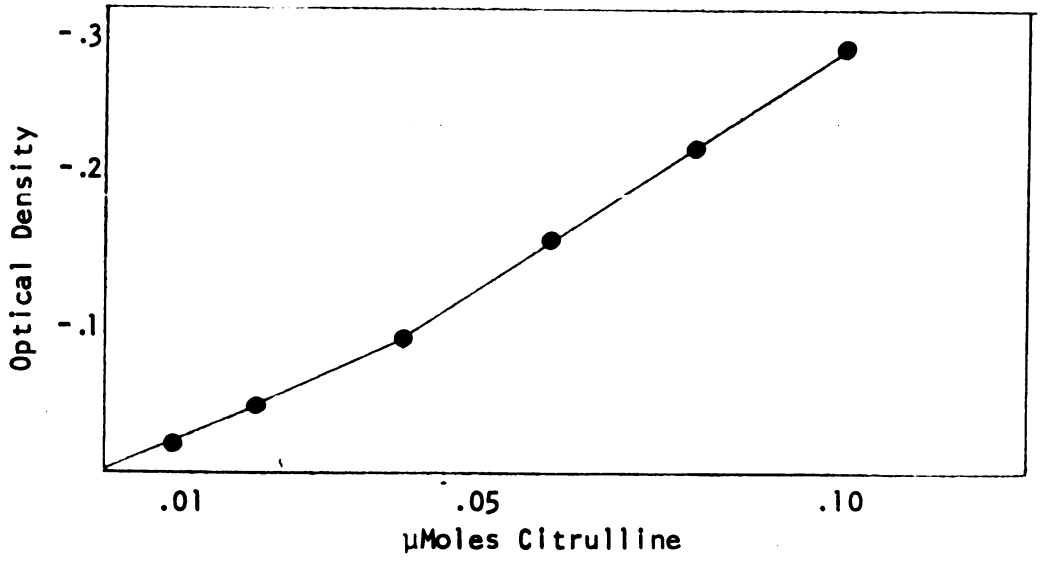


Fig. 3b.--Optical density vs µMoles Citrulline.

Ornithine Carbamoyl Transferase

Other than the liver homogenate or lymphocyte lysate,* the ingredients in the OCT assay were ornithine, carbamoyl phosphate, and glycylglycine buffer. The assay was stopped with 15% perchloric acid.

It was noted that preboiled blanks in the OCT experiments produced color. Experiments were run to determine whether color was developed by the various ingredients in the assay mixture independently of the amount of citrulline. Varying amounts of carbamoyl phosphate (0.0, 2.5, 5.0, 7.5, 10 µMoles) were tested and no trace of color was produced in the color reaction experiment. Citrulline added to a solution of 5 µMoles of carbamoyl phosphate in amounts of .00, .01, .02, .03, and .04 µMoles gave the same results as citrulline added to the color reagents in water. Perchloric acid, which was used to stop the reaction, gave no color development. However, the total mixture, but without enzyme, incubated 15 minutes, produced color. When 50 µl samples of an enzyme-free system were used for color development, optical density readings were in the 0.01 O.D. range. When 500 µl

*because of the method of preparation the suspension of liver in water or buffer is called homogenate, and the lymphocyte suspension is called lysate.

were used, a typical O.D. reading was .250. This was nearly half of the color development shown by the lymphocyte assays. Table 3 shows a sample series of optical density readings, and the net production of citrulline by the enzyme. At time zero, an O.D. reading of .250 would be equivalent to .11 μM of citrulline.

Figure 4a is a graph showing the increased color production in a near-linear fashion when glycylglycine buffer and carbamoyl phosphate were held constant, and ornithine was increased. It should be noted that color was produced even at zero levels of ornithine in the presence of high levels (.1 M) of carbamoyl phosphate. Although carbamoyl phosphate did not produce color in a previously described experiment, the fifteen minute incubation time probably allowed a color-producing reaction of carbamoyl phosphate with glycylglycine buffer. The addition of perchloric acid to the reaction mixture did not prevent the continued nonenzymatic color production.

Figure 4b shows similar results when ornithine is held at a constant .1 M concentration and carbamoyl phosphate is increased. It should be noted that between zero and 1 mM concentrations of carbamoyl phosphate, there was

TABLE 3.--The production of color by carbamoyl phosphate and ornithine, with and without enzyme.

Number of lymphocytes X 10^5	Optical densities (mean of three values)	Net production of by enzyme, µMoles
0	.249	--
1.25	.369	.06
2.5	.435	.09
5.0	.575	.16
10.	.723	.23

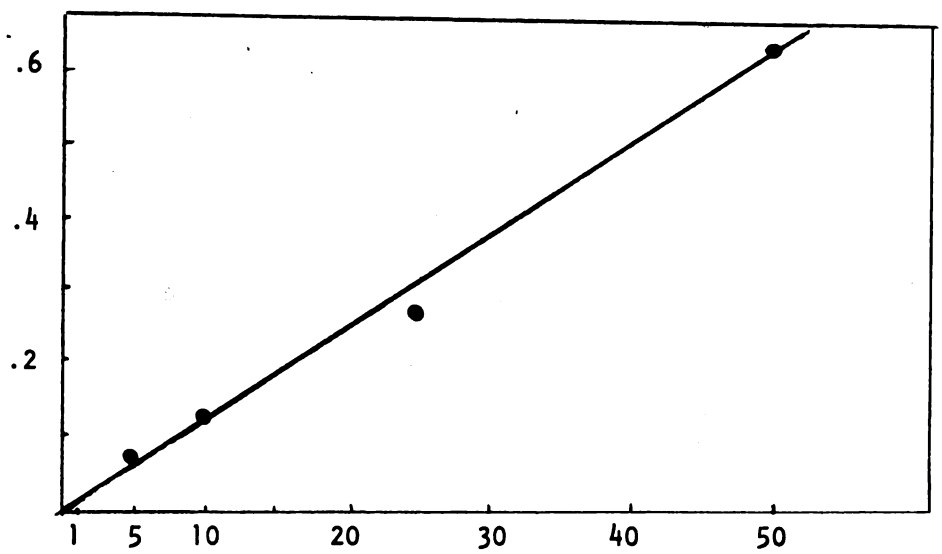


Fig. 4a.--Carbamoyl phosphate (mM) (ornithine at 100 mM).

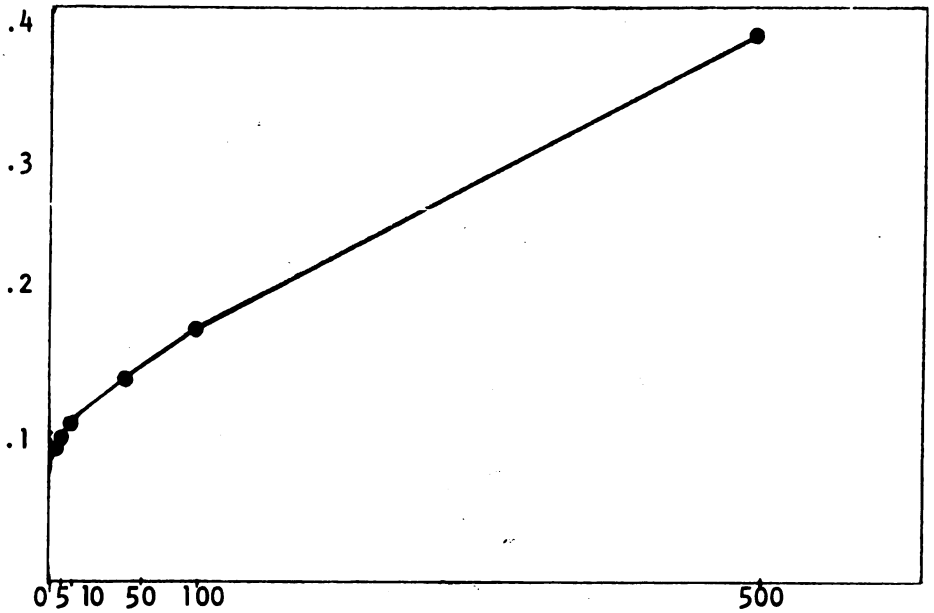


Fig. 4b.--Ornithine (mM) (carbamoyl phosphate at 50 mM)

Fig. 4.--Non-enzymatic color development by the OCT assay mixture.

no discernible color development, confirming the prior conclusion that ornithine alone or with glycylglycine buffer produces no color reaction.

Because of the nature of the nonenzymatic color development, the experiments with OCT were run with reagent blanks as controls and the reactions were started by the addition of carbamoyl phosphate in buffer to previously equilibrated test tubes. The appropriate subtractions for background color were then made.

Endogenous citrulline or interference from other constituents of the cell lysate could have contributed to the determination. Table 4 shows that very small amounts of color were present, approximately .01 O.D. units for about 6 X 10^5 lymphocytes. Therefore, no preboiled controls were used.

The Enzyme Assays for Ornithine Carbamoyl Transferase

Activity of OCT was demonstrated in lymphocyte lines 43, 54, and 61. Lines 43 and 61 had the highest activities: 10^6 lymphocytes produced .8 µMoles citrulline in a 15 minute incubation.

TABLE 4.--Color development from lymphocyte lysate in citrulline determinations.

Numbers of lymphocytes X 10^5	Optical density	µM citrulline
3	.005	.001
6	.012	.002
9	.017	.0025
1.2	.021	.003
1.5	.026	.005

Lability at -80°C

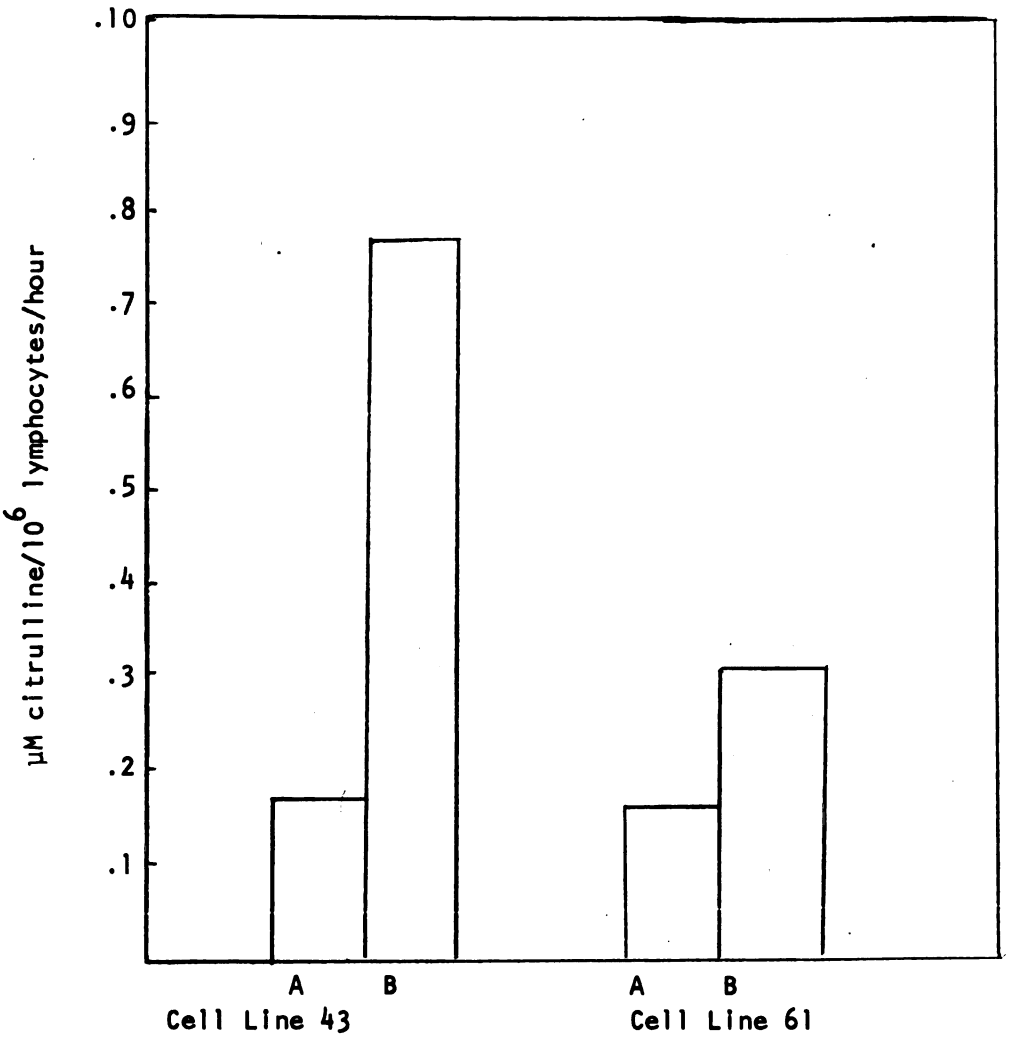
It is often stated that OCT loses much of its activity when stored overnight at -15°C (Levin, 1971). Lability was tested on samples stored overnight at -80°C. Figure 5 shows that the activity of a crude homogenate prepared from a button stored overnight was greater than that of a fresh sample. Since line 43 had the higher activity after being frozen, subsequent experiments were done on this line.

Time Dependency

The production of citrulline was shown to be time dependent (Figure 6). At times of 0, 5, 10, and 20 minutes 0, .26, .51, and 1.1 μMoles of citrulline were produced per 10^6 lymphocytes.

Protein Dependency

To establish that a reaction is enzymatic it is necessary to show that the rate of product formation is



A Activity when tested immediately after harvest.
B Activity when tested after 24 hours at -80°C.

Fig. 5.--Ornithine carbamoyl transferase activities before and after storage at -80°C.

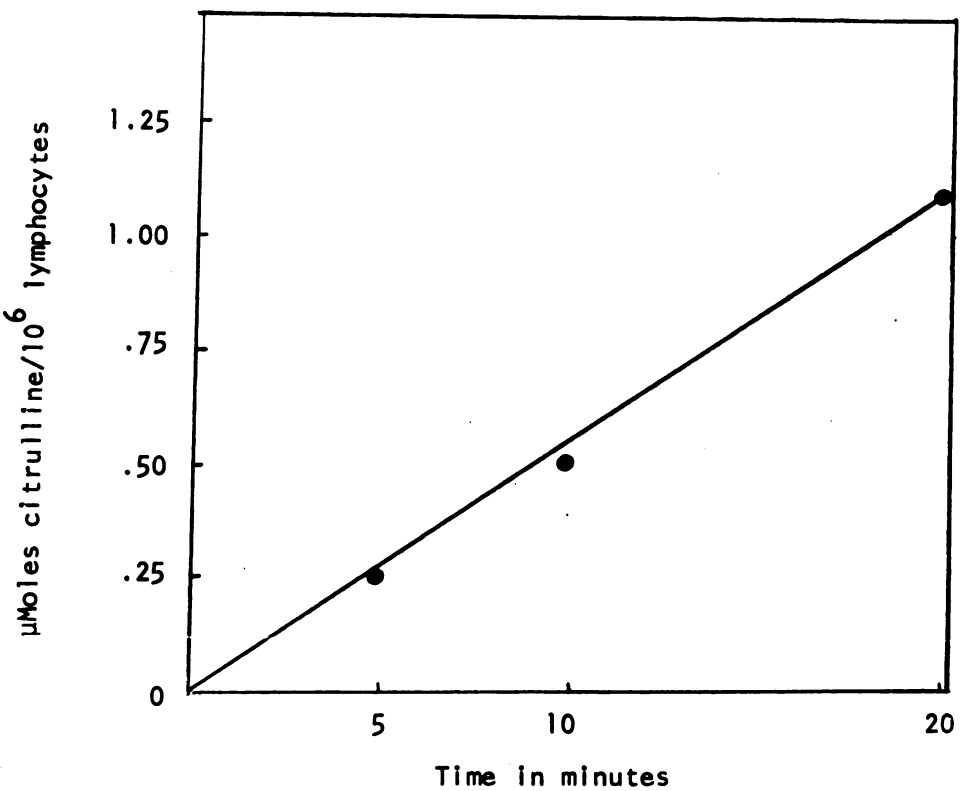


Fig. 6.--Citrulline production by OCT as a function of time by lymphocytes.

linearly dependent upon the amount of protein in the system. Figure 7 shows that for OCT the rate of citrulline production does increase linearly with increasing amounts of protein, expressed in numbers of lymphocytes (2.5, 5, and 10 X 10^5).

Specific Activity

Specific activity for the lysate was .41 µMoles citrulline per milligram protein per hour. In the same experiment, the specific activity of liver was 107 µMoles citrulline per milligram protein per hour. Therefore, the activity of the lymphocytes was about 1/260 that of the activity on the liver.

Location of the Enzyme

When the lysate was centrifuged before assay, twice the activity was found in the supernatant as in the button resuspended in an equal amount of water. This may indicate that some cell organelles were not completely ruptured during the lysing process and that OCT is located within these organelles.

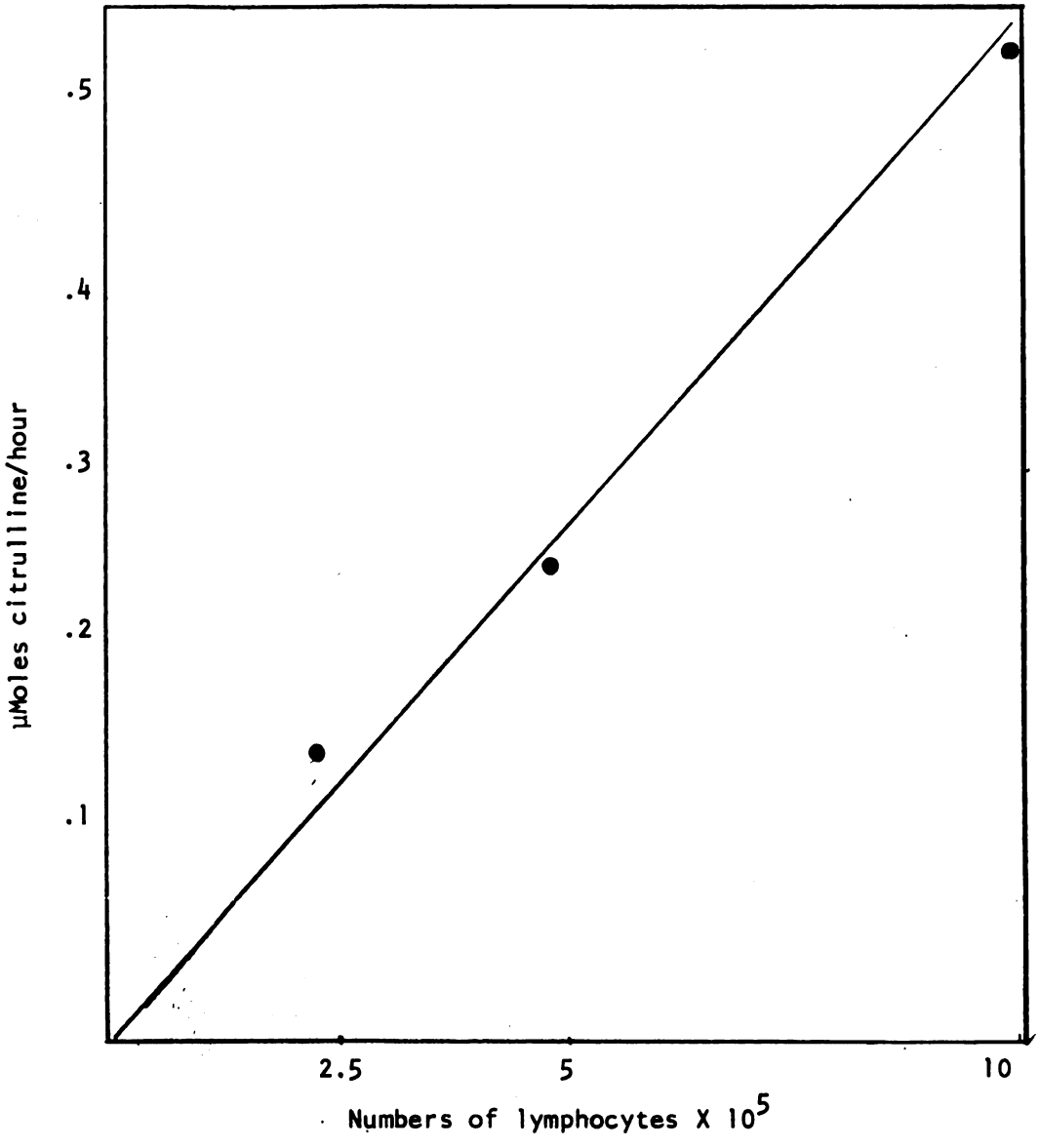


Fig. 7.--Citrulline production by OCT as a function of lymphocyte protein concentration.

Establishment of Michaelis-Menten Constants (Km) for OCT

Michaelis-Menten constants were derived for both substrates on the whole lysate and compared with laboratory values for crude liver homogenate. The experiments were done in triplicate and repeated at least once for each Km. Graphs are shown from one representative experiment in each group.

Ornithine Km for OCT

The velocity vs substrate curve for liver homogenate shows a linear rate to 2 mM and a flattening above 5 mM of ornithine (Figure 8). (This indicates substrate inhibition.) The velocity vs substrate curve for the lymphocyte lysate increases as substrate concentration rises up to 0.1 M ornithine (Figure 9). Not included on the graph are concentrations of ornithine at 500 and 1000 mM. These values are less than the value at 100 mM, also indicating substrate inhibition with the lymphocyte enzyme. These Km's were run at 50 mM carbamoyl phosphate.

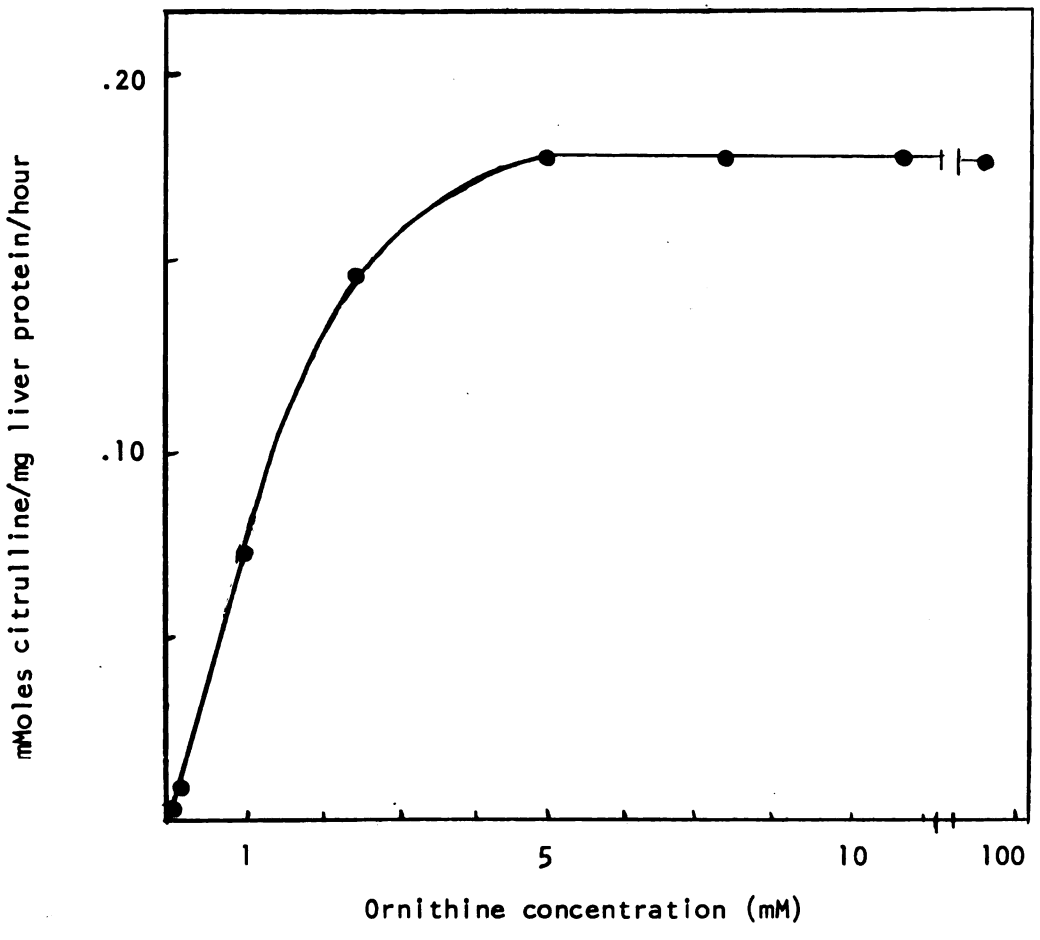


Fig. 8.--Rate of citrulline production by OCT in liver as a function of ornithine concentration.

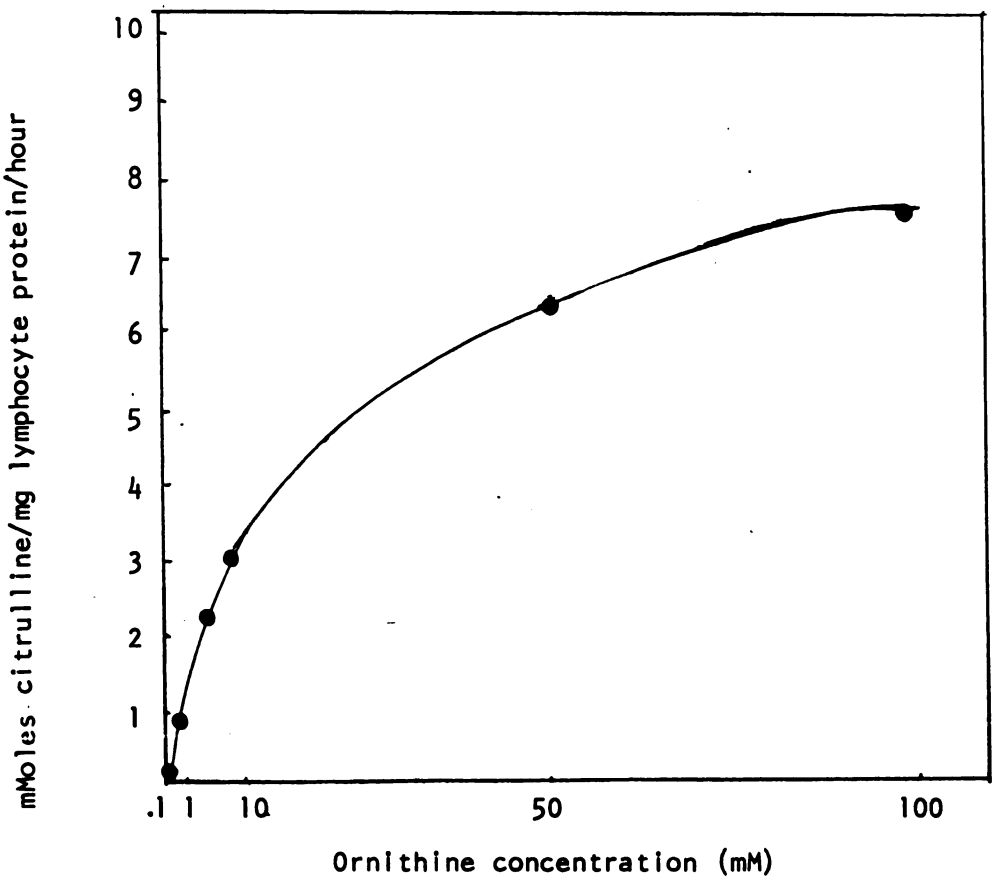


Fig. 9.--Rate of citrulline production by OCT in lymphocytes as a function of ornithine concentration.

Lineweaver-Burk Plots

Lineweaver-Burk plots (1/V vs 1/S) were done for both crude liver homogenate and lymphocyte lysate (Figures 10 and 11). The Michaelis-Menten constant (Km) for liver was 3 mM ornithine (Figure 10). The plot shows that as the concentration increases, there is substrate inhibition, because the theoretical maximum velocity of the reaction (V max) is greater than the actual figure. Repetition again produced 3 mM ornithine as the Km. The value at 1 mM has the greatest likelihood of error. It is the minimum substrate level read at the lowest optical density. The inversion of this figure results in small errors being greatly magnified.

The Km for cells was estimated at 15 mM ornithine from a Lineweaver-Burk plot (Figure 11). The value at 1 mM has the greatest likelihood of error. It is the minimum substrate level read at the lowest optical density. The inversion of this figure results in small errors being greatly magnified. Repetition of the experiment produced a Km of 17.5 mM ornithine.

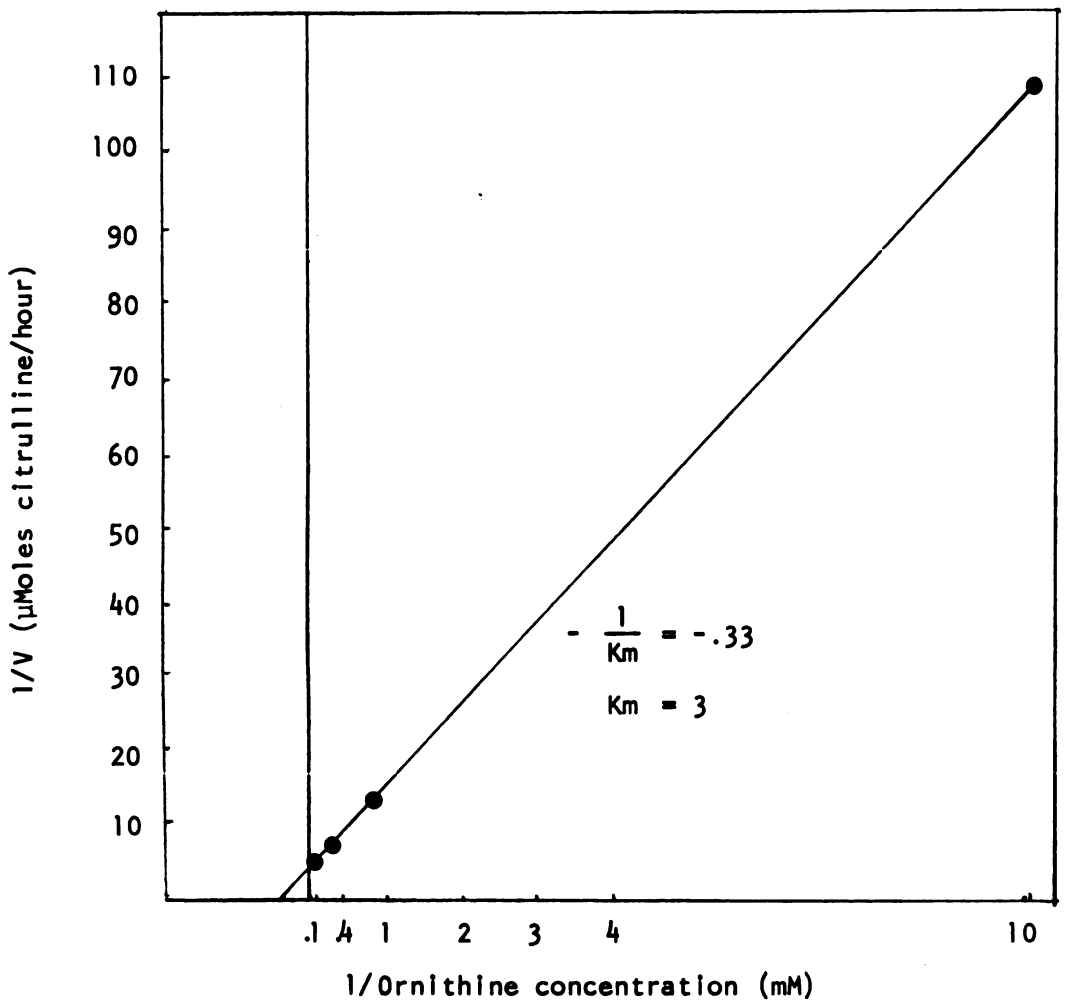


Fig. 10.--Lineweaver-Burk plot (1/V vs 1/S) of OCT in liver as a function of ornithine concentration.

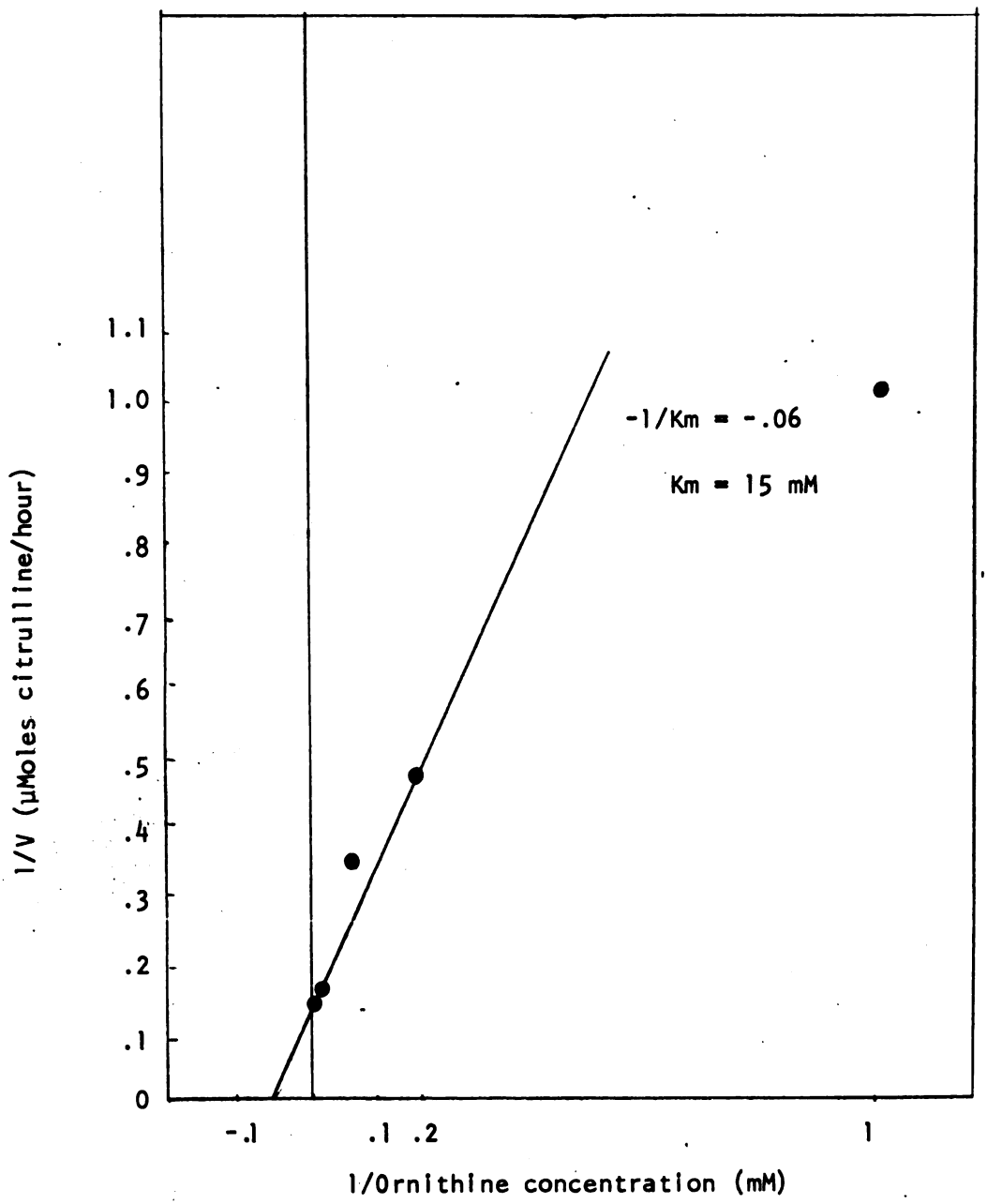


Fig. 11.--Lineweaver-Burk plot (1/V vs 1/S) of OCT in lymphocytes as a function of ornithine concentration.

Hanes Plots

Hanes plots (S/V vs S) were done for liver (Figure 12) and lymphocytes (Figure 13). The Hanes plot attempts to correct for errors at low concentrations by plotting the substrate levels directly. Km's estimated from each of these plots were the same as those estimated from the Lineweaver-Burk plots: 3 mM for liver, and 15 and 17.5 mM for lymphocytes.

Carbamoyl Phosphate Km for OCT

The velocity vs substrate curve for liver shows nearly linear increases to 5 mM and inhibition above 10 mM (Figure 14). The same curve for lymphocytes (Figure 15) shows a linear increase to 7.5 mM and a slower rate of increase after 20 mM.

Lineweaver-Burk and Hanes Plots

A Lineweaver-Burk plot of 1/V vs 1/S for liver gives a Km estimate of 3.3 mM (Figure 16), while a Hanes plot on the same data gives an estimate of 2.4 (Figure 18).

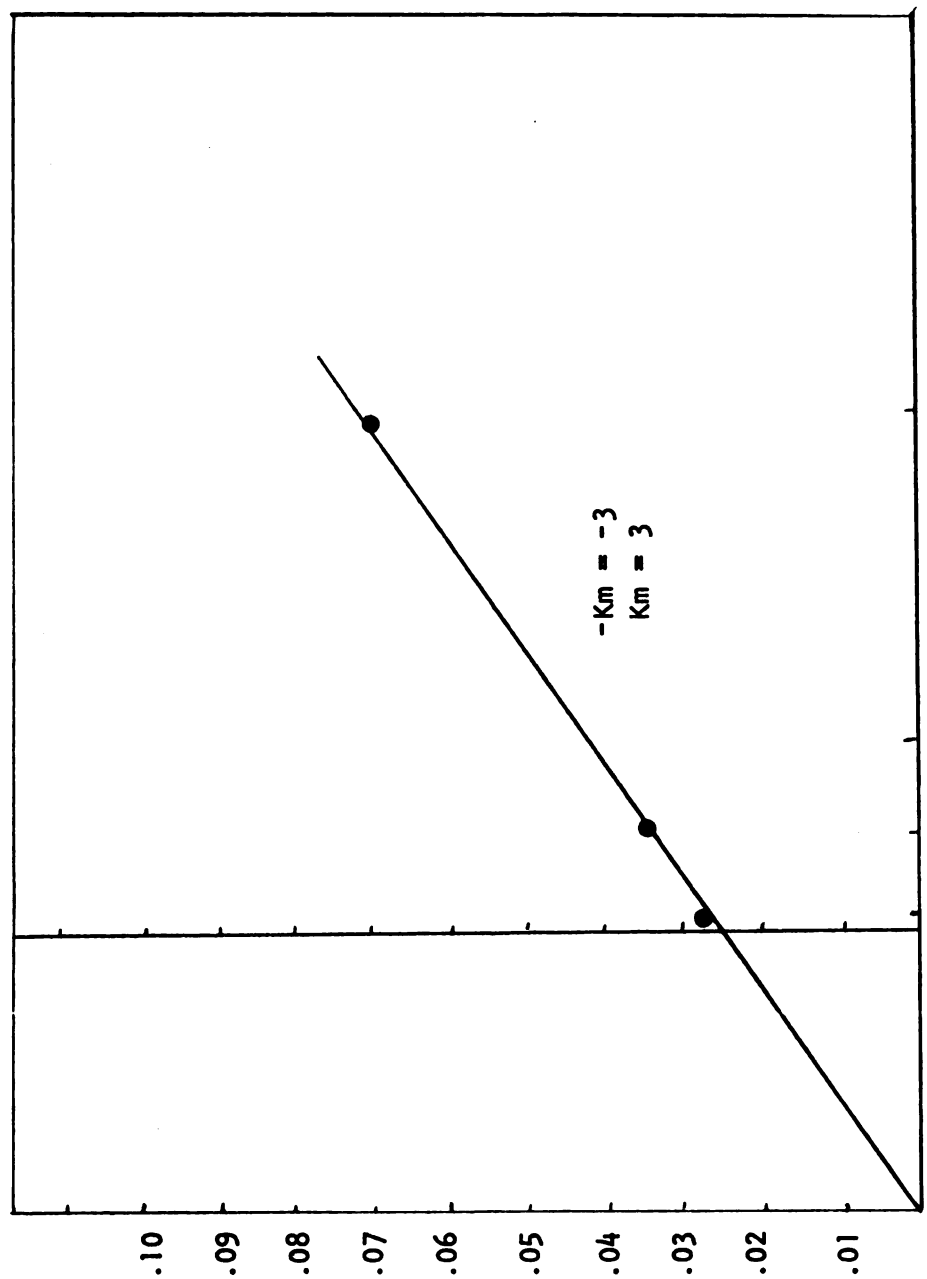


Fig. 12.--Hanes plot (S/V vs S) of OCT in liver as a function of ornithine concentration.

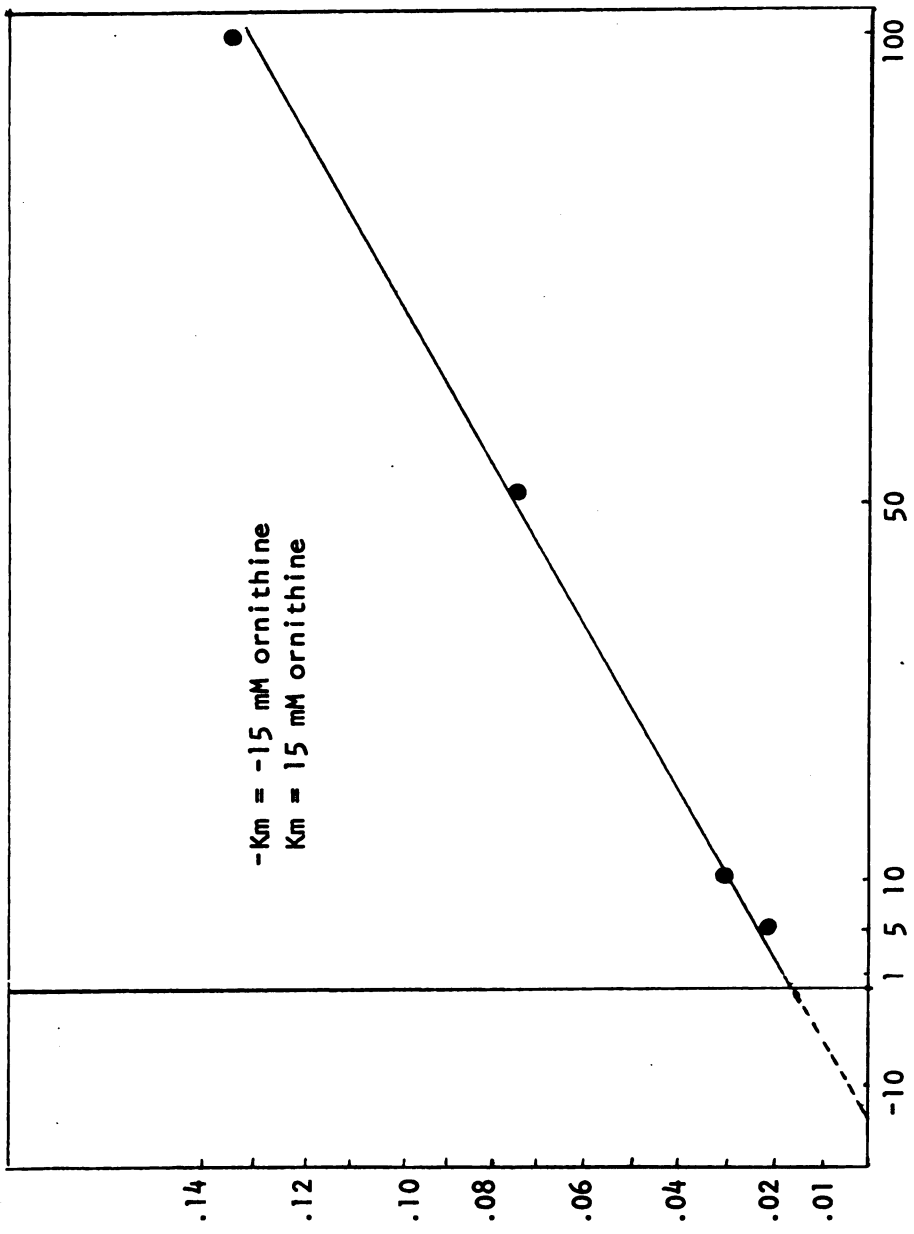


Fig. 13.--Hanes plot (S/V vs S) of OCT in lymphocytes as a function of ornithine concentration.

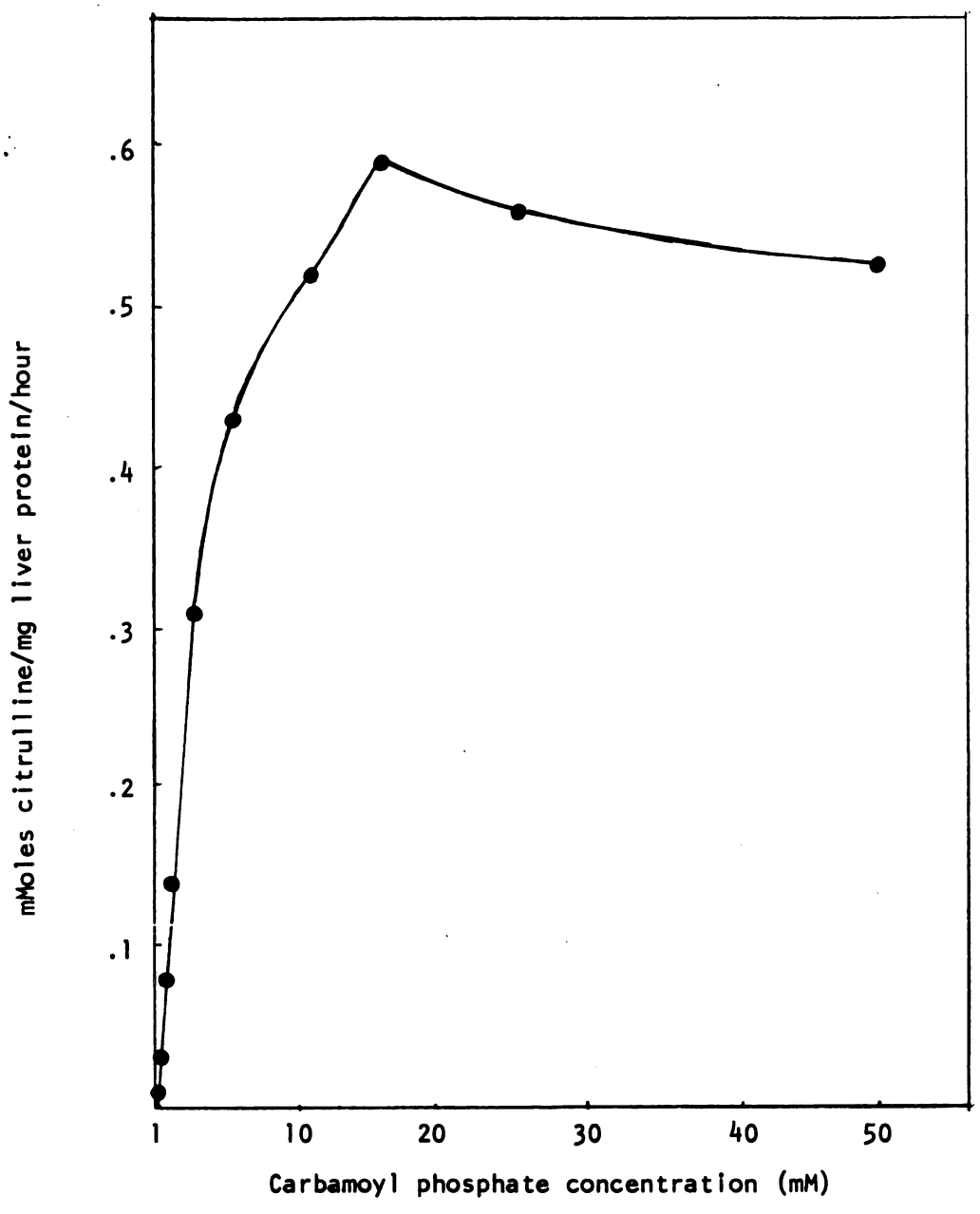


Fig. 14.--Rate of citrulline production by OCT in liver as a function of carbamoyl phosphate concentration.

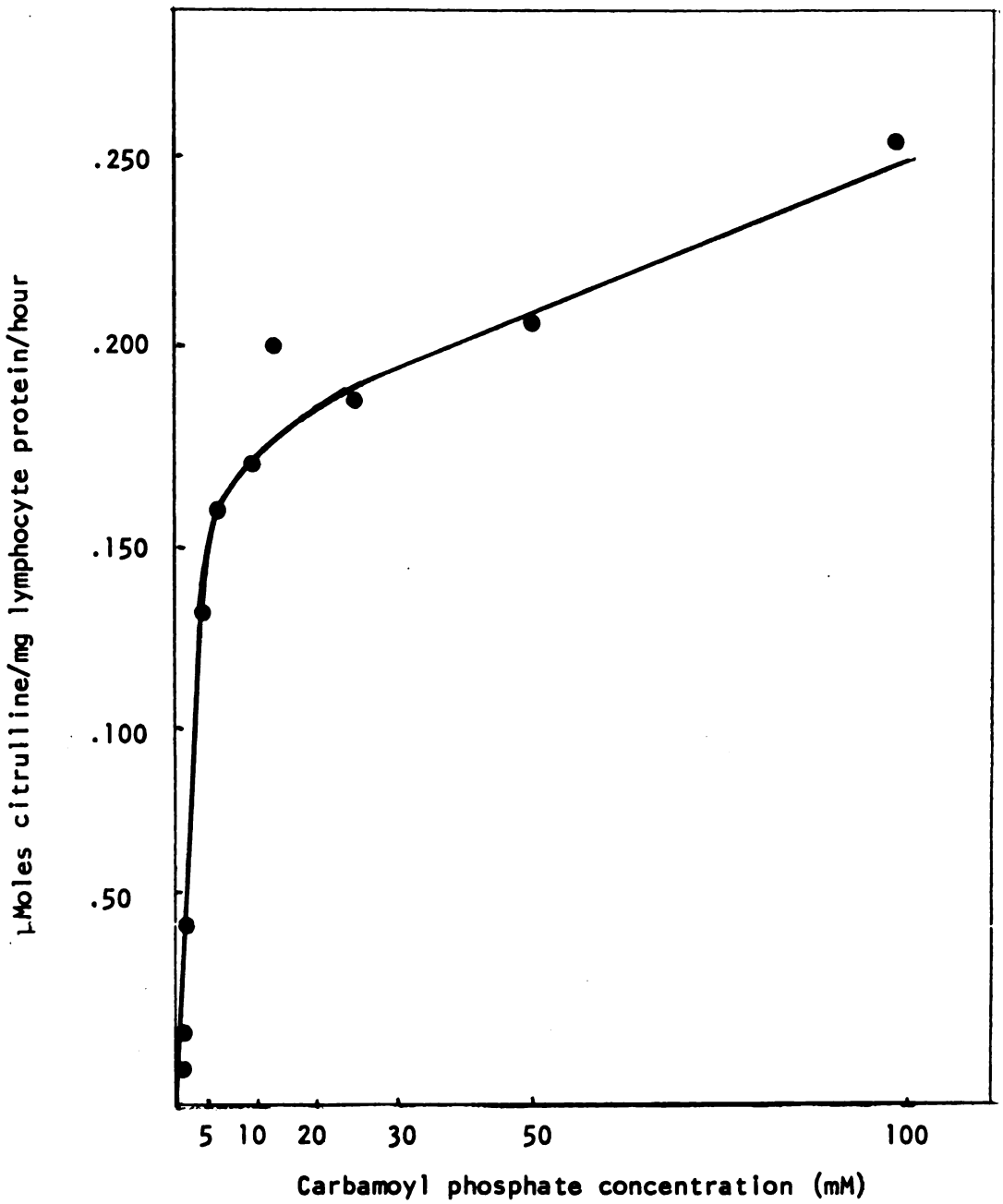


Fig. 15.--Rate of citrulline production by OCT in lymphocytes as a function of carbamoyl phosphate concentration.

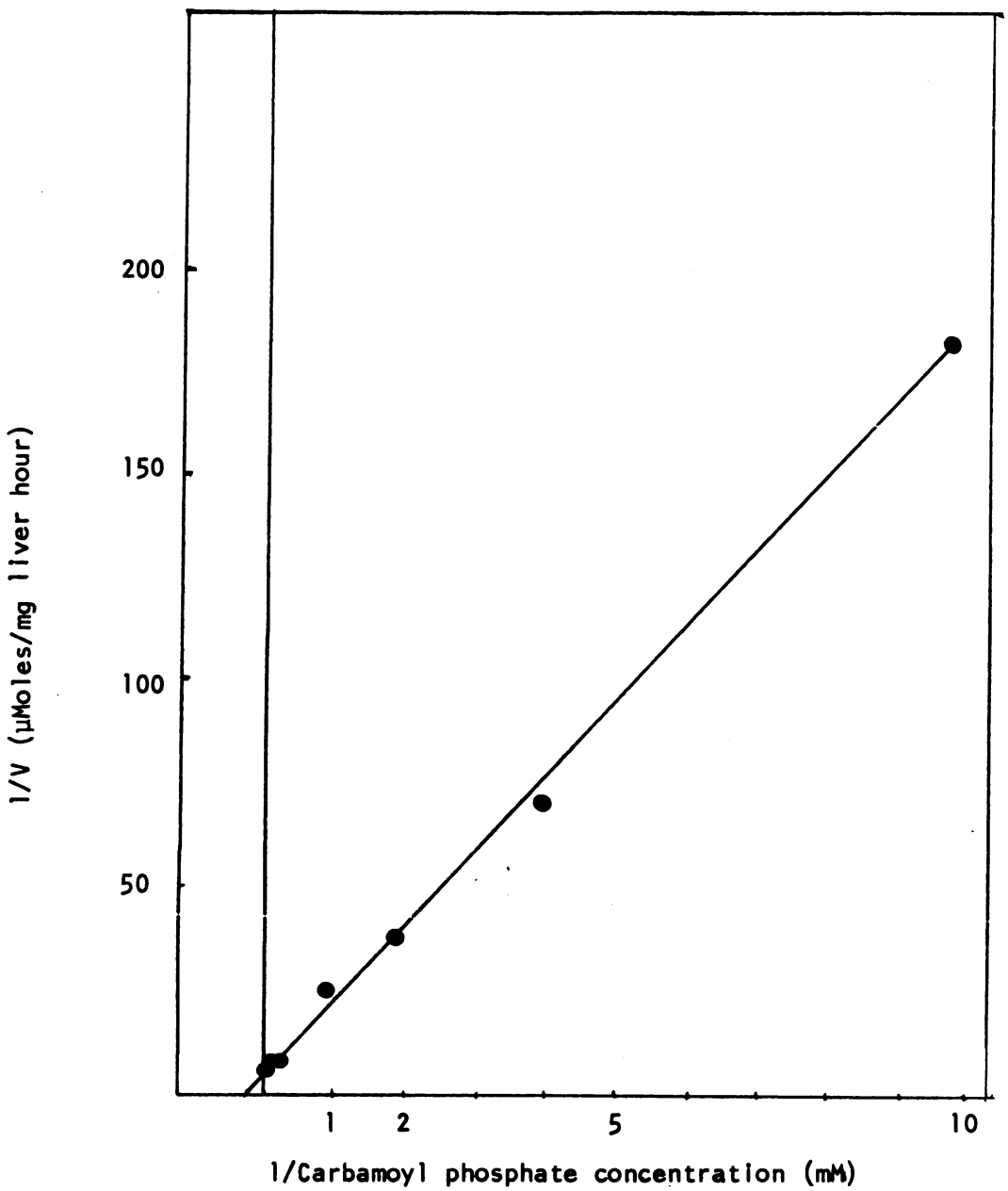


Fig. 16.--Lineweaver-Burk plot (1/V vs 1/S) for OCT in liver as a function of carbamoyl phosphate concentration.

Repetition produced a value of 3.3 mM for liver on both the Lineweaver-Burk plot and the Hanes plot.

The Lineweaver-Burk plot for the lymphocytes gives a Km estimate of 2.2 mM carbamoyl phosphate (Figure 17). Repetition produced a value of 1.5 mM. A Hanes plot for the same data gives an estimate of 2.5 mM for one experiment and 1.5 mM for the repeat (Figure 19).

Argininosuccinic Acid Synthetase

Argininosuccinic acid is produced from citrulline, aspartic acid, and ATP. The assay was based upon the depletion of the substrate citrulline. The assays of Brown and Coehn (1959) and Schimke (1961) were run with a 1:40 dilution of liver homogenate. The enzymes ASase and arginase were added to prevent product inhibition by argininosuccinic acid. Citrulline determination was by the method of Ratner (1955).

An assay mixture containing 5 mM citrulline and the 1:40 dilution of liver homogenate produced optical density shifts of 0.1 O.D. units after incubation for one hour. To obtain comparable results with the lymphocytes in the ASAS assay, it would have been necessary to use approximately

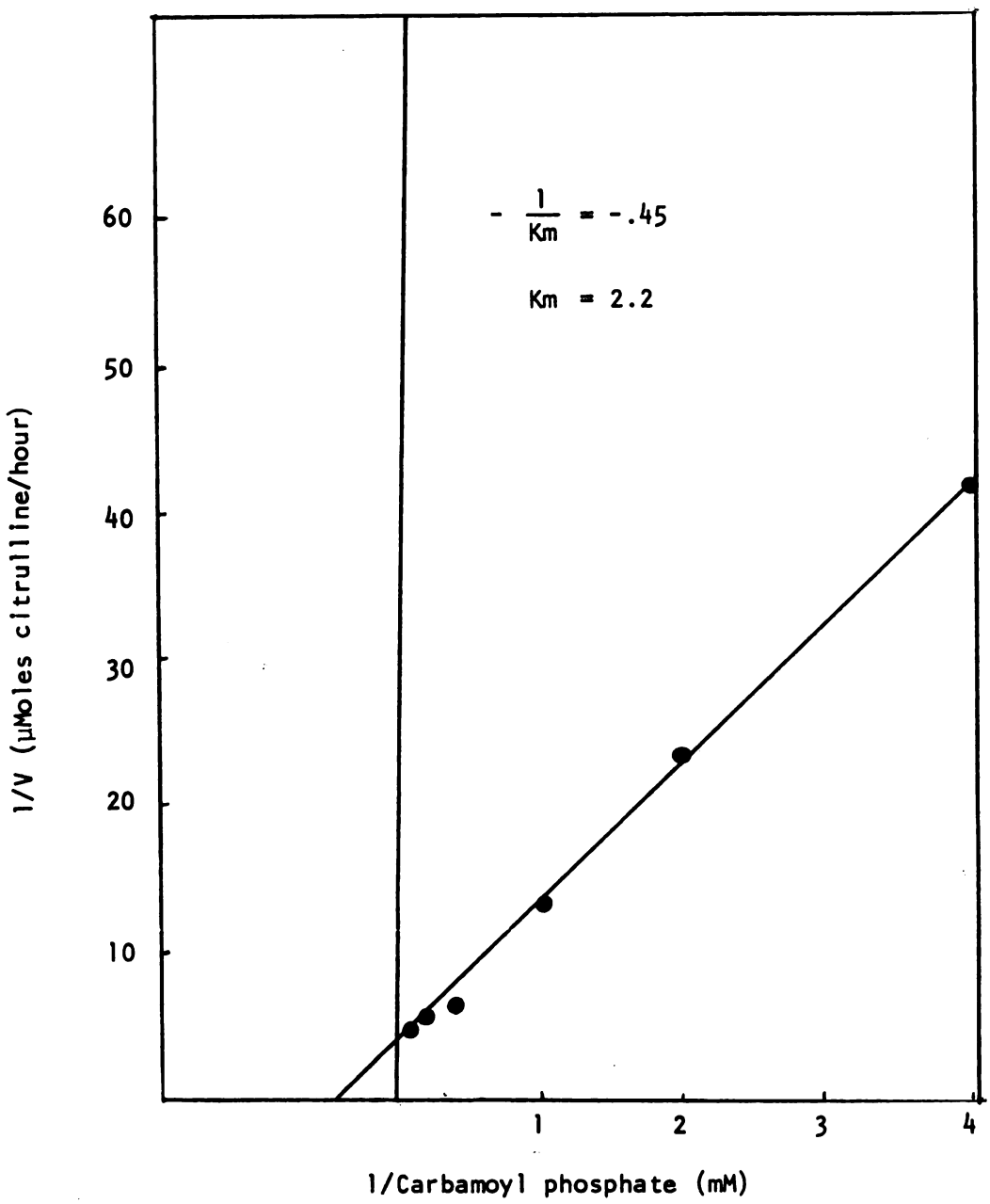


Fig. 17.--Lineweaver-Burk plot (1/V vs 1/S) for OCT in lymphocytes as a function of carbamoyl phosphate concentration.

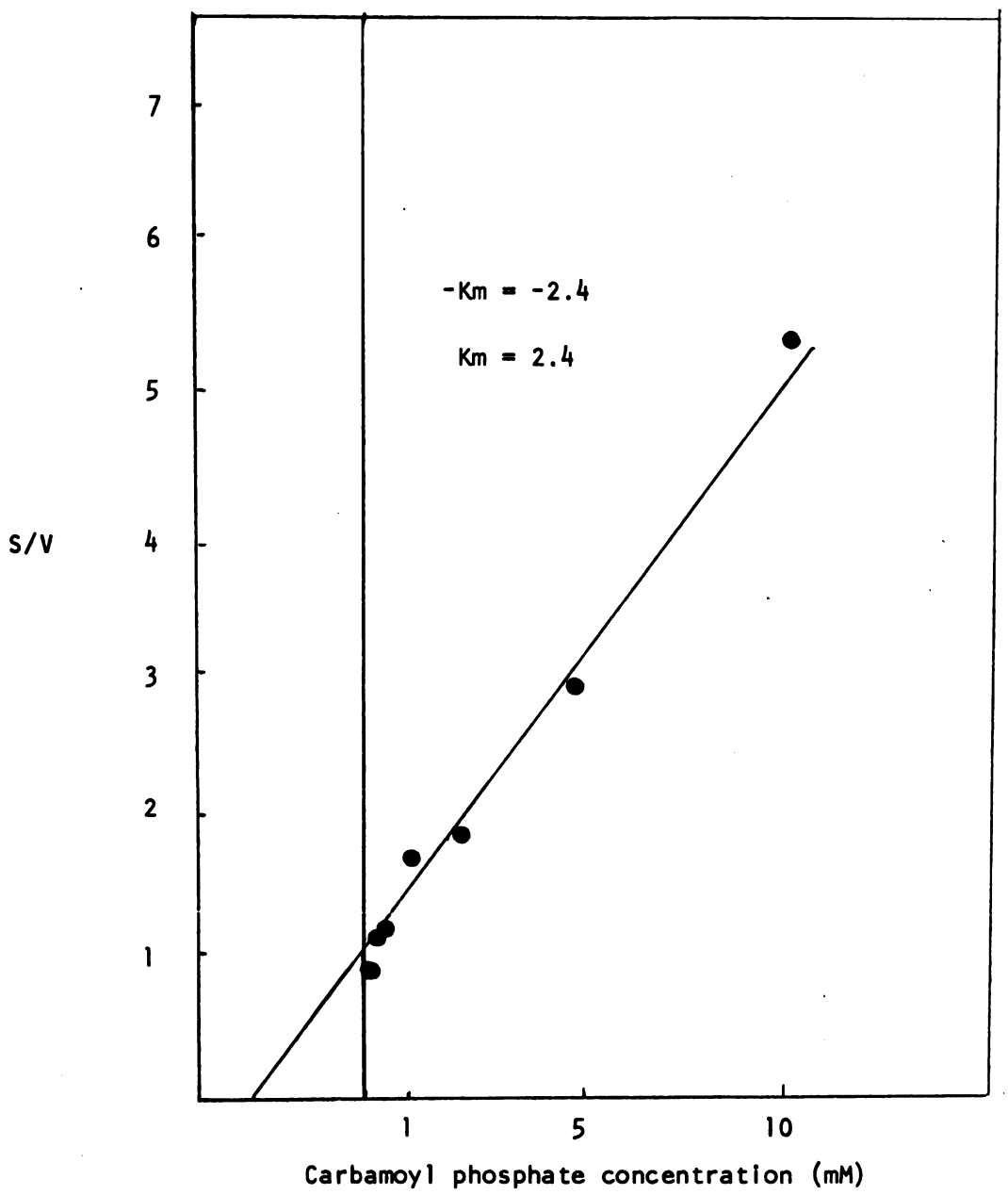


Fig. 18.--Hanes plot (S/V vs S) of OCT in liver as a function of carbamoyl phosphate concentration.

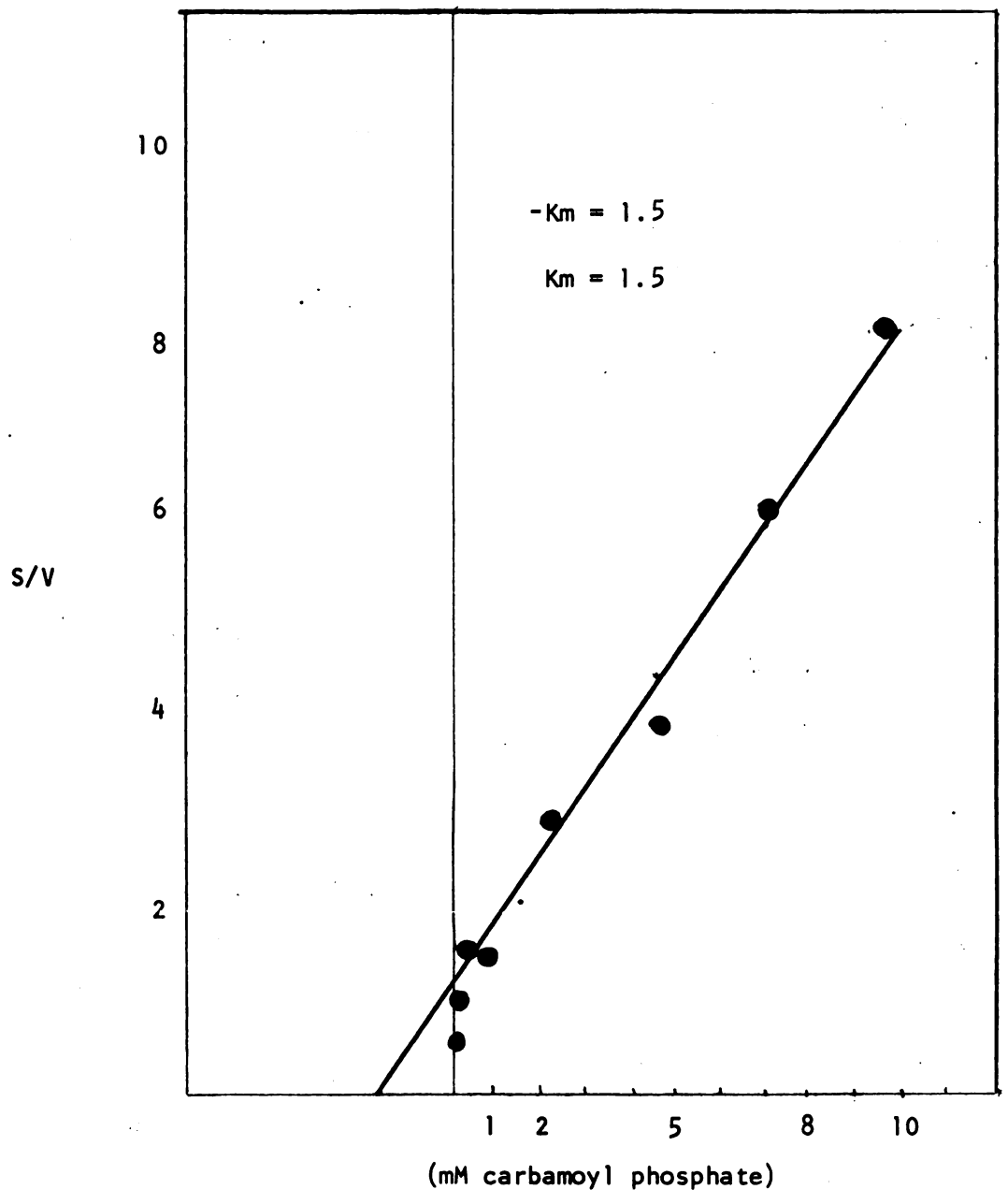


Fig. 19.--Hanes plot (S/V vs S) of OCT in lymphocytes as a function of carbamoyl phosphate concentration.

4×10^9 lymphocytes or 5.2 g protein per ml, and therefore could not be performed.

Urea is produced in the reaction mixture described above. The ureido group ($-\overset{\overset{O}{\|}}{C}-NH_2$), which is free in both citrulline and urea, produces color in the citrulline determination method. Therefore, urea interferes with the determination of the citrulline concentration. The addition of urease to the reaction mixture to eliminate the urea has been reported to increase the sensitivity some threefold (Wixom et al., 1972).

Urease Effects

To confirm that the addition of urease would increase the sensitivity, three replicate experiments of the reaction were performed. The summary of these experiments is seen in Table 5. The experiment included the following treatments, each step of which was terminated by boiling for five minutes:

A. preboiled enzyme incubated with the assay mixture without urease.

TABLE 5.--The effects of urease on optical density shifts in the ASAS assay with 1 mM citrulline in assay medium.

Time	Optical Density Readings						
	A	B	C	D	A-B	C-D	B-D
5	.282	.272	.270	.242	.010	.028	.030
10	.271	.267	.253	.230	.004	.023	.037
20	.251	.249	.245	.219	.002	.026	.030
30	.243	.223	.221	.180	.020	.041	.043
60	.231	.220	.173	.118	.011	.055	.102

Treatments:

A. Preboiled enzyme, boiled after incubation, no urease

B. Aliquot of A. Urease added, incubated 10 minutes, stopped by boiling.

C. Fresh homogenate, boiled after incubation, no urease.

D. Aliquot of C. Urease added, incubated 10 minutes, stopped by boiling.

B. an aliquot of A to which urease was added and incubated for ten minutes.

C. freshly prepared homogenate incubated with the assay mixture without urease.

D. an aliquot of C incubated with urease for ten minutes.

Treatment A controlled the effect of time on color development. Treatment B controlled the level of endogenous urea. Treatment C showed the effects of urea in the assay. Treatment D removed the endogenous and enzyme-produced urea. The change in optical density seen in the preboiled control indicates that some free ureido group present in the assay mixture deteriorates with incubation time in the absence of active enzyme. Therefore, preboiled incubated controls must be run with each experiment. The results of the experiment indicated that .18 mμMoles of citrulline per mg per hour was converted. By adding urease to the reaction mixture, it was possible to increase the sensitivity of the assay fourfold. This small increase in sensitivity was not sufficient to make the lymphocyte assay possible.

Citrulline Concentrations

Because it was desirable to use some 20% of the citrulline to register appropriate optical density shifts (Brown and Cohen, 1959), various concentrations of citrulline were attempted. With a concentration of 1 mM citrulline and 20 minutes incubation, an O.D. shift of 0.2 O.D. units was obtained. The lower citrulline level thus increased the sensitivity sixfold.

Time and Dilution Effects

To test the effects of time and dilution on the homogenate, replicate experiments were performed using dilutions of 1:20, 1:60, 1:100, 1:500, and 1:1000, and incubations of 1 hour and 2 hours. The results are shown in Table 6. In these experiments, the 1/20 and 1/60 dilutions of liver homogenate produced similar O.D. shifts, while the 1:100 and the 1:180 dilutions resulted in less citrulline reduction. The 1/500 and 1/1000 dilutions produced lower rates of citrulline utilization. The rates of utilization were not linear over the range. The effect may be due to the presence of ATPases in the homogenate

TABLE 6.--Optical density shifts recorded in ASAS assay after incubation of serial dilutions of liver homogenate in an assay mixture containing 1 mM citrulline and urease.

Experiment number	Time	Dilution of homogenate	Optical density shift
1	1 hr	1:20	.110
		1:60	.097
		1:180	.068
2	1 hr	1:20	.132
		1:60	.134
		1:180	.075
3	1 hr	1:100	.070
		1:500	.049
		1:1000	.050
	2 hr	1:100	.101
		1:500	.058
		1:1000	.058

and have caused ATP to become rate limiting at the higher concentrations of homogenate.

The detection of activity in a 1:500 dilution of liver homogenate is a 250 fold increase in the sensitivity of the assay as reported commonly for colorimetric assays of ASAS. With this level of sensitivity, activity should be detectable with about 10^7 lymphocytes per tube. The experiments were done repeatedly with lymphocytes, using liver homogenate as a control. However, no activity was detectable in the lymphocytes.

Argininosuccinic Acid Lyase

This enzyme catalyzes the splitting of ASA into arginine and fumerate, and was measured as urea production in the presence of excess arginase. Urea was measured by the method of Archibald (1944).

Activity for this second-least-active enzyme of the urea cycle was detectable when there were 4×10^7 lymphocytes per ml of reaction medium. The experiment was run with reagent blanks and controls of preboiled enzyme. The reagent blanks showed no color development, indicating that

spontaneous conversion did not occur significantly and that the commercial arginase was not contaminated with ASase. The preboiled controls showed endogenous urea to be present in both liver and lymphocytes. The assay mixture with preboiled enzyme showed 5 mμM endogenous urea per ml for the liver assay, which was 5.6 mμM endogenous urea per mg protein. The lymphocytes had 100 mμM endogenous urea per ml, or 1.78 mμM endogenous urea per mg protein (Table 7).

Specific Activities

After ten minutes, the liver homogenate showed production of urea at 2000 mμMoles urea per mg protein per hour. After 60 minutes, the rate of production was 1440 mμMoles urea per mg protein per hour, indicating that the enzyme activity is reduced with time. The lymphocytes showed 5.25 mμMoles urea per mg protein per hour. The lymphocytes had 1/275 of the ASase activity of the liver in this experiment.

TABLE 7.--Argininosuccinic acid lyase activity.

Enzyme Source	Time	mμMoles urea per hour per mg protein
Liver	0	5.6
	10 min	2000
	60 min	1440
Lymphocytes	0	1.78
	60 min	5.25

Arginase

Arginase is the enzyme which catalyzes the splitting of arginine to urea and ornithine. Its activity was measured by the appearance of urea in a solution of arginine. Urea determinations were by the method of Archibald (1944).

As it had been found that when samples of 500 µl were taken for color development for OCT determinations, significant amounts of citrulline were discovered, zero enzyme controls were tested for color development. When 1.6% isonitrosopropriophenone was used for the color-producing reagent, no background color appeared. Reagent blanks were used for controls for the Km studies, but no significant color development occurred even at very high concentrations of arginine (1 M).

Arginase activity was established in all four lymphocyte lines. Line 61 had the highest activity.

Time Dependency

Experiments with time periods of one half, one, and two hours showed the production of urea to be time

dependent (Figure 21). The production of urea was not linear over the time period of two hours. Therefore, it appeared that the enzyme activity diminished with time, with about 75% activity after one hour.

Protein Dependency

The production of urea was shown to be linearly dependent upon protein concentration (Figure 20): 1×10^6 lymphocytes produced 400 mμM urea per hour, 2×10^6 lymphocytes produced 860 mμM per hour, and 3×10^6 lymphocytes produced 1200 mμM urea per hour.

Specific Activity

The arginase activity of liver was 9,340 mμM per mg protein per hour. The activity of the lymphocytes was 23 mμM per mg protein per hour. Therefore, in this experiment the specific activity of the lymphocytes was about 1/400 of the specific activity of the liver.

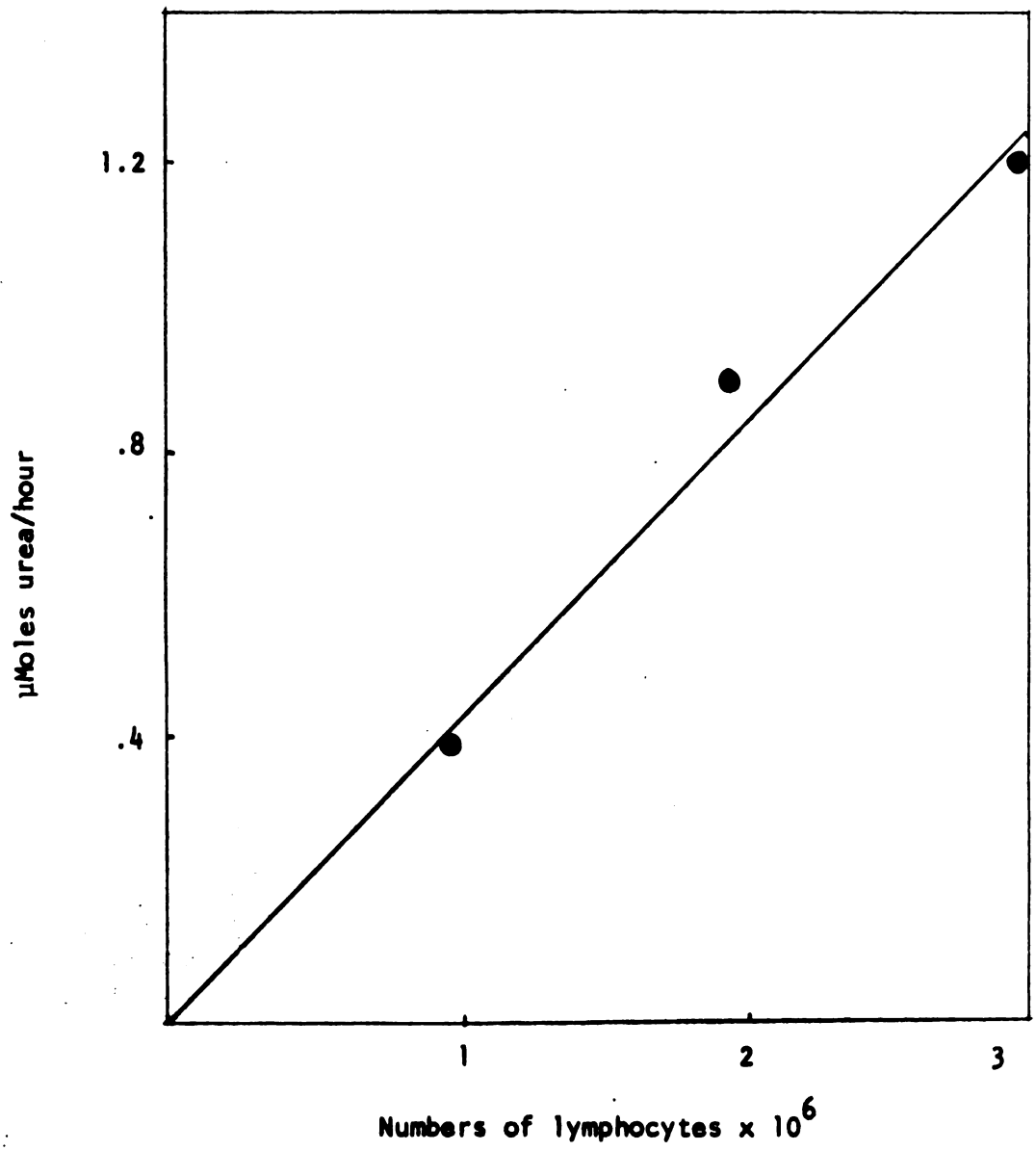


Fig. 20.--Urea production by arginase as a function of lymphocyte protein concentration.

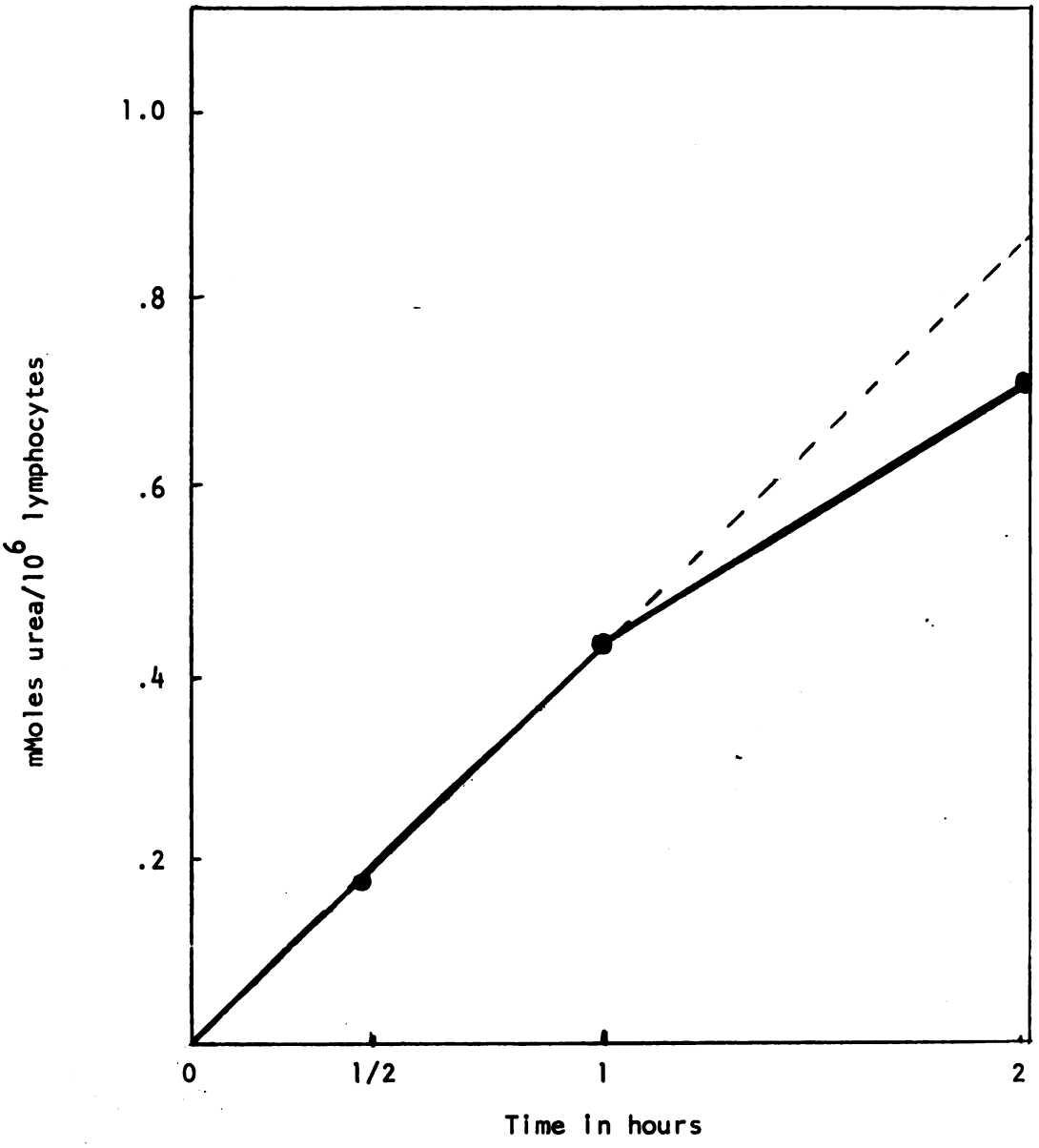


Fig. 21.--Urea production by arginase in liver as a function of time.

Establishment of Michaelis-Menten Constants (Km)

Michaelis-Menten constants were measured and compared between liver and lymphocytes. Figure 22 shows the velocity as a function of the substrate level for the liver enzyme. The velocity was linear with respect to substrate to about 10 mM and then gradually leveled off. The substrate vs velocity curve for the lymphocytes (Figure 23) is linear to about 5 mM, and then approaches a level.

Lineweaver-Burk Plots

Lineweaver-Burk plots (1/V vs 1/S) were done for liver and lymphocytes. From the Lineweaver-Burk plot for the liver, the Km is estimated as 10 mM (Figure 24). The same plot for lymphocytes results in a Km estimate of 2.0 mM (Figure 25). When the experiments were repeated, the Km estimates were 11.1 mM for liver and 2.2 mM for lymphocytes.

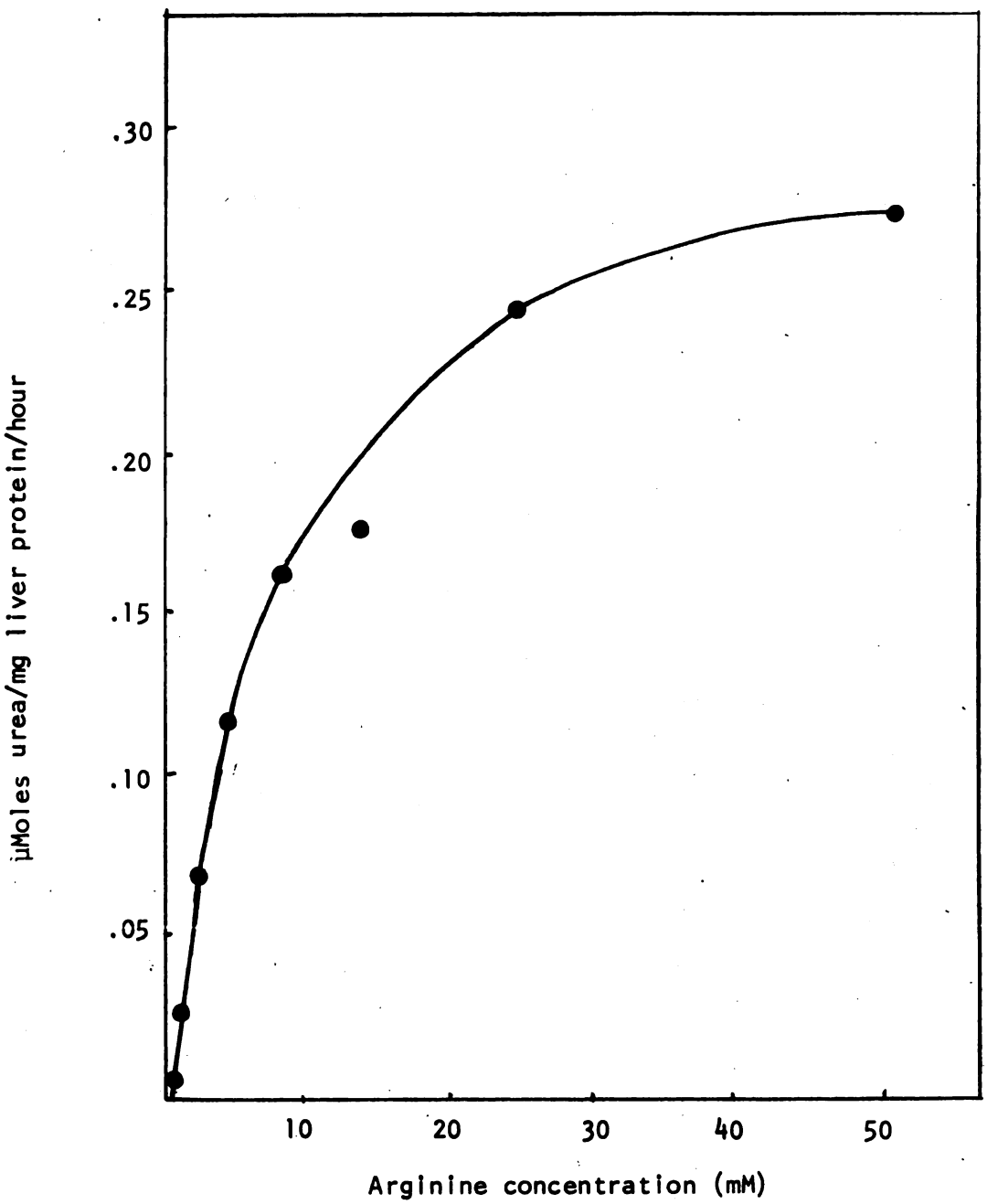


Fig. 22.--Rate of urea production by arginase in liver as a function of arginine concentration.

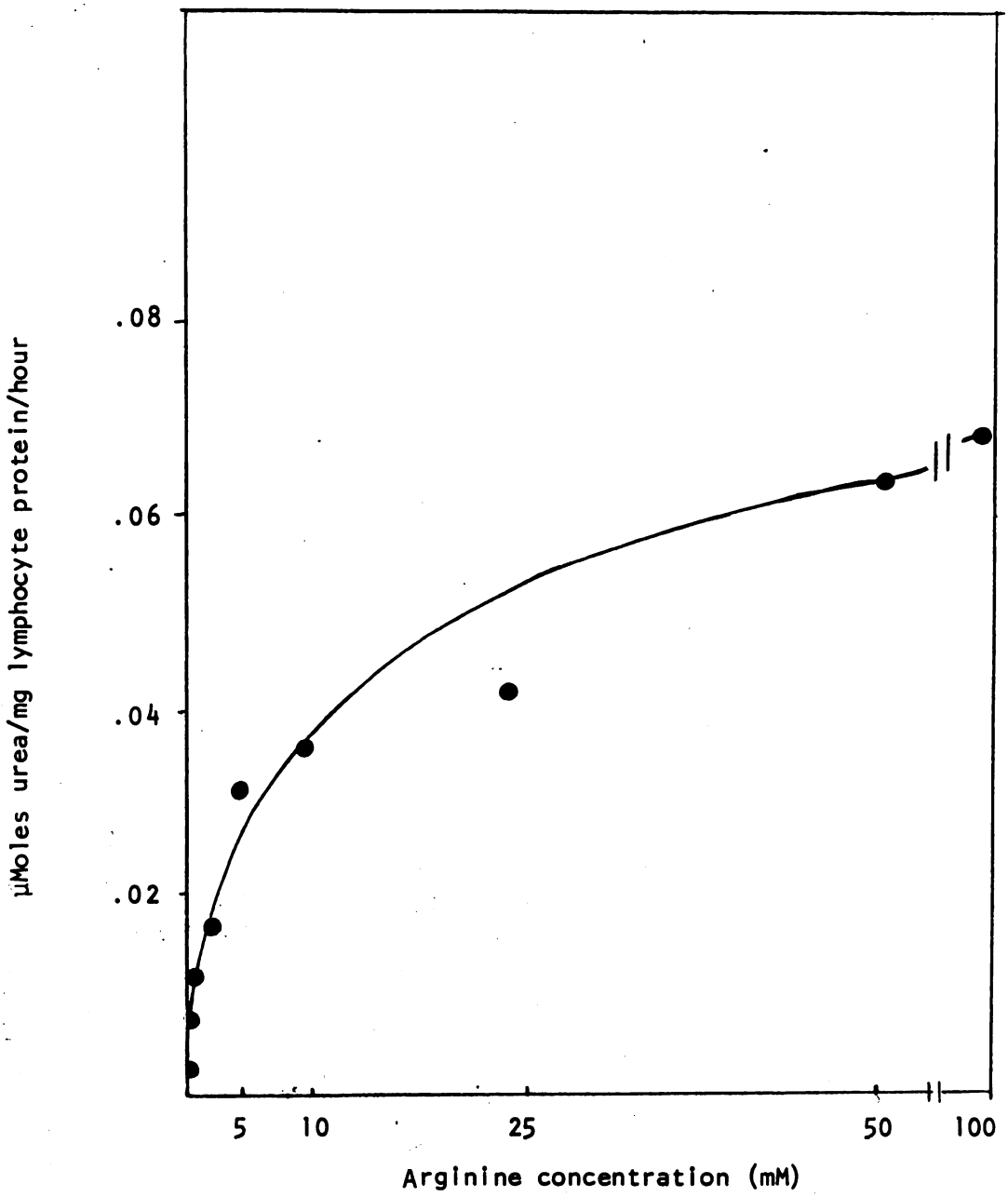


Fig. 23.--Rate of urea production by arginase in lymphocytes as a function of arginine concentration.

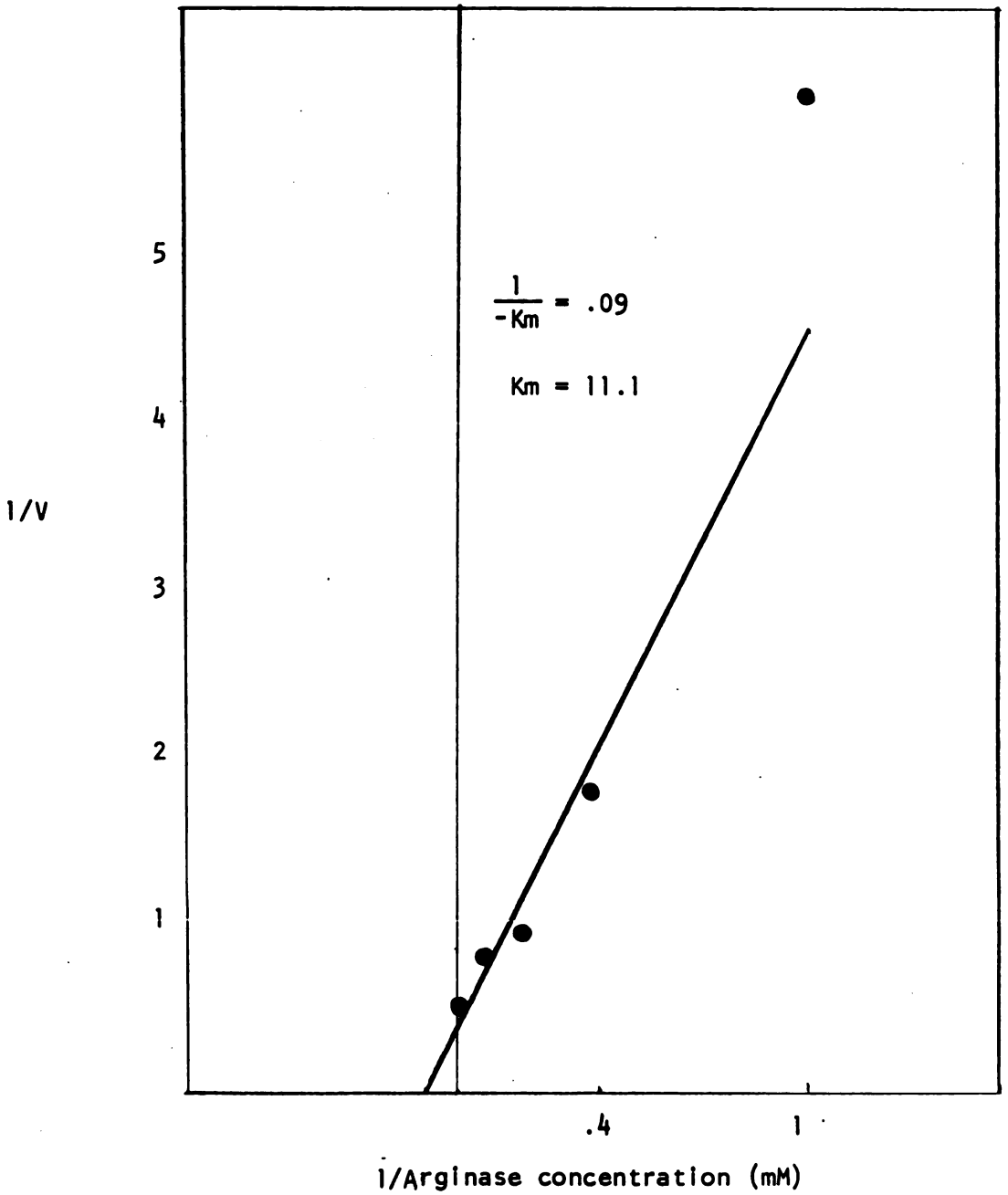


Fig. 24.--Lineweaver-Burk plot (1/V vs 1/S) of arginase in liver as a function of arginine concentration.

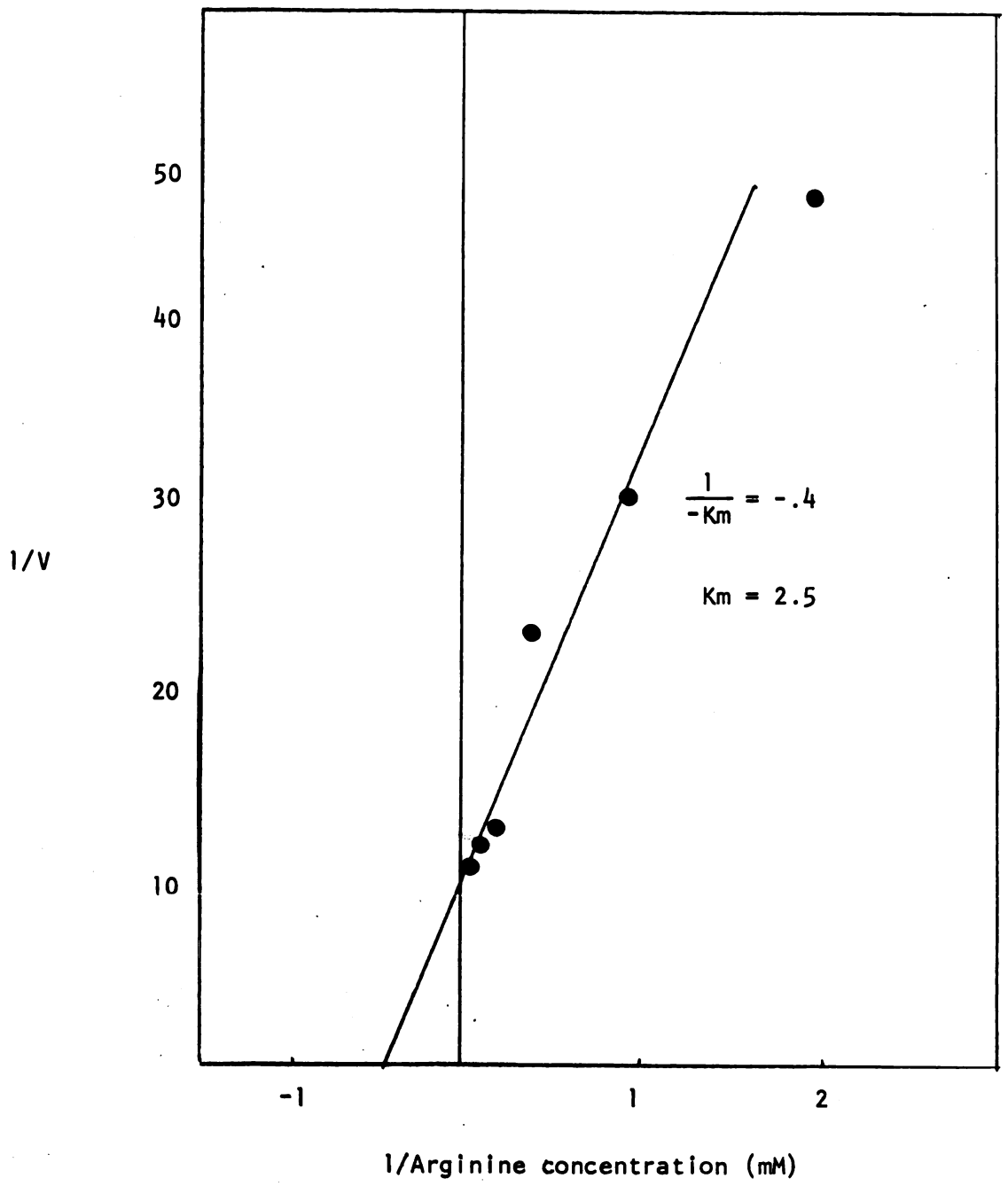


Fig. 25.--Lineweaver-Burk plot (1/V vs 1/S) of arginase in lymphocytes as a function of arginine concentration.

Hanes Plots

Hanes plots (S/V vs S) were done on the same data as the Lineweaver-Burk plots. Both experiments for liver gave Km estimates of 11.1 mM arginine (Figure 26). One experiment gave a Km estimate of 2.0 mM arginine for lymphocytes (Figure 27) and the second gave a Km estimate of 2.2 mM arginine.

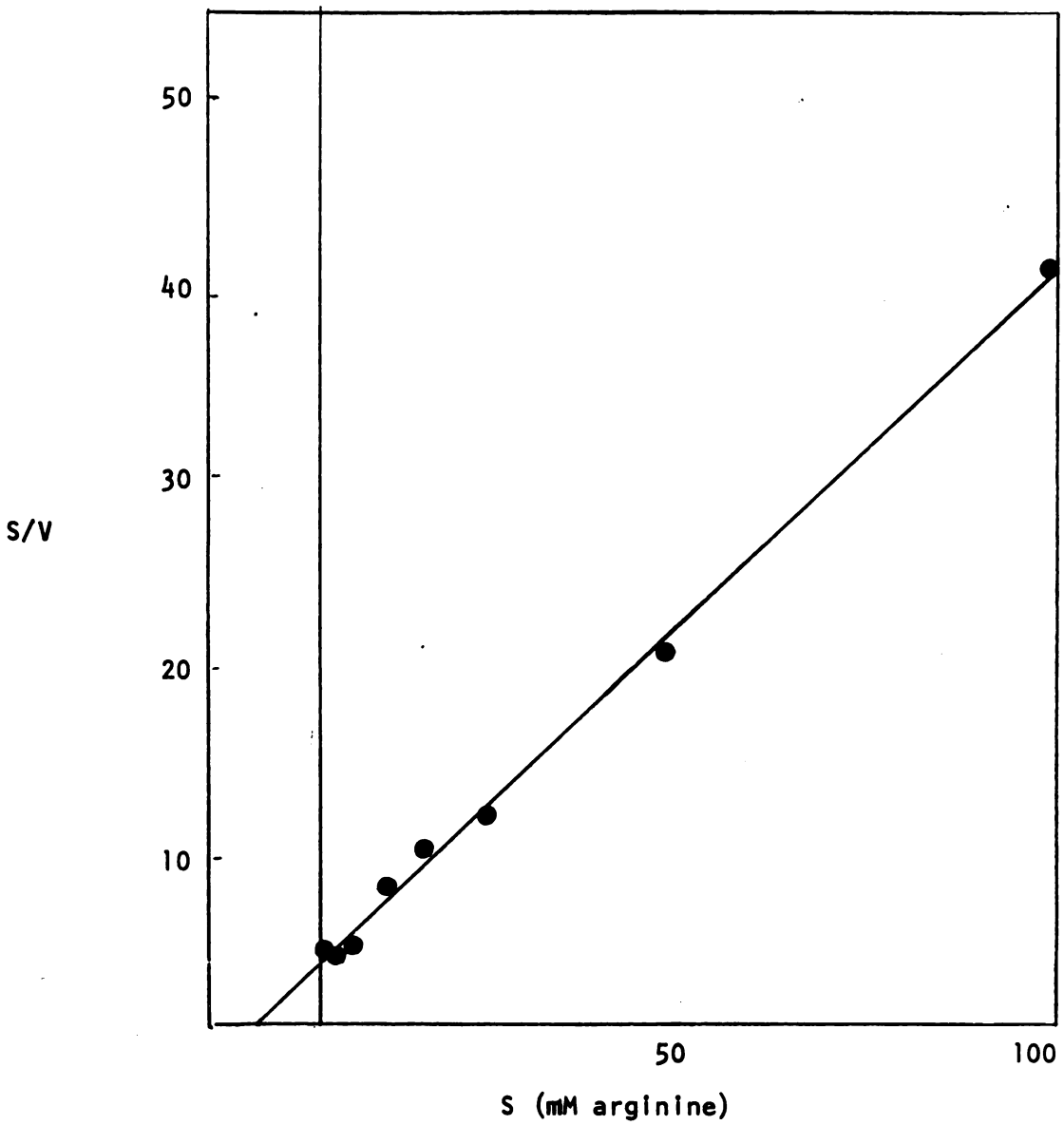


Fig. 26.--Hanes plot (S/V vs S) of arginase in liver as a function of arginine concentration.

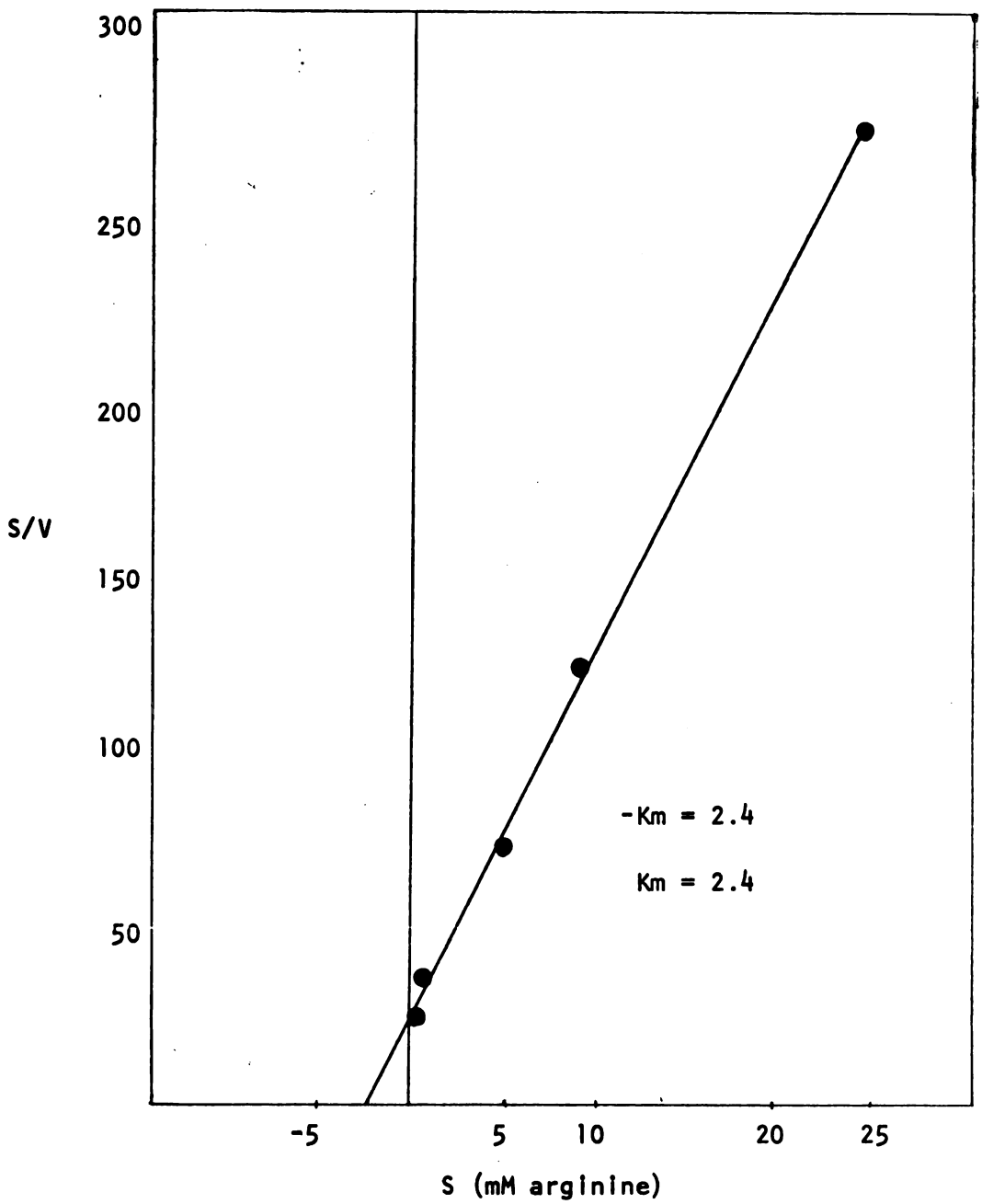


Fig. 27.--Hanes plot (S/V vs S) of arginase in lymphocytes as a function of arginine concentration.

DISCUSSION

Lymphocytes

The advent of long-term lymphocyte cultures and the laboratory interest in errors of the urea cycle led to the study of the enzymes of the ornithine-urea cycle in these lymphocytes.

Growth Rates and Viability of Lymphocytes

Streeter et al. (1973) in studies on optimal growth conditions for lymphocytes reported maximum levels of 1.6 to 1.8 X 10^6 lymphocytes per ml with 30% nonviable cells. In the present study, line 43 had .075 X 10^6 lymphocytes per ml at harvest, with 20% nonviable cells. Line 61 had .75 X 10^6 lymphocytes per ml at harvest, about half of that reported by Streeter et al. However, the viability of the present study was greater, with 85% of the lymphocytes viable. Because the desire was to have maximum viability

and logarithmic growth rates for the enzyme assays, our population densities were reduced.

Growth on Arginine-Free D' Medium

Spector and Bloom (1973) have reported that cultured lymphocytes from normal people could utilize citrulline, in arginine-free medium, to form the needed arginine for normal cell growth. If the complete ornithine-urea cycle were present in cultured lymphocytes, then ornithine, as well as citrulline, could serve as an arginine source. Arginine-free RPMI 1640 (Appendix I) was not readily available; therefore, an attempt was made to adapt the lymphocytes to D' medium (Appendix II). This medium was available in both arginine-free and arginine-added forms. This medium has high concentrations of all other amino acids except glutamic acid, plus high concentrations of vitamins and additional glucose; however, less fetal calf serum had been added than was used with RPMI 1640. The lymphocytes did not grow in D' whether arginine was present or was substituted by ornithine. It is possible that such high concentrations of the ingredients have an

inhibiting effect on the growth of the lymphocytes, or that the change in available nutrients did not allow adaptation to take place. Chu (personal communication) is successful with D' medium in plating experiments with lymphocytes. The reasons for the difference in the reaction of the lymphocytes in culture and in plating experiments is presently unknown. Since the lymphocytes in this laboratory are preadapted to RPMI 1640, culturing the cells in arginine-free 1640 medium with ornithine substituted for arginine, would establish whether the enzyme activities described in this study are sufficient to produce arginine for growth.

The Enzyme Assays

The four enzymes studied were ornithine carbamoyl transferase (OCT), argininosuccinic acid synthetase (ASAS), argininosuccinic acid lyase (ASase), and arginase. By colorimetric methods, all but ASAS were determined to be present. ASAS activity has been demonstrated by radiochemical methods in another laboratory (Kennaway, as reported by Spector and Bloom, 1973).

Ornithine Carbamoyl Transferase

Nonenzymatic Reaction

Brown and Cohen (1959) stated that "Some nonenzymatic transcarbamylation accompanies the enzymatic transcarbamylation" (p. 1771). Schimke (1961) noted the instability of carbamoyl phosphate, but did not suggest the extent of the spontaneous reaction. Levin (1971) noted that besides this reaction, the carbamoyl phosphate also reacts with the glycylglycine buffer. Because most assays use the high activities of liver, the relative differences between the reagent blanks and the experimental tubes are large. However, studies done on OCT levels in serum indicated that the nonenzymatic conversion of carbamoyl phosphate and ornithine to citrulline in blood probably equalled the amount converted enzymatically (Snodgrass and Parry, 1969). In the present study the nonenzymatic conversion was of the same order of magnitude as the conversion by 10^6 lymphocytes, but at low substrate levels the enzymatic conversion was much greater in proportion. In studies of lymphocytes, in which specific activity is low, reagent blanks become essential for each assay level.

OCT activity had not previously been reported in lymphocytes. In the present study, specific activity was shown to be .41 μmoles of citrulline per milligram protein per hour. Subsequently, the Michaelis-Menten constants were established for each substrate.

Potentially, showing OCT activity in lymphocytes in long-term culture could solve the problem of identifying carriers of OCT deficiency. If, as Short et al. (1973) have suggested, the gene is sex-linked, then two populations of lymphocytes are possible: one with the normal gene being active, the other with only the abnormal gene. The detection of the presence of enzymatic activity in one clone and the absence in another from the same person would be strong evidence for carrier status. Identifying heterozygotes at present is unsuccessful because the activity levels of OCT activity in them overlap normal levels when determined on liver samples obtained by needle or surgical biopsy.

Table 8 lists the KM's established for OCT from several sources, including the present study.

TABLE 8.--Michaelis-Menten constants for ornithine carbamoyl transferase.

Source	ORN Km (mM)	CP Km (mM)	pH	Reference
E. coli	1.5	.196	8.5	Rogers and Novelli, 1962
S. lactis	2	3.7	8.5	Cohen and Marshall, 1962
Rat liver	1.4	.41	7.5	Reichard, 1957
"purified liver"	3.0	1.2	8.0	Burnett and Cohen, 1957
Ox liver	1.4	1.7	8.3	Joseph et al., 1963
Human liver	1.26-1.47	1.45-1.67	?	Matsuda et al., 1971
Human liver (mutant)	1.25	6.65		Matsuda et al., 1971
Human serum	.069	.97-.95	7.9	Snodgrass and Parry, 1969
Rat liver	3.0	3.3	8.3	Present study
Human lymphocytes	15-17.5	1.5-2.2	8.3	Present study

ORN = ornithine

CP = carbamoyl phosphate

Ornithine Km for OCT

It can be seen that the ornithine Km's established for human lymphocytes are an order of magnitude different from those established for human liver (Matsuda et al., 1971). It is not clear from the table what effect the range of pH has on the recorded Km. Though it is not included in the table, most of the assays were buffered by TRIS rather than by glycylglycine buffer. Therefore, the conditions of the assay are not directly comparable. Even so, the magnitude of the Km difference found is not easily explained. The low concentration of glycylglycine buffer (.05 M) used in these experiments must be considered. This concentration is used to minimize the reaction of glycylglycine with carbamoyl phosphate, but may not be sufficient to maintain the pH throughout the experimental period (Levin, 1971).

The large amount of protein has its own buffering effect, perhaps overwhelming the effect of the glycylglycine buffer and producing a pH very different from the one with which the experiment was initiated. There are two methods to overcome this difficulty. Enzyme purification (Burnett and Cohen, 1957) on large amounts of

lymphocytes could produce higher specific activities, and reduce the level of extraneous protein. The second method would be to use radioisotopes. Using ^{14}C-labeled carbamoyl phosphate would allow the use of a higher concentration of glycylglycine buffer, as citrulline would be separated from the other constituents in the assay mixture before determination (Schimke, 1963). By this separation, the spontaneous reaction of carbamoyl phosphate with glycylglycine buffer would not contribute to spurious product formation.

Isozymes for OCT

There may be isozymes of OCT present in the lymphocytes that have not been detected in the liver. Isozymes are proteins with similar catalytic properties which differ from each other in one or more ways. They may be as similar as Hemoglobin A is to Hemoglobin S, differing by only one amino acid (See McKusick, 1972, for a recent list of all known hemoglobin differences in humans discovered to date), or they may be different combinations of polypeptide chains, such as occurs with lactate dehydrogenase (Harris, 1971). Lactate dehydrogenase consists of

all possible tetrameric combinations of subunits A and B, each with its own isoelectric point. Skeletal muscle LDH consists almost entirely of A4, while heart muscle LDH consists mostly of B4. Other tissues have other combinations.

There are at least two isozymes of human OCT: liver and serum. If human serum OCT has a Km of 1/50 of that of human liver OCT for the substrate ornithine (.069 mM vs 1.26 mM), another Km of 10 times the liver Km in another tissue may not be surprising.

Different species usually have different molecular forms of the same enzyme. The more widely divergent the species, the more the isozymes differ. The lymphocytes which are in permanent culture presumably are infected with an Epstein-Barr virus (Gerber, 1973).

> Viral-induced enzymes generally differ from enzymes present in uninfected cells in kinetic, chromatographic, and immunological properties. Frequently, these altered characteristics have been observed even after extensive enzyme purifications . . . if the properties of the new enzyme differ from those of the normal host enzyme, then it is unlikely that a normally functioning host gene is "derepressed" or induced to synthesize increased amounts of the same enzyme species (Kit and Dubbs, 1969, p. 55).

Although it cannot be proven at present, the viral infection may have induced the enzymatic activity observed

in the lymphocytes. The first steps in solving this problem could involve establishing that there is, indeed, an isozyme of OCT in cultured lymphocytes that is not in liver; or, short-term cultured lymphocytes (without virus) could be tested for enzymatic activity of OCT. If the short-term cultured lymphocytes do not have OCT activity, and the enzyme in the lymphocytes in long-term culture is an isozyme of OCT, it is more likely that the virus is responsible for the enzymatic activity than the host lymphocyte. If lymphocytes in short-term culture show OCT activity then most likely the isozyme is the product of the lymphocytic DNA.

If lymphocytes from patients who have altered OCT characteristics in their liver could be induced to grow, the activities of those cell lines would establish whether liver changes were concurrent with changes in cultured lymphocytes. Concurrent changes of lymphocytic and liver enzymes would suggest common genetic control.

Carbamoyl phosphate Km for OCT

The Km estimates as listed in Table 8 for carbamoyl phosphate for OCT in lymphocytes are very near to

that established for human liver (Matsuda et al., 1971). They are slightly different from that mentioned for human serum OCT (Snodgrass and Parry, 1969): 1.5-2.2 mM vs .97-.95 mM. However, all Km's of carbamoyl phosphate in OCT are similar except for that of E. coli (Rogers and Novelli, 1962), and for rat liver (Reichard, 1957). The former was .196 mM, the latter .41 mM. In the present study the rat liver Km (3.3 mM) differs significantly from that of Reichard (1957), but it is only double that for "purified liver" (Burnett and Cohen, 1957), ox liver (Joseph et al., 1963), and human liver (Matsuda et al., 1971). Either differences in strains of laboratory rats or differences in experimental conditions may have affected the Km determination slightly. But it is difficult to explain the large Km differences between the liver value given by Reichard and that determined in the present study.

Argininosuccinic Acid Lyase

ASase had been shown indirectly in the lymphocytes by Spector and Bloom (1973), but the enzyme activity had never been measured. Spector and Bloom (1973) showed that

the cultured lymphocytes could incorporate the label from ^{14}C-labeled citrulline into the TCA precipitable fraction. This was interpreted as the lymphocytes having changed citrulline into arginine, through ASAS and ASase. While tests for OCT and arginase required 2 X 10^6 cells per ml of assay medium, the ASase assay required 4 X 10^7 cells per ml of assay medium, a 20-fold increase. In addition, the small amount of background color from the lysate that was present in the OCT assays became a significant factor in the ASase assays. Therefore, preboiled lymphocyte lysate was an essential control. As the ASA did not break down spontaneously nor contribute color to the assay, no allowance for extraneous color was necessary. Approximately 600 X 10^6 lymphocytes would have been required for a Km determination on ASase. Three months would have been required to accumulate the cells for one Km determination; cost of media precluded this assay.

Arginase

Although arginase activity had been shown in freshly drawn "leukocytes" by Reynolds et al. (1957), it had not

been previously shown in lymphocytes in long-term culture. Therefore, when the presence of arginase activity was established, it was decided to determine the Km for the lymphocyte arginase.

Arginine Km for Arginase

The published Km for bovine liver arginase is 11.6 mM arginine (Hunter and Downs, 1945). The experiments herein described produced a Km for rat liver of 11.1 mM arginine, virtually the same as for bovine liver. The Km for the lymphocytes was 2.1 mM arginine in these experiments.

Isozymes for Arginase

There may be isozymes of arginase in the lymphocytes that have not been detected in liver. However, it is not known that isozymes of arginase exist within the same organism. The genetic evidence is that arginase activity is produced by the same protein in liver and in red blood cells, as the elimination of arginase activity in liver is accompanied by a similar loss in RBC's

(Terheggen et al., 1969). The difference in the Km's of the liver and lymphocytes in these experiments requires consideration of isozymal activities as previously described for OCT.

SUMMARY

The present study was undertaken to determine which of the enzymes of the ornithine-urea cycle occur in lymphocytes in long-term culture. Three enzymes: ornithine carbamoyl transferase, argininosuccinic acid lyase, and arginase were demonstrated colorimetrically in the lymphocytes. Kinetic studies of ornithine carbamoyl transferase, using ornithine as substrate, and arginase indicated that the isozymes found in the lymphocytes were distinct from those in rat liver. It is highly probable that the lymphocytes in long-term culture were infected with Epstein-Barr virus; therefore, it cannot be stated whether the isozymes are the product of lymphocytic or viral DNA.

APPENDICES

APPENDIX I

RPMI-1640

COMPONENT	mg/L
$Ca(NO_3)_2 \cdot 4H_2O$	100
Glucose	2000
$MgSO_4 \cdot 7H_2O$	100
KCL	400
$Na_2HPO_4 \cdot 7H_2O$	1512
NaCl	6000
L-Arginine (free base)	200
L-Asparagine	50
L-Aspartic Acid	20
L-Cystine	50
L-Glutamic acid	20
L-Glutamine	300
Glutathione (reduced)	1
Glycine	10
L-Histidine (free base)	15
L-Hydroxyproline	20
L-Isoleucine (Allo free)	50
L-Leucine (Methionine free)	50
L-Lysine HCL	40
L-Methionine	15

COMPONENT	mg/l
L-Phenylalanine	15
L-Proline (Hydroxy L-Proline free)	20
L-Serine	30
L-Threonine (Allo free)	20
L-Tryptophane	5
L-Tyrosine	20
L-Valine	20
Biotin	.2
Vitamine B12	.005
D-Ca pantothenate	.25
Choline Cl	3
Folic Acid	1
i-Insitol	35
Nicotinamide	1
Para Aminobenzoic Acid	1
Pyridoxine HCl	1
Riboflavin	.2
Thiamine HCL	1
Phenol red	5
$NaHCO_3$	2000

APPENDIX II

D' MEDIUM

COMPONENT	mg/L	COMPONENT	mg/L
NaCl	6800	L-Methionine	22.5
KCl	400	L-Phenylalanine	48
$NaH_2PO_4 \cdot H_2O$	140	L-Proline	230
$MgSO_4 \cdot 7H_2O$	200	L-Serine	210
$CaCl_2$ (anhyd.)	200	L-Threonine	72
Glucose	3333	L-Tryptophan	15
L-Alanine	178	L-Tyrosine	54
L-Arginine	36	L-Valine	69
L-Asparagine$\cdot H_2O$	300	Choline C	1.5
L-Aspartic acid	266	Folic acid	1.5
L-Cystine	36	i-Inositol	3.0
L-Glutamic acid	294	Nicotinamide	1.5
L-Glutamine	292	D-Ca pantothenate	1.5
Glycine	150	Pyridoxal HCl	1.5
L-Histidine	46.5	Riboflavin	15
L-Isoleucine	78.75	Thiamine HCl	1.5
L-Leucine	78.60	$NaHCO_3$	2200
L-Lysine	87	Na Pyruvate	.36

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Allan, J. D., D. C. Cusworth, C. E. Dent, and V. D. Wilson. 1958. "A disease probably hereditary, characterized by severe mental deficiency and a constant gross abnormality of amino acid metabolism." Lancet i: 182-187.

2. Anon. 1970. Cellular probing for genetic information. J. AMA 213: 289-90.

3. Anon. 1972. Gene therapy for human genetic disease. Science 178: 648-9.

4. Archibald, R. M. 1945. Colorimetric determination of urea. J. Biol. Chem 157: 507.

5. Armstrong, M. D., K. N. Yates, and M. G. Stemmermann. 1964. "An occurrence of argininosuccinic aciduria." Pediatrics 33: 280-282.

6. Berenberg, W., E. S. Kang. 1971. The congenital hyperammonemic syndrome. Dev. Med. Child. Neurob. 13: 355-61.

7. Bickel, H., D. Feist, H. Muller, G. Quadbeck, and M. Kekomaki. 1969. "Ornithinaemia. Another disorder of amino acid metabolism associated with brain damage." Gen. Med. Monthly 14: 101-105.

8. Bickel, H. 1969. "Hyperornithinaemia due to a defect of ornithine ketoacid transaminase." Proc. of the 6th Annual Symp. of the Society for the Study of Inborn Errors of Metabolism. Zurich, 1968, pp. 145-146. Livingstone, Edinburgh.

9. Brown, G. W., Jr., and P. P. Cohen. 1959. Comparative biochemistry of urea synthesis I. Methods for the quantitative assay of urea cycle in liver. J. Biol. Chem. 234: 1764.

10. Brown, H. 1970. Hepatic Failure. pp. 55-92; 116-124.

11. Bruton, O. J., J. A. N. Corsellis, and A. Russell. 1969. "Hereditary hyperammonaemia." Brain 93: 423-434.

12. Buergi, W., R. Richterich, J. P. Colombo. 1966. L-lysine dehydrogenase deficiency in a patient with congenital lysine intolerance. Nature (London) 211: 854-855.

13. Bumgartner, R., S. Scheideyger, G. Stalder, and A. Hottinger. 1968. Arginibersteinsauer-Krankhei des neugeborenen mit latelem verlaux. (Neonatal death due to argininosuccinic aciduria) Helv. Paediat. Acta 23: 77-106.

14. Burnett, G. H., P. P. Cohen. 1957. Studies of carbamoyl phosphate-ornithine transcarbamylase. J. Biol. Chem. 299: 337-344.

15. Campbell, A. G. M., L. E. Rosenberg, P. J. Snodgrass, C. T. Nuzum. 1971. Lethal neonatal hyperammonemia due to complete ornithine transcarbamylase deficiency. Lancet 2: 217-8.

16. Campbell, A. G., L. E. Rosenberg, P. J. Snodgrass, C. T. Nuzum. 1973. Ornithine transcarbamylase deficiency: a cause of lethal neonatal hyperammonemia in males. N. Eng. J. Med. 288: 1-6.

17. Carlisky, N. J., V. Botbol, C. A. Garcia-Argiz, A. Barrio, V. L. Leev. 1968. Properties and subcellular distribution of ornithine cycle enzymes in amphibian kidney. Comp. Biochem. Physiol. 25: 835-48.

18. Carson, N. A. J., D. W. Neill. 1962. Metabolic abnormalities detected in a survey of mentally backward children in Northern Ireland. Arch Dis. Childhood 37: 505-513.

19. Carton, D., F. DeSchryver, J. Kint, J. Van Durme, C. Hooft. 1969. Argininosuccinic aciduria. Neonatal variant with rapid fatal course. Acta. Pediat. Scond. 58: 528-534.

20. Cederbaum, S. D., K. N. F. Shaw, M. Valente, M. E. Cotton. 1973. Argininsuccinic aciduria. Am. J. Ment D. 77: 395.

21. Choi, K. W., A. D. Bloom. 1970. Biochemically marked lymphocytoid lines establishment of Lesch Nyhan cells. Science 170: 89.

22. Cohen, P. P., J. H. Sallach. 1961. In Metabolic Pathways (D. M. Greenberg, ed) New York, Acad. Press 2: 1.

23. Cohen, P. P., and M. Marshall. 1962. In The Enzymes. 6: 327.

24. Colombo, J. P. 1971. Congenital Disorders of the Urea Cycle and Ammonia Detoxication. S. Karger (Basel) Monographs in Paediatrics, Vol. 1.

25. Colombo, J. P., W. Buergi, R. Richterich, and E. Rossi. 1967. Congenital lysine intolerance with periodic amino acid intoxication: a defect in L-lysine degradation. Metab. Clin. Exp. 16: 910-925.

26. Colombo, J. P., F. Vassella, R. Humbel, W. Buergi. 1967. Lysine intolerance with periodic ammonia intoxication. Amer. J. Dis Child 113: 138-141.

27. Corbeel, L. M., J. P. Colombo, M. van Sande, A. Weber. 1969. Periodic attacks of lethargy in a baby with ammonia intoxication due to a congenital defect in ureogenesis. Arch. Dis. Childhood 44: 681-687.

28. Corbeel, L., J. P. Colombo, M. van Sande, and A. Weber. 1968. Periodic attacks of lethargy in a baby with ammonia intoxication and defect in ureogenesis. Proceedings of the 6th Annual Symposium of the Society for the Study of Inborn Errors of Metabolism, Zurich, pp. 115-118.

29. Coryell, M. E., W. K. Hall, T. G. Theraos, D. A. Welter, A. J. Gatz, B. F. Horton, B. D. Sisson, J. W. Loopey Jr., and R. T. Farrow. 1964. A familial study of a human enzyme defect, argininosuccinic acidurea. Biochem Biophys Res. Commun. 14: 307-314.

30. Crane, C. W., W. M. Gay, and F. A. Jenner. 1969. Urea production from labelled ammonia in argininosuccinic acidurea. Clin. Chim. Acta 24: 445.

31. Davidson, R. G., H. M. Nitowski, B. Childs. 1963. Demonstration of two populations of cells in the human female heterozygous for glucose-6 phosphate dehydrogenase variants. PNAS, USA. 50: 481-485.

32. Dent, C. E. 1959. Argininosuccinic acidurea: A new form of mental deficiency due to metabolic causes. Proc. Roy. Soc. Med. 52: 885.

33. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. Science 130: 432.

34. Efron, M. L., D. Young, H. W. Moser, and R. A. MacCready. 1964. A simple chromatographic screening test for the detection of disorders of amino acid metabolism. New England J. Med. 270: 1378-1383.

35. Freeman, J. M., J. F. Nicholson, W. S. Masland, L. P. Rowland, S. Carter. 1964. Ammonia intoxication due to a congenital defect in urea synthesis. J. Pediat. 65: 1039-1640. Abstr.

36. Garrod, A. E. 1909. Inborn errors of metabolism. Oxford Univ. Press, London.

37. Gerraerts, F., L. Schimpfessel, N. VanTieghem, J. DeBruyne, and R. Crockaert. 1972. Lactate dehydrogenase isoenzymes in normal and virus-transformed hamster cells. Archives Internationales de Physiologie et de Biochimie. 80: 396.

38. Gerber, P. 1973. The role of Epstein-Barr virus in establishing long-term human lymphocyte cultures. In: Long-Term Lymphocyte Cultures in Human Genetics, D. Bergsma, ed., Birth Defects: Original Article Series, Vol. IV, No. 1. The National Foundation-March of Dimes.

39. Greenberg, D. M. 1955. "Arginase." In: Methods in Enzymology, Vol. II, p. 368.

40. Harris, H. 1971. The Principles of Human Biochemical Genetics. American Elsevier Publ. Co., Inc. New York.

41. Harris, H. 1973. Potentialities of lymphocytoid lines in human biochemical genetics. Birth Defects 9: 132-7.

42. Havir, E. A., H. Tamir, S. Ratner, R. C. Warner. 1963. Biocynthesis of urea XL \ Preparation and properties of chrystalline orgininosuccinase. J. BC 240: 3079.

43. Hirschhorn, K., H. L. Nadler, W. I. Waithe, B. I. Brown, and R. Hirschhorn. 1969. Pompe's disease: Detection of heterozygotes of lymphocyte stimulation. Science 166: 1632-3.

44. Hommes, F. A., C. J. Degroot, C. W. Wilmink, J. H. P. Jonnis. 1969. Carbamyl phosphate synthetase deficiency in an infant with severe cerebral damage. Arch. Dis. Childhood 44: 688-693.

45. Hopkins, I. J., J. F. Conelly, A. G. Dawson, F. J. R. Herd, I. T. G. Maddison. 1969. Hyperammonaemia due to ornithine transcarbamylase deficiency. Arch. Dis. Childhood 144: 143-148.

46. Hsia, D. Y. 1972. Utilization of leukocytes for the study of inborn errors of metabolism. Enzyme 13: 161-8.

47. Hunter, A. and C. E. Downs. 1945. The inhibition of arginase by aminoacids. JBC 157: 427-446.

48. Jacob, J. and J. Monod. 1961. Genetic regulatory mechanism in the synthesis of protein. J. Mol. Biol. 3: 318-356.

49. Jacoby, L. B., J. W. Littlefield, A. Milunsky, V. E. Shih, and R. S. Wilroy, Jr. 1972. A microassay for argininosuccinase in culture cells. Amer. J. Hum. Genetics 24: 321-324.

50. Joseph, R. L., E. Baldwin, and D. C. Watts. 1963. Studies on carbamoyl phosphate-L-ornithine carbamoyltransferase from ox liver. Bioch. J. 87: 409.

51. Joseph, R. L., D. C. Watts, and E. Baldwin. 1964. The comparative biochemistry of carbamoyl phosphate-L-ornithine carbamoyltransferases in relation to their evolution. Comp. Biochem. Physiol. 11: 119.

52. Kang, E., P. J. Snodgrass. 1973. Lethal ornithine transcarbamylase deficiency in the newborn. Pediatrics.

53. Katunuma, N., N. Okada, T. Natsuzawa, Y. Otuka. 1965. Studies on ornithine keto-acid transaminase II. Role in metabolic pathway. J. Biochem. (Tokyo) 57: 445-449.

54. Katunuma, N., N. Okada, Y. Nishi. 1966. Regulation of the urea cycle and TCA cycle by ammonia. Advan. Enzyme Regul. 4: 317-335.

55. Keating, J. P., R. D. Feigin, S. M. Tenenbau, and R. E. Hillman. 1972. Hyperglycinemia with ketosis due to a defect in isoleusine metabolism--Preliminary report. Pediatrics 50: 890.

56. Kekomäki M. P., N. C. Räikä, H. Bickel. 1969. Ornithine ketoacid aminotransferase in human liver with reference to patient with hyperornithinaemia and familial protein intolerance. Clin. Chim. Acta 23: 203-208.

57. Kekomäki, N. P. 1968. Citrullinaemia. Pediatrics 41: 690.

58. Kennan, A. L. and P. P. Cohen. 1961. Ammonia detoxication in the liver. Proc. Soc. Exp. Biol. Med. 106: 170-173.

59. Kim, S., W. K. Paik, and P. P. Cohen. 1972. Ammonia intoxication in rats. PNAS, USA 69: 3530.

60. Kint, J., and D. Carton. 1968. Deficient argininosuccinase activity in brain in argininosuccinic acidurea. Lancet ii: 635.

61. Kirkman, H. N., and J. L. Kiesel. 1969. Congenital hyperammonemia. Pediat. Res. 3: 358-9. Abstr.

62. Kit, S., and D. R. Dubbs. 1969. Enzyme Induction by Viruses. Monographs in Virology, Vol. 2. J. L. Melnick, ed. S. Karger, New York.

63. Kleczkowski, K., and P. P. Cohen. 1964. Purification of ornithine transcarbamylase from pea seedlings. Arch Biochim Biophys 107: 271.

64. Krebs, A. A., H. Henseliet. 1932. Untersuchunger über die harnstaff-bildung im Tierkörper. Hoppe-Seyler's. Z. Physiol. Chem. 210: 33-36.

65. Kulhanek, V., and V. Vojtiskova. 1964. On the determination of ornithine carbamyltransferase activity. Clin. Chim. Acta 9: 95-96.

66. Levin, B., and R. H. Dobbs. 1968. Hereditary metabolic disorders involving the urea cycle. Proc. Roy. Soc. Med. 61: 773-774.

67. Levin, B. 1967. Argininosuccinic aciduria. Amer. J. Dis. Child. 113: 162-165.

68. Levin, B., H. N. N. Mackay, V. G. Oberholzer. 1961. Argininosuccinic aciduria: An inborn error of amino acid metabolism. Arch. Dis. Childhood 36: 662-632.

69. Levin, B. 1968. Hyperammonaemia. Proceedings of the 6th Annual Symposium of the Society for the Study of Inborn Errors of Metabolism, Zurich. pp. 123-130. Livingston, Edinburgh, 1969.

70. Levin, B., and A. Russell. 1967. Treatment of hyperammonemia. Amer J. Dis. Child. 113: 142-145.

71. Levin, B., J. M. Abraham, V. G. Oberholzer, and E. A. Burgess. 1969. Hyperammonaemia: A deficiency of liver ornithine transcarbamylase occurrence in mother and child. Arch. Dis. Childhood 44: 152-161.

72. Levin, B., R. H. Dobbs, E. A. Burgess, and T. Palmer. 1969. Hyperammonaemia. A variant type of deficiency of liver ornithine transcarbamylase. Arch. Dis. Childhood 44: 162-169.

73. Levin, B., V. G. Oberholzer, and L. Sinclain. 1969. Biochemical investigations of hyperammonaemia. Lancet ii: 170-174.

74. Levin, B. 1971. Hereditary metabolic disorders of the urea cycle. Adv. Clin. Chem. 14: 65-143.

75. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265.

76. Lundgren, E. 1972. Isozyme variations in human cells grown in vitro. VI Further data on the amino acid naphthylamidase isozymes. Human Heredity 21: 580.

77. Lyon, M. F. 1961. Gene action in the S-chromosome of the mouse (Mus musculus L.). Nature 190: 372.

78. MacLeod, P., S. Mackenzie, and C. R. Scriver. 1972. Partial ornithine carbamyl transferase deficiency: An inborn error of the urea cycle presenting as orotic aciduria in a male infant. Can. Med. Assoc. J. 107: 405.

79. Malmquist, J., R. Jadenburg, and G. Lindstedt. 1971. Familial protein intolerance. Possible nature of enzyme defect. N. Eng. J. Med. 284: 997-1002.

80. Matsuda, J. S. Arashima, H. Nanbu, Y. Takekoshi, and M. Anakura. 1971. Hyperammonemia due to a mutant enzyme of ornithine transcarbamylase. Pediatrics 48: 595-600.

81. McLean, P., and M. W. Gurney. 1963. Effect of adrenalectomy and of growth hormone on enzymes concerned with urea synthesis in rat liver. Biochem. J. 87: 96-140.

82. McKusick, V. A. 1971. Mendelian Inheritance in Man. Catalogs of Autosomal Dominant, Autosomal Recessive, and X-Linked Phenotypes. 3rd ed. (The Johns Hopkins Press, Baltimore).

83. McMurray, W. C., F. Mohyuddin, S. M. Bayer, J. C. Rathbun. 1964. Citrullinuria: A disorder of amino acid metabolism associated with mental retardation. Proceedings of the Third International Copenhagen Congr. Sci. Study Ment. Retard. 1: 117.

84. McMurray, W. C., J. C. Rathbun, F. Mohyuddin, and S. J. Koegler. 1963. Citrullinuria. Pediatrics 32: 347-357.

85. McMurray, W. C., F. Mohyuddin, R. J. Rossiter, J. C. Rathbun, G. H. Valentine, and S. J. Kroegler. 1962. Citrullinuria: A new aminoaciduria associated with mental retardation. Lancet i: 138.

86. Mellman, W. J., and G. Kohn. 1970. Human cell cultures: Their use in the investigation and diagnosis of disease. Med. Clin. Amer. 54: 701-712.

87. Mohyuddin, F., J. C. Rathbun, and W. C. McMurray. 1967. Studies on amino acid metabolism in citrullinuria. Amer. J. Dis. Child. 113: 152-156.

88. Moore, P. T., M. C. Martin, V. P. Coffey, and B. M. Stokes. 1968. Argininosuccinicaciduria. A (case) report on a rare condition. J. Ir. Med. Ass. 61: 172-178.

89. Mora, J., J. Martuscelli, J. Ortig-Peneda, and G. Soberson. 1965. The regulation of urea-biosynthesis enzymes in vertebrates. Biochem. J. 96: 28-35.

90. Morrow, G., L. A. Barness, and M. L. Efron. 1967. Citrullinemia with defective urea production. Pediatrics 40: 565-574.

91. Morrow, G. 1967. Citrullinemia. A preliminary case report. Amer. J. Dis. Child. 113: 157-159.

92. Moser, H. W., M. L. Efron, H. Brown, R. Diamond, and C. G. Newman. 1967. Argininosuccinic aciduria: Report of two cases and demonstration of intermittent elevation of blood ammonia. Amer. J. Med. 42: 9-26.

93. Nadler, H. L., and T. J. Egan. 1970. Deficiency of lysomal acid phosphatase. A new familial metabolic disorder. New Eng. J. Med. 282: 302-307.

94. Nagayama, E., T. Kitayama, and H. Oguchi. 1970. Hyperammonemia: A deficiency of liver ornithine transcarbamylase. Paediatr. Univ. Tokyl 18: 167-173.

95. Nuzum, C. T., and P. J. Snodgrass. 1971. Urea cycle enzyme adaptation to dietary protein in primates. Science 172: 1042-1043.

96. Péralta-Serrano, A. 1965. Argininuria, convulsiones y oligofrenia: a nuevo error innato del metabolismo. Rev. Clin. Espan. 97: 176-184. Referred to in: B. Levin, 1971.

97. Perheentupa, J., and J. K. Visakorpi. 1965. Protein intolerance with deficient transport of basic amino acids. Lancet ii: 813-816.

98. Ratner, S. 1955. Enzymatic synthesis of arginine (condensing and splitting enzyme) In: Methods in Enzymology, Vol. II, S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York. p. 356.

99. Ratner, S., W. P. Anslow, Jr., and B. Petrack. 1953. Biocynthesis of urea VI. Enzymatic cleavage of argininosuccinic acid to arginine and fumaric acid. J. Biol. Chem. 204: 115.

100. Ratner, S., H. Morell, and E. Cervalho. 1960. Enzymes of arginine metabolism in brain. Arch. Biochem. Biophys. 91: 280-289.

101. Ratner, S., and A. Pappas. 1949. Biocynthesis of urea, Part I. Enzymatic mechanism of arginine synthesis from citrulline. J. Biol. Chem. 179: 1183-1198.

102. Ratner, S., and B. Petrack. 1951. Biosynthesis of urea III. Further studies of arginine synthesis from citrulline. J. Biol. Chem. 191: 693-705.

103. Ratner, S., and B. Petrack. 1953. Biosynthesis of urea IV. Further studies on arginine synthesis from citrulline. J. Biol. Chem. 200: 161.

104. Ratner, S., and B. Petrack. 1954. Urea synthesis and metabolism of arginine and citrulline. Advan. Enzymol. Relat. Subj. Biochem. 15: 319-387.

105. Ratner, S., B. Petrack, and O. Rochovansky. 1953. Biosynthesis of urea, Part V. Isolation and properties of argininosuccinic acid. J. Biol. Chem. 204: 95-113.

106. Ravel, J. M., M. L. Grona, J. S. Humphreys, and W. Shive. 1959. Properties and biotin content of purified preparations of the ornithine-citrulline enzyme of Streptococcus lactis. J. Biol. Chem. 234: 1452.

107. Reichard, H. 1960. Ornithine carbamoyl transferase activity in human tissue homogenates. J. Lab. Clin. Med. 56: 218-221.

108. Reichard, P. 1957. Ornithine carbamoyl transferase from rat liver. Acta Chem. Scand. 11: 523.

109. Reynolds, J., J. H. Follette, and W. N. Valentine. 1957. The arginase activity of erythrocytes and leukocytes with particular reference to pernicious anemia and thalassemia major. J. Lab. Clin. Med. 50: 78.

110. Rogers, P., and G. D. Novelli. 1962. Purification of ornithine transcarbamylase from derepressed cells of Escherichia coli W. Arch Biochem. Biophys. 96: 398.

111. Russell, A., B. Levin, V. G. Oberholzer, and L. Sinclair. 1962. Hyperammonaemia--a new instance of an inborn error of the urea cycle. Lancet ii: 699-700.

112. Salle, B., and B. Levin. 1970. Hyperammoniemie congenitale par deficit en ornithine carbayltransferase. Paper presented to la Societe Francaise de Pediatrie. Quoted by Levin, B., 1971.

113. Salzman, J., R. DeMars, and P. Benke. 1968. Single allele expression at an X-linked hyperuricemia locus in heterozygous human cells. PNAS-USA 60: 545-553.

114. Schimke, R. T. 1962. Adaptive characteristics of urea cycle enzymes in the rat. J. Biol. Chem. 237: 459.

115. Schimke, R. T. 1963. Enzymes of arginine metabolism in mammalian cell culture I. Repression of argininosuccinic synthetase and argininosuccinase. J. Biol. Chem. 239: 136-145.

116. Schneider, S., J. F. Nicholson, and A. B. Chutorian. 1970. Sex-linked hyperammonemia response to reduced dietary protein. Trans. Am. Neurol. Assoc. 95: 86-90.

117. Scott-Emuakpor, A., J. V. Higgins, and A. F. Kohrman. 1972. Citrullinemia: a new case, with implications concerning adaptation to defective urea synthesis. Pediat. Res. 6: 626.

118. Shih, V. E. 1972. Early dietary management in an infant with argininosuccinase deficiency: A preliminary report. J. Pediatr. 80: 645-648.

119. Shih, V. E., and M. L. Efron. 1972. Urea Cycle disorders. In: The Metabolic Basis of Inherited Disease, 3rd ed. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, eds. McGraw-Hill.

120. Shih, V. E., M. L. Efron, and H. W. Moser. 1969. Hyperornithinemia, hyperammonemia, homocitrullinuria. Amer. J. Dis. Child. 117: 83-92.

121. Short, E. M., H. O. Conn, P. J. Snodgrass, A. G. M. Campbell, and L. E. Rosenberg. 1973. Evidence for X-linked dominant inheritance of ornithine transcarbamylase deficiency. New Engl. J. Med. 288: 7.

122. Snodgrass, P. J. 1968. The effects of pH on the kinetics of human liver ornithine carbamyl transferase. Biochemistry 7: 3047-3051.

123. Snodgrass, P. J., and D. J. Parry. 1969. The kinetics of serum ornithine transcarbamylase. J. Lab. Clin. Med. 73: 940-950.

124. Solitare, G. B., V. E. Shih, D. J. Nelligan, and T. F. Dolan, Jr. 1969. Argininosuccinic aciduria: Clinical, biochemical, anatomical, and neuropathological observations. J. Ment. Defic. Res. 13: 153-170.

125. Spector, E. B., and A. Bloom. 1973. Citrullinemic lymphocytes in long-term culture. Pediat. Res. 7: 700.

126. Starer, F., and R. Couch. 1963. Cerebral atrophy in hyperammonaemia. Clin. Radiol. 14: 353-355.

127. Streeter, S., E. Spector, and A. Bloom. 1973. Growth of established lymphocytes in selective media: Application to studies of inborn metabolic errors and somatic mutations in long-term lymphocytes cultures in human genetics. Birth Defects: Original Article Series 9 (1): 138.

128. Sunshine, P., J. E. Lindenbaum, H. L. Levy, and J. M. Freeman. 1972. Hyperammonemia due to a defect in hepatic ornithine transcarbamylase. Pediatrics 50: 110-11.

129. Tedesco, T. A., and W. J. Mellman. 1967. Argininosuccinate synthetase activity and citrulline metabolism in cells cultured from a citrullinemic subject. Proc. N.A.S.--USA 57: 829.

130. Terheggen, H. G., A. Schwent, A. Lowenthal, M. van Sande, and J. P. Colombo. 1970. Hyperargininamie mit arginasedefke. Eine neue familiare Stoffwechselstorung. 2. Biochemiscke Untersuchunger. Z. Kinderheilk 107: 313-323.

131. Terheggen, H. A., A. Schwenk, A. Lowenthal, M. van Sande, and J. P. Colombo. 1969. Argininaemia with arginase deficiency. Lancet ii: 748-749.

132. Tomlinson, S., and R. G. Westall. 1964. Argininosuccinic aciduria: argininosuccinase and arginase in normal human blood cells. Clin. Sci. 26: 261-269.

133. Vidailhet, M., B. Levin, M. Dautrevaux, P. Paysant, S. Gelot, Y. Badonnel, M. Pierson, and N. Neimann. 1971. Citrullinemie. Arch. Fr. Pediat. 28: 521-532.

134. Visakorpi, J. K. 1962. Citrullinuria. Lancet i: 1357-1358.

135. Walker, C. O., and S. Schenker. 1970. Pathogenesis of hepatic encephalopathy. Amer. J. Clin. Nutr. 23: 619-632.

136. Wallis, K., S. Beer, and J. Fischl. 1963. A family affected by argininosuccinic aciduria. Helv. Paediat. Acta 18: 339-343.

137. Westall, R. G. 1967. Treatment of argininosuccinic aciduria. Amer. J. Dis. Child. 113: 160-161.

138. Westall, R. G. 1960. Argininosuccinic aciduria. Identification of the metabolic defect. A newly described form of mental disease, with some preliminary metabolic studies. Biochem. J. 77: 135-144.

139. Wixom, R. L., M. K. Reddy, and P. P. Cohen. 1972. A concerted response of the enzymes of urea biosynthesis during thyroxine-induced metamorphosis of Rana catesbeiana. J. Biol. Chem. 247: 3681.

140. Woody, N. C. 1964. Hyperlysinemia. Amer. J. Dis. Child. 108: 543-553.

141. Woody, N. C., J. Hutzler, and J. Dnacis. 1966. Further studies of hyperlysinemia. Amer. J. Dis. Child. 112: 557-580.