Factors affecting utilization of aspartic acid by Leuconostoc Mesenteroides P-60
by Richard S Clark

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of Master of Science in Chemistry at Montana State College
Montana State University
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Approved:

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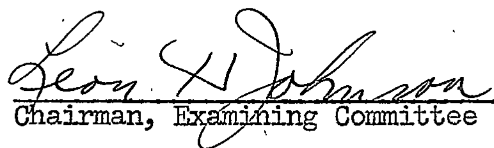

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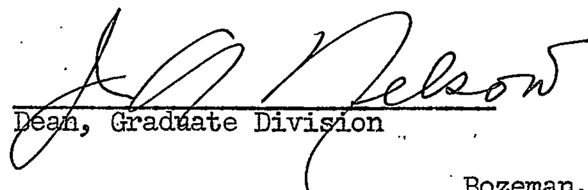

Dean, Graduate Division

Bozeman, Montana
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TABLE OF CONTENTS

	Page
ABSTRACT	3
INTRODUCTION	4
HISTORICAL RÉSUMÉ	5
MATERIALS, METHODS AND DATA	9
EXPERIMENTAL RESULTS	15
DISCUSSION	29
SUMMARY	35
ACKNOWLEDGEMENT	36
LITERATURE CITED AND CONSULTED	37

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ABSTRACT

A study of the utilization by Leuconostoc mesenteroides P-60 of the factors affecting this utilization was made. It was shown that the D-isomer is utilized. An indication of enzymatic adaptation appeared. A difference in the type and amount of buffer affected the response to D-aspartic acid. Varying amounts of alanine had no appreciable affect and D- and L-asparagine would not substitute for D- and L-aspartic acid.

INTRODUCTION

The D-isomers of amino acids have long been considered the synthetic or "unnatural" form. The possibility of their utilization by microorganisms has been recognized only within the past few years. The question of whether these forms occur as components of living tissue or only as extracellular metabolic products has been debated during the past few years and is still being debated.

It now seems apparent that a great many different microorganisms can utilize various D-isomers of the amino acids. The D-isomers of all but five or six amino acids have been reported as being utilized partially or completely by various microorganisms. The six reported as most generally utilized are D-alanine, D-valine, D-leucine, D-aspartic acid and D-glutamic acid. Of these, D-glutamic acid has been, perhaps, the most extensively studied and D-aspartic acid the least.

According to Rydon (1948) the only lactic acid producing organism found capable of utilizing D-aspartic acid is *Lactobacillus delbrückii*. Assays run in the laboratory of the Department of Chemistry Research, Montana State College Agricultural Experiment Station indicated the D-aspartic acid was substituting, at least partially, for L-aspartic acid with *Leuconostoc mesenteroides* P-60. This organism is of particular interest since it has been used as the principal assay organism for aspartic acid. Because of peculiarities which had been noted in its response to this amino acid further study seemed advisable.

HISTORICAL RÉSUMÉ

Early investigators believed that the L-forms of amino acids were the only naturally occurring forms of amino acis. The D-forms were considered as synthetic compounds and metabolically inert. These concepts were based on Fischer and Abderhalden's (1905) report that only the peptide linkages built up from amino acids of the L-series were hydrolyzed by enzymes of the pancreas and intestine.

The first experiments with the utilization of D-amino acids were performed by Wohlgemuth (1905). He fed racemic tyrosine, leucine, aspartic acid and glutamic acid to rabbits. He reported that the corresponding D-amino acids were excreted in the urine. Dakin (1909) injected 8 grams of optically inactive phenylalanine into a cat and recovered 2 grams of inactive phenylalanine from the urine. Dakin (1910) also injected inactive tyrosine into a cat. He stated as a result of this experiment, "the naturally occurring laevo-acid was in every case more readily decomposed but the rates of decomposition of the d and l forms cannot be very widely different since when smaller doses of the inactive acid are given, no unchanged tyrosine may be found in the urine." Berg and Potgieter (1931) observed that rats fed on a tryptophan deficient diet responded as well to racemic tryptophan as to L-tryptophan. Whipple and Robscheit-Robbins (1937) showed that D-histidine and D-tyrosine are utilized by an anemic dog for the regeneration of hemoglobin to the same extent as the L-series. Conrad and Berg (1937) noted that rats fed a histidine

deficient diet supplemented with D-histidine were able to maintain normal activity. When the tissues of these rats were examined they were shown to contain only L-histidine. The natural occurrence of a D-isomer of an amino acid was first demonstrated by Jacobs and Craig (1935) who obtained a methyl ester of D-proline as a cleavage product of ergotine. Smith and Timmis (1937) confirmed the findings of Jacobs and Craig. Ivanovics and Brúckner (1937) isolated D-glutamic acid from Bacillus mesentericus vulgatus. Heinsen (1936) reported the isolation of DL-arginine from a kidney autolysate. Kotake and Goto (1937) using rat kidney slices found an inversion of D-tryptophan into L-tryptophan. Hotchkiss and Dubos (1940) reported that nearly half of the amino acids in the acid hydrolysate of gramicidin and gramidinic acid occur as the D-isomer. Lipmann, Hotchkiss and Dubos (1941) and Christensen, Edwards and Piersma, (1941) isolated D-leucine from gramicidin. Bovarnick (1942) reported a strain of Bacillus subtilis that produces a polypeptide composed solely of D-glutamic acid. Hanby and Rydon (1946) showed that the capsular polypeptide of Bacillus anthracis contained large amounts of D-glutamic acid. Kogl and Erxeleben (1939) claimed to have isolated the D-form of glutamic acid from hydrolysates of tumour tissue in amounts of up to 48 per cent of the total glutamic acid. They postulated that the growth controlling enzymes are deprived of their controlling ability when they have the D-form in their structure. The findings and theory of Kogl and Erxeleben had been postulated in 1907 by Margaret A. Cleaves (1939). Arnow and Opsahl (1939), Schroeder (1939), White and White (1939), and others confirmed the findings of Kogl and Erxeleben whereas Chibnall, Rees, Tristam, Williams and Boyland

(1939), Graff (1939), Chargaff (1939), and others were unable to find any D-glutamic acid in tumour tissue. Kogl and Erxeleben (1939 A) attributed their failure to find D-glutamic acid to differences in analytical procedure. Johnson reported finding D-glutamic acid in normal rat liver and Chibnall found the compound in a number of plant proteins as well as in normal animal tissue (1940). The utilization of D-isomers of amino acids by microorganisms was reported by Webster and Bernheim (1936) when they stated that Bacillus pyocyaneus (Pseudomonas aeruginsa) utilized both isomers of tyrosine, proline, and to a slight extent those of valine and phenylalanine. Dunn, Camien, Rockland, Shankman and Goldberg (1944) suggested that D-glutamic acid might be utilized by Lactobacillus arabinosus 17-5. Lymann and Kuiken (1948) found that the ability of Lactobacillus arabinosus to utilize the D- forms of the amino acids was greatly increased by the substitution of pyridoxamine for pyridoxine. They also found that Streptococcus faecalis did not utilize D-methionine in a medium buffered with acetate but utilized substantial amounts in a medium buffered with citrate. Camien and Dunn (1949) reported that D- and DL-glutamic acids under some conditions are more active than L-glutamic acid in promoting growth of Lactobacillus arabinosus 17-5, but that increased amounts of aspartic acid in the basal medium partly or completely suppressed the activity of D-glutamic acid for this organism. Camien and Dunn (1950) showed that Lactobacillus arabinosus 17-5 utilized D-methionine and DL-methionine equally as well as L-methionine in a synthetic

medium containing at least one per cent of pyridoxamine or 10 per cent of pyridoxal. At lower concentrations of these vitamins, the utilization of D-methionine was reduced or eliminated. Pyridoxine and other nutrients in relatively high concentrations were ineffective in promoting utilization of D-methionine.

MATERIALS, METHODS AND DATA

A culture of Leuconostoc mesenteroides P-60 was obtained from the American Type Culture Collection. In order to have a pure strain, it was necessary to isolate a single cell of the organism. For this purpose a Chambers micromanipulator was used. The general procedure followed for single cell isolation was that outlined by Hildebrand (1938). Two modifications were made on this method. The first was to use a different moist chamber than that described by Hildebrand. Hildebrand's moist chamber did not provide sufficient moisture for the dry air of this atmosphere. The moist chamber outlined by Richter (1948) proved much more satisfactory. The dilution was made greater than that described by Hildebrand. The dilution was such that rarely were two organisms contained in a single drop. A drop was placed on the cover plate, examined and, if it had only one organism, the cover plate was transferred to a hanging drop slide for incubation. If the drop had no organism another drop was deposited and examined for a single organism. This was repeated until a drop was deposited with a single organism. If more than one organism appeared in a drop the cover plate was discarded.

After incubation in the hanging drop slide the organisms were transferred to a tryptose phosphate medium and maintained until identification. The organisms were identified according to the procedure outlined for Leuconostoc mesenteroides in Bergey's Manual (1948).

After identification the organisms were transferred to an agar stab. This stab consisted of basal medium I, enriched according to Henderson and Snell (1948) by 0.2 per cent Bacto-tryptone, 0.5 per cent Bacto-yeast extract and 2 per cent Bacto-agar. The cultures were incubated at 35°C for 36 hours when heavy growth was observed. After incubation the cultures were stored in a refrigerator until used. The inoculum medium was the same as the above, except that agar was omitted.

Three different basal media were used. Basal medium I (Johnson, Leon H., unpublished data) is shown in Table I.

Basal Medium II is the same as that outlined in Table I except that 20 grams of sodium acetate was substituted for the 20 grams of sodium citrate.

Basal Medium III was the medium described by Horn, Jones and Blum (1950). It is shown in Table II.

These media were adjusted to a pH of 6.8 as described by Henderson and Snell (1948), and 4 ml was pipetted into each tube with an automatic pipette. The volume was doubled by addition of 4 ml of a solution of aspartic acid or water. Forty tubes were placed in a rack and covered with a metal cover about two inches deep. The cover was loosely lined with gauze to permit it to fit tightly over each tube. These media were steam sterilized under 12 pounds pressure for 10 minutes.

Before inoculation into the assay medium, the organisms were revitalized by passing through five transfers. The cultures were incubated 24

TABLE I

BASAL MEDIUM I

Amino acids in mg

DL-alpha alanine	500	L-Methionine	100
L-cysteine	100	DL-Phenylalanine	100
L-arginine HCL	200	L-Proline	50
L-glutamic	500	DL-Serine	100
Glycine	100	DL-Threonine	100
L-Histidine	100	L-Tyrosine	100
DL-Isoleucine	200	DL-Tryptophan	100
L-Leucine	100	DL-Valine	200

Glucose	20 grams

Salts in grams

Sodium citrate	20	KH_2PO_4	1
Sodium acetate	1	$MgSO_4$	0.2
Ammonium chloride	3	$FeSO_4$	0.01
K_2HPO_4	1	NaCL	0.01

Purines in mg

Adenine	10	Uracil	10
Guanine	10	Xanthine	10

Vitamins in mg

Thiamine	0.5	Ca pantothanate	0.5
Riboflavin	0.5	Niacin	1.0
Pyridoxal	0.3	p-aminobenzoic acid	0.1

Micrograms of the following

Biotin	10	Folic acid	10
Reticulogen	0.2		

Quantities are based on 500 ml volume

hours at 35°C between transfers. After the fifth transfer a centrifuge tube containing 3.5 ml of medium was inoculated and incubated for 24 hours at 35°C. Following incubation, the cells were centrifuged and washed twice with a normal physiological saline solution. After the final washing the cells were again centrifuged and suspended in 7 ml of normal saline solution. Approximately 0.1 ml of this suspension was placed in each tube of assay medium.

Assays were run with various concentrations of D- and L-aspartic acid with all three media, and with basal medium I varying the amounts of glutamic acid and alanine. Varying incubation periods of 12, 24, 36, 48, 60, 72, and 84 hours were employed.

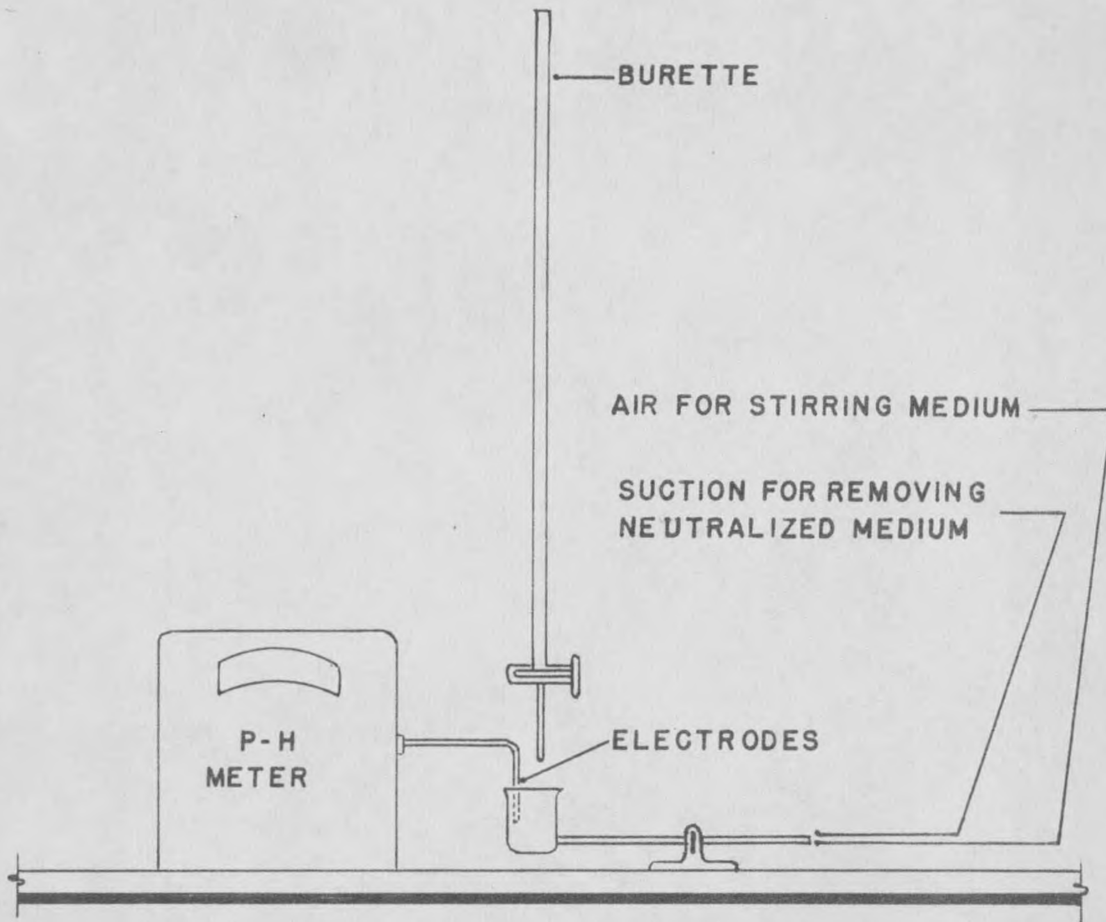
TABLE II

BASAL MEDIUM III

Nutrient	Amount	Nutrient	Amount
Glucose	20gm	Biotin	.01mg
Sodium acetate (anhy-		Folic acid	.002mg
drous)	12gm	DL-Alanine	80mg
Salts A:		L-Arginine hydro-	
K_2HPO_4	1gm	chloride	96mg
KH_2PO_4	1gm	DL-Aspartic acid	240mg
Salts B:		L-Cystine	400mg
$MgSO_4 7H_2O$	400mg	DL-Glutamic acid H_2O	940mg
$MnSO_4 4H_2O$	20mg	Glycine	400mg
NaCL	20mg	L-Histidine hydro-	
$FeSO_4 7H_2O$	20mg	chloride H_2O	54mg
Adenine	100mg	L-Hydroxyproline	20mg
Guanine	100mg	DL-Isoleucine	50mg
Uracil	100mg	DL-Leucine	400mg
Thiamine chloride	2.0mg	DL-Lysine hydro-	
Pyridoxamine dihydro-		chloride	300mg
chloride	.4mg	DL-Methionine	200mg
Calcium pantothenate	.4mg	DL-Norleucine	400mg
Riboflavin	.4mg	DL-Phenylalanine	160mg
Nicotinic acid	.8mg	L-Proline	140mg
p-Aminobenzoic acid	.4mg	DL-Serine	240mg
DL-Tryptophane	400mg	DL-Threonine	180mg
DL-Valine	240mg	L-Tyrosine	130mg

Solution brought to 1,000 ml volume, pH 6.8.

FIG. I



TITRATION APPARATUS

EXPERIMENTAL RESULTS

The response of Leuconostoc mesenteroides P-60 in each of the three basal media to D and L aspartic acid, varying periods of incubation and concentration of aspartic acid, are reported in Tables III to IX. The lactic acid produced was titrated with 0.092 N NaOH. All values are corrected to zero blank value. Figures 2 to 18 illustrate the results expressed in these Tables graphically.

TABLE III

BASAL MEDIUM I

ug of aspartic acid per tube	36 hour culture ml NaOH		ml NaOH		60 hour culture ml NaOH		ml NaOH	
	D	L	D	L	D	L	D	L
40	0.7	1.4	0.6	1.7	1.2	2.6	1.3	2.3
80	1.6	2.8	1.5	3.0	2.9	4.6	3.1	4.3
160	2.4	3.7	2.3	3.6	4.6	6.4	4.1	6.1
240	3.3	5.1	3.3	4.8	5.3	7.4	5.3	7.5
320	3.7	5.2	3.6	4.9	6.7	8.1	6.6	8.1

TABLE IV

BASAL MEDIUM I

ug of aspartic acid per tube	12 hour culture ml NaOH		18 hour culture ml NaOH		36 hour culture ml NaOH		60 hour culture ml NaOH		84 hour culture ml NaOH	
	D	L	D	L	D	L	D	L	D	L
40	0.0	0.7	0.4	1.0	0.5	1.8	1.1	2.4	2.2	3.5
80	0.0	1.1	0.4	1.8	1.4	3.0	2.9	4.4	4.4	5.8
160	0.7	1.3	0.4	2.4	2.3	3.9	4.7	6.3	6.3	7.4
320	0.8	1.2	1.5	2.6	3.4	5.1	6.5	7.7	7.1	8.5
640	0.8	1.1	2.4	2.0	5.0	5.2	6.6	8.1	7.7	8.6

TABLE V

BASAL MEDIUM I

ug of aspartic acid per tube	24 hour culture ml NaOH		36 hour culture ml NaOH		48 hour culture ml NaOH		60 hour culture ml NaOH	
	D	L	D	L	D	L	D	L
40	0.6	1.4	0.9	2.1	1.3	2.7	2.4	3.5
160	2.5	4.0	3.8	5.5	5.5	6.6	6.6	7.5
640	4.4	5.5	6.2	6.7	7.5	8.0	9.0	9.9
1280	4.9	5.0	6.4	6.4	8.3	7.8	9.7	8.9

TABLE VI

BASAL MEDIUM I

ORGANISMS PASSED THROUGH 10 TRANSFERS IN MEDIUM CONTAINING ONLY THE D-FORM OF ASPARTIC ACID

60 hour culture

ug of aspartic acid per tube	ml NaOH D	ml NaOH L
40	1.8	2.7
160	6.8	7.3
320	8.2	8.9
640	10.4	10.6
960	11.0	11.1
1280	11.1	10.3

TABLE VII

BASAL MEDIUM II

ug of aspartic acid per tube	12 hour culture ml NaOH		24 hour culture ml NaOH		36 hour culture ml NaOH		60 hour culture ml NaOH	
	D	L	D	L	D	L	D	L
40	0.2	0.8	0.5	1.3	1.1	2.3	2.2	2.6
160	0.4	2.1	2.1	3.5	2.8	4.7	4.8	5.7
640	0.4	2.2	2.2	4.0	3.2	5.8	5.3	7.9
1280	0.6	2.2	2.3	4.0	3.3	5.8	5.3	7.7

TABLE VIII.

BASAL MEDIUM III

ug of aspartic acid per tube	36 hour culture ml NaOH		60 hour culture ml NaOH	
	D	L	D	L
40	0.4	1.6	0.4	2.4
80	0.9	1.9	1.0	2.9
160	1.5	2.1	1.6	2.9
240	1.8	2.1	1.7	2.9
320	1.9	2.1	2.2	2.9

TABLE IX

BASAL MEDIUM I

Glutamic acid varied ---- 60 hour culture

Amount of glutamic acid per tube

ug of aspartic acid per tube	50 ug ml NaOH		100 ug ml NaOH		500 ug ml NaOH		1 mg ml NaOH		4 mg ml NaOH	
	D	L	D	L	D	L	D	L	D	L
40	0.8	1.7	1.5	2.0	1.2	2.0	0.9	2.0	1.5	2.0
80	1.2	1.6	2.2	2.8	1.8	3.0	2.2	3.4	2.7	3.5
120	0.8	1.2	2.6	3.1	2.5	4.3	2.8	4.3	3.8	4.7
160	0.9	0.9	2.8	3.3	2.8	4.9	3.7	5.2	3.9	5.3

TABLE X

BASAL MEDIUM I

Per cent Response of D-Aspartic Acid in Terms of L-Aspartic Acid at Concentrations of 40 to 320 ug per Tube

ug of aspartic acid per tube	36 hour culture per cent	60 hour culture per cent
40	50	60
80	56	70
160	43	45
240	42	46
320	47	54

TABLE XI

BASAL MEDIUM I

Per cent Response of D-Aspartic Acid in Terms of L-Aspartic Acid At Concentrations of 40 to 640 ug per Tube

ug of aspartic acid per tube	12 hour culture per cent	18 hour culture per cent	36 hour culture per cent	60 hour culture per cent
40	0	40	40	50
80	0	20	46	65
160	25	10	30	50
320	15	15	15	43
640	7.5	-	-	-

TABLE XII

BASAL MEDIUM II

Per cent Response of D-Aspartic Acid in Terms of L-Aspartic Acid At Concentrations of 40 to 160 ug per Tube

ug of aspartic acid per tube	36 hour culture per cent	60 hour culture per cent
40	40	90
80	80	75
160	25	45

TABLE XIII

BASAL MEDIUM III

Per cent Response of D-Aspartic Acid in Terms of L-Aspartic Acid At Concentrations of 40 to 320 ug per Tube

ug of aspartic acid per tube	36 hour culture per cent	60 hour culture per cent
40	18	15
80	20	18
160	23	14
240	25	10
320	25	11

TABLE XIV

BASAL MEDIUM I

Per cent Response of D-Aspartic Acid in Terms of L-Aspartic Acid at Concentrations of 40 to 320 ug per Tube With Glutamic acid varied

	Amount of glutamic acid per tube				
ug of aspartic acid per tube	50 ug per cent	100 ug per cent	500 ug per cent	1 mg per cent	4 mg per cent
40	30	60	62	41	70
80		60	40	58	71
120		57	53	53	75
160		50	50	60	63

BASAL MEDIUM I
40 TO 320 ug ASPARTIC ACID / TUBE
60 HOUR CULTURE

L ASP
D ASP

0 40 80 160 240 320

FIG. III

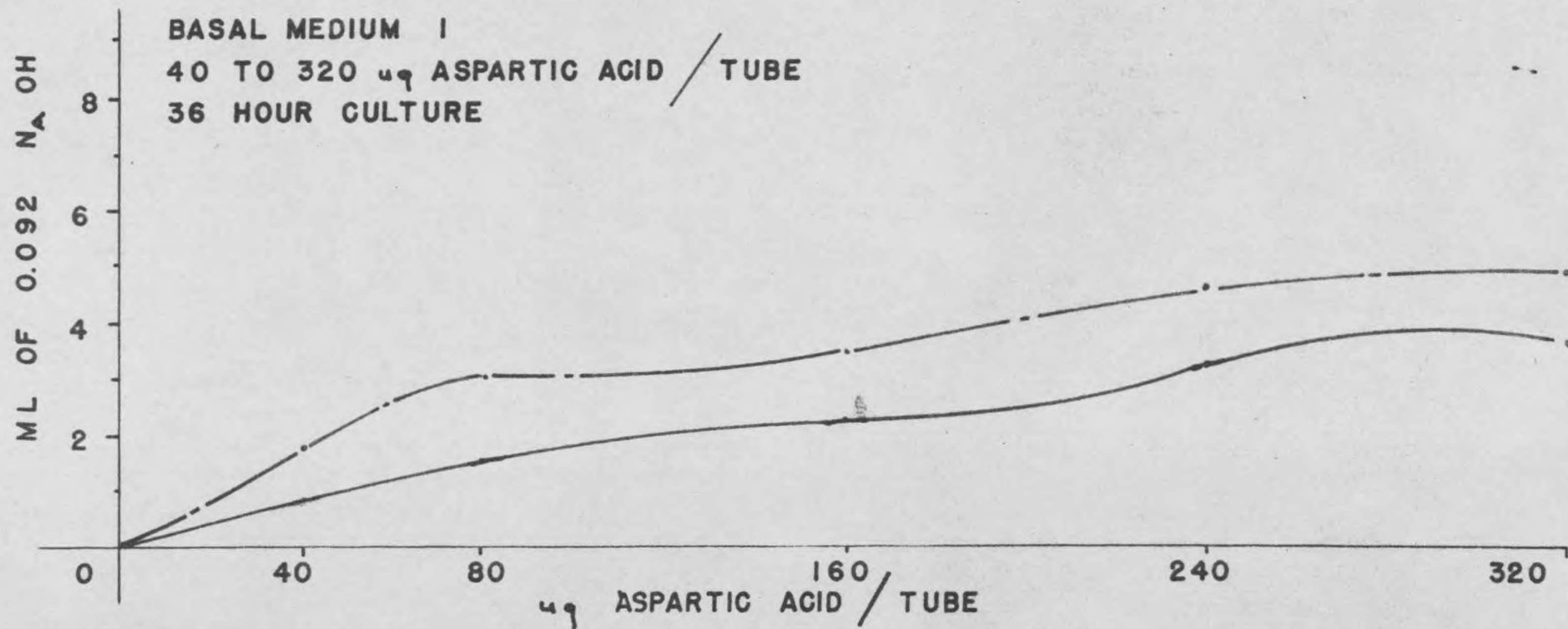


FIG. II

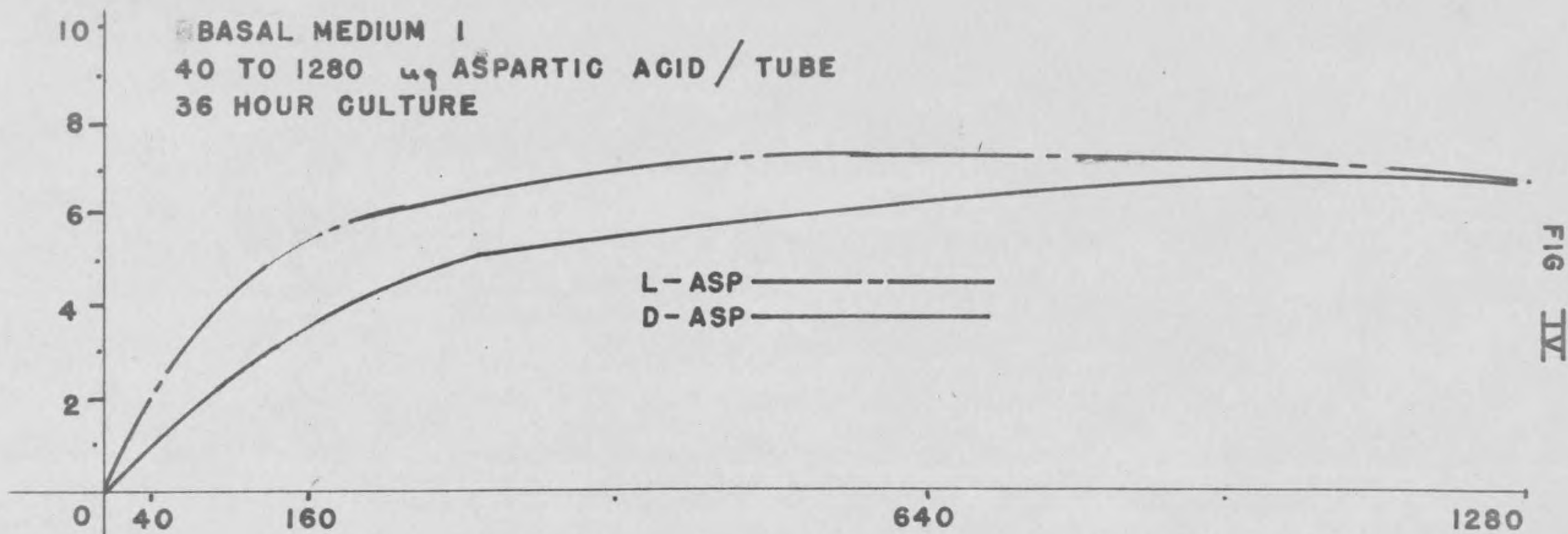


FIG IV

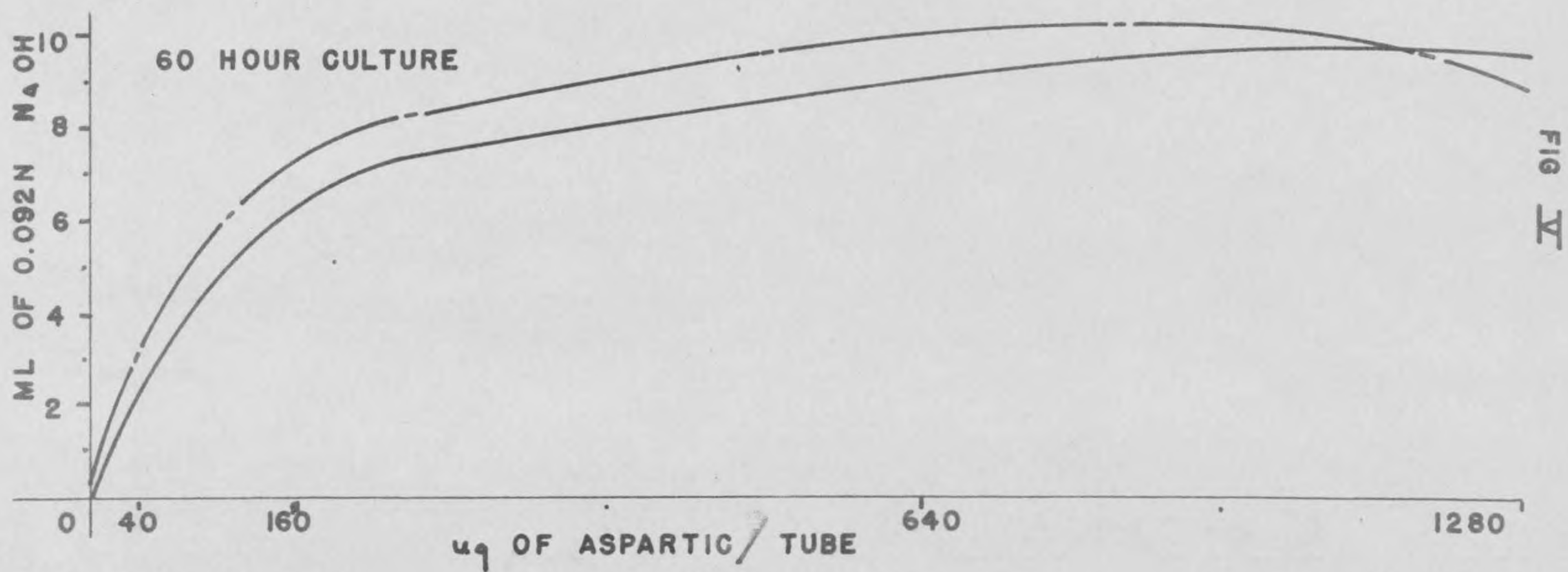


FIG V

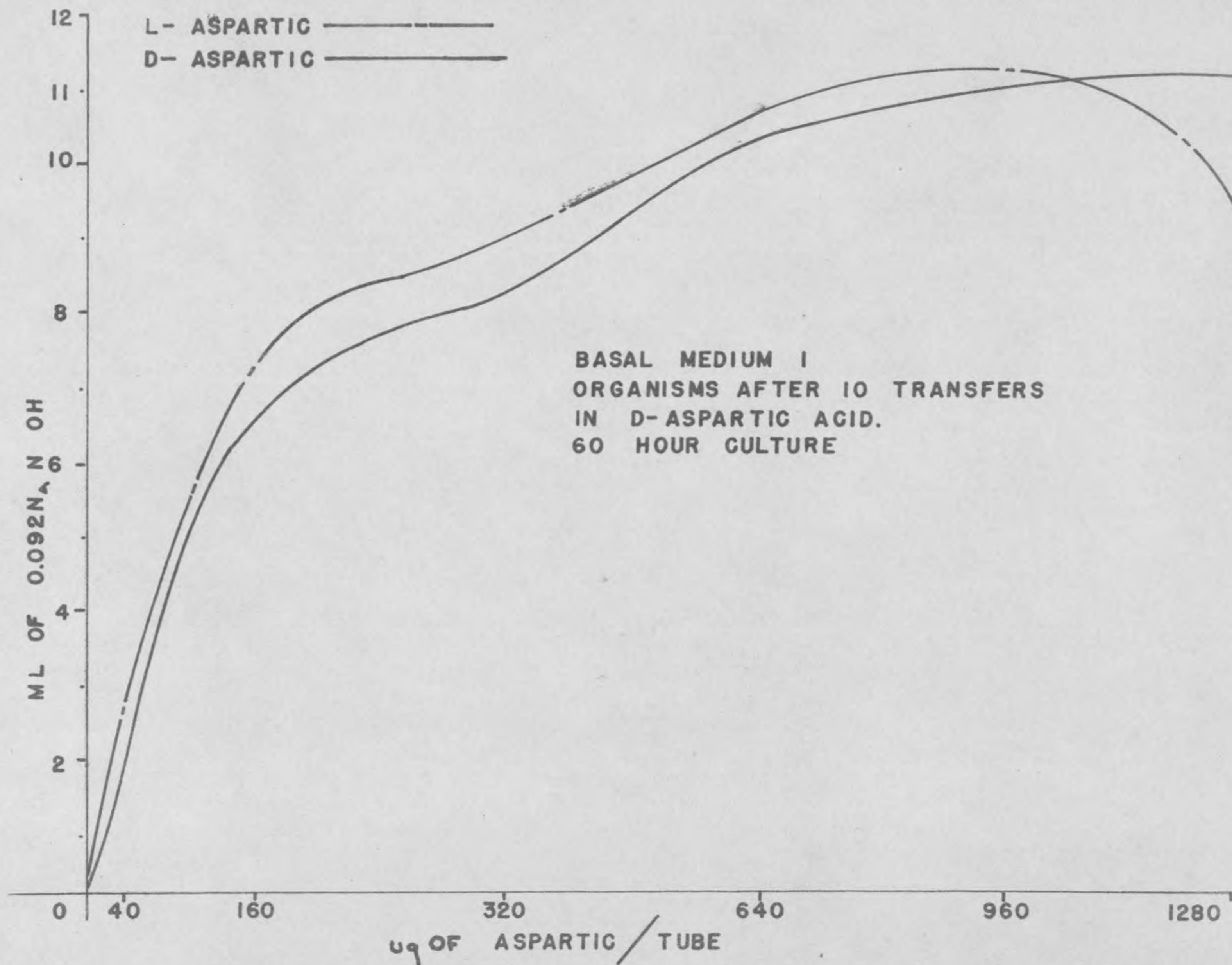


FIG. VI

BASAL MEDIUM III
40 TO 320 ug ASPARTIC ACID/TUBE
36 HOUR CULTURE

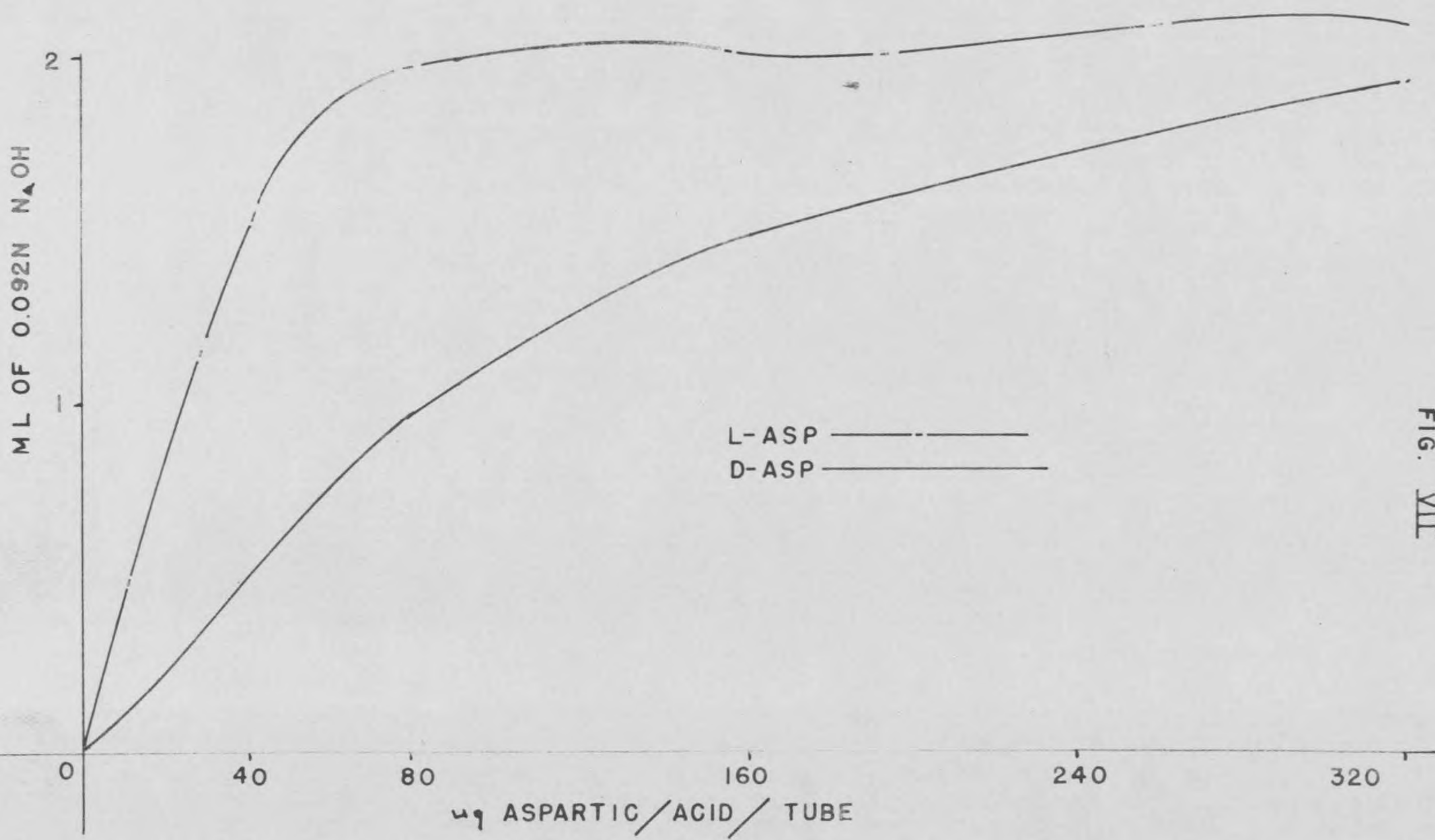


FIG. VII

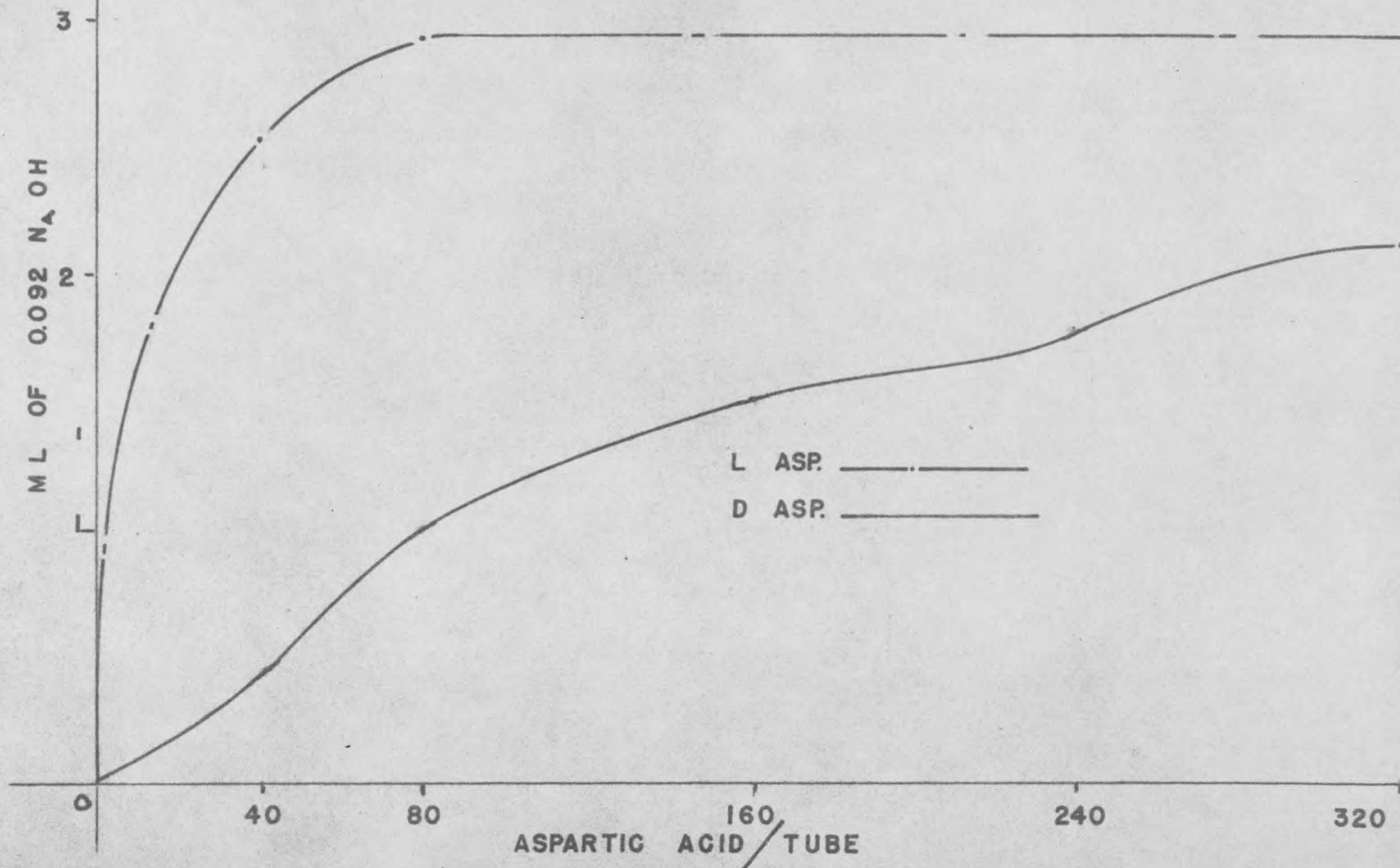
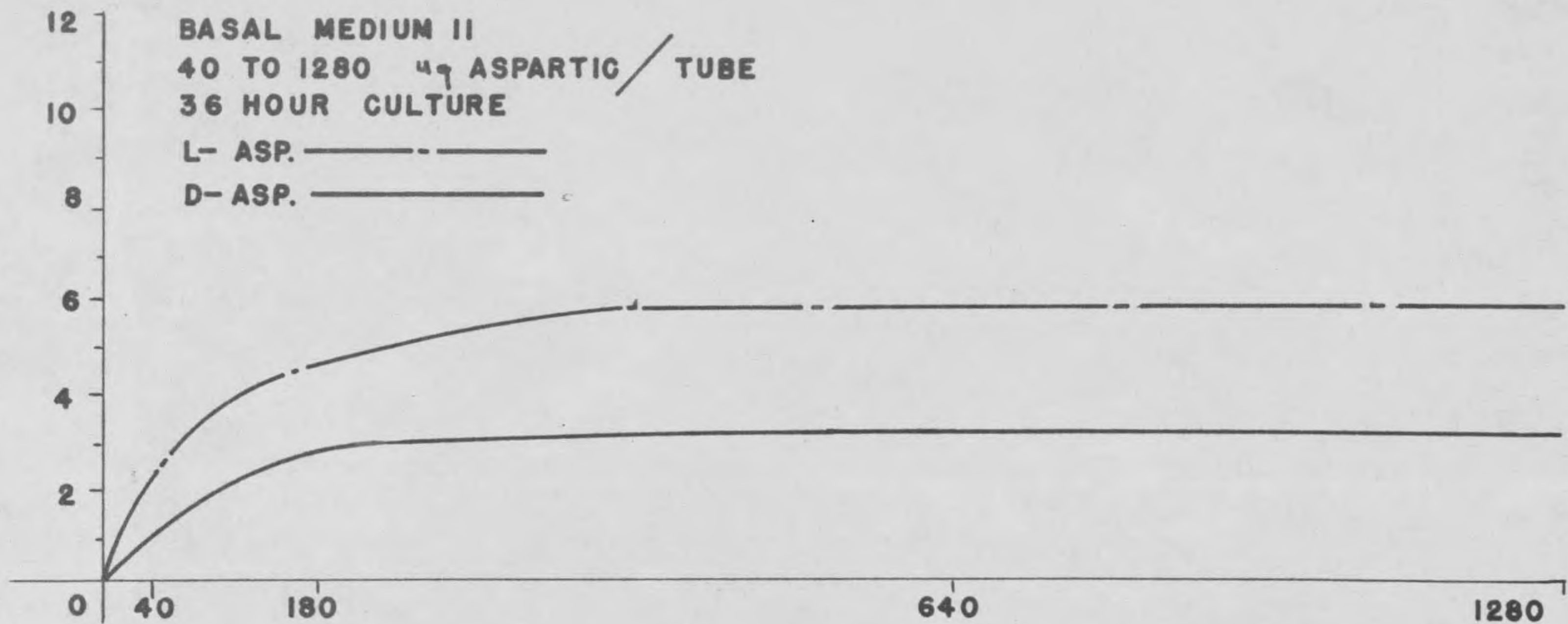
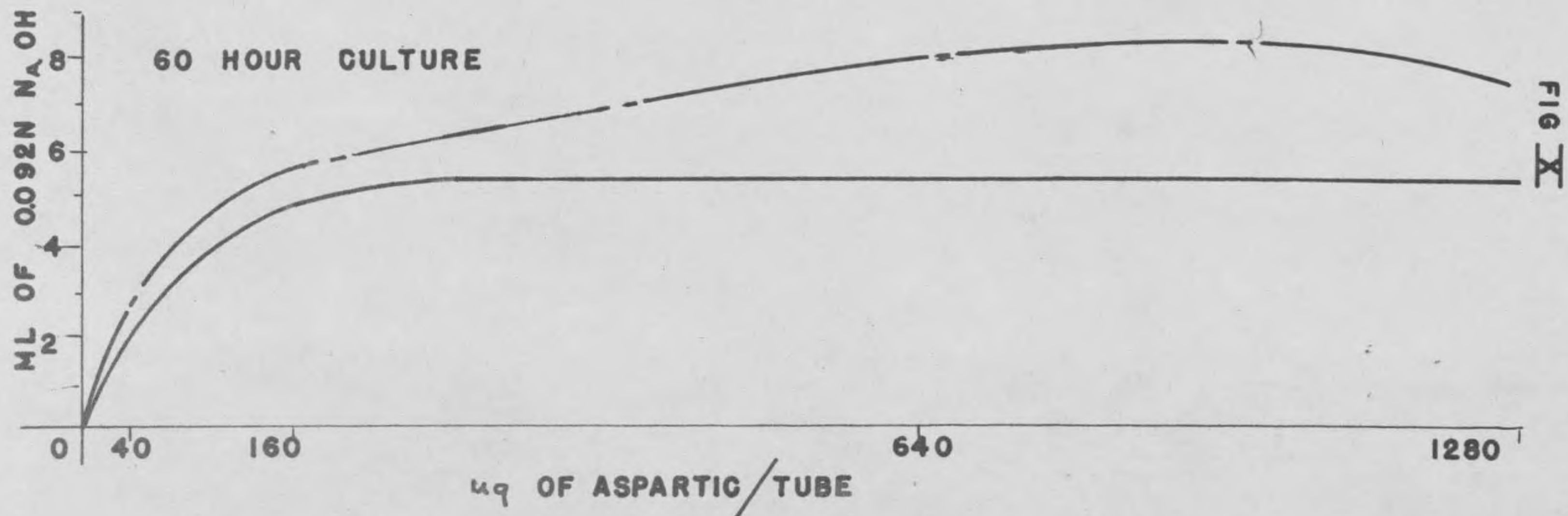


FIG. VIII



BASAL MEDIUM II
40 TO 1280 ug ASPARTIC / TUBE
36 HOUR CULTURE
L- ASP.
D- ASP.
12
10
8
6
4
2
0
40
180
640
1280
FIG. IX



60 HOUR CULTURE
ML OF 0.092N NaOH
8
6
4
2
0
40
160
640
1280
ug OF ASPARTIC/TUBE
FIG X