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Degradation of the Inducible Enzyme Tryptophan Pyrrolase by Lysosomes from Rat Liver Tissue

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B. S., University of Illinois, Chicago Circle Campus 1970

A Thesis

Submitted in Partial Fulfillment of the

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ABSTRACT

Attempts were made to induce the synthesis of the enzyme tryptophan pyrrolase in rat liver tissue, using tryptophan injections. After injection of radioactive ^{14}C-L-Leucine, the incorporation of the labeled amino acid into the inducible enzyme, tryptophan pyrrolase, was determined. The isolation of purified lysosomes was accomplished by centrifugal fractionation and the radioactivity level of the lysosomal fraction was used to follow the degradation of the induced enzyme, tryptophan pyrrolase, during the post-induction period.

Although the results from these experiments are somewhat promising, they do not definitely answer the questions concerning the intracellular function of lysosomes. The lysosomal fractions isolated from the induced rat liver tissue indicated an increase in radioactivity over the lysosomal fractions of the control animals. The expected decrease in the level of radioactivity in the lysosomal fractions of the induced and stabilized animals was not observed.

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INTRODUCTION

In 1949 at the University of Louvain, Belgium, Christian de Duve discovered latency for the enzyme acid phosphatase, i.e. the activity of the enzyme was released over a period of time (1). De Duve presented his discovery at the Second International Congress of Biochemistry in Paris, in 1952 (2). At this meeting he accounted for the latency of acid phosphatase by suggesting that this enzyme may belong to a special type of cytoplasmic particle. He hypothesized that these particles contained a membrane like barrier, limiting the accessibility of the enzyme (acid phosphatase) to its substrate (1). At this meeting also, P.G. Walker indicated to de Duve that he had observed the same latency effects for the enzyme β-glucuronidase (3). R. Gianetto, while working with de Duve, duplicated Walker's work and found that β-glucuronidase exhibited latency patterns similar to acid phosphatase.

Studies involving subcellular fractionation by centrifugation, led de Duve and Berthlet to examine the distributions of the activity of several acid hydrolases in subcellular fractions (1). From these studies de Duve et. al. found that several acid hydrolases exhibited similar sedimentation properties. De Duve speculated that this property was associated with membrane bound granules, containing the several acid hydrolases in their latent form (1). He suggested the name "lysosomes" for these particles (4). The particles (lysosomes) have been found in a variety of animal (4,5,6) and plant tissues (7,8).

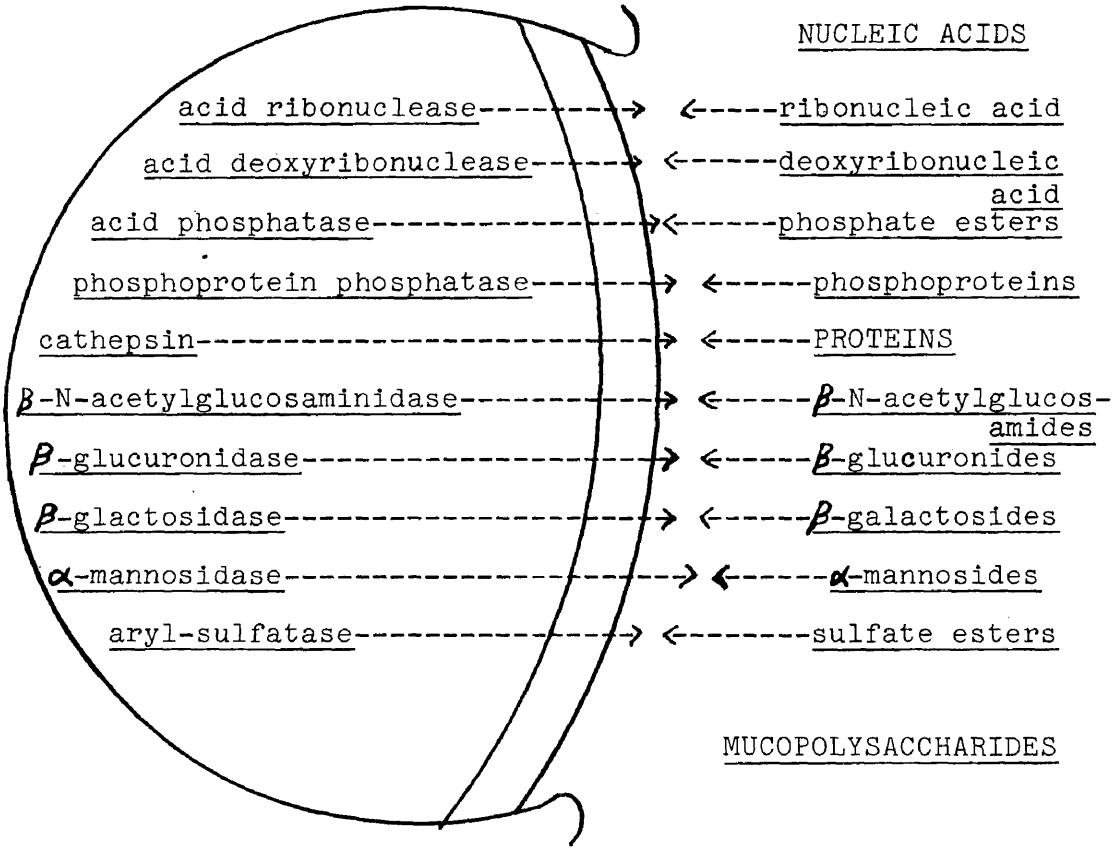
De Duve hypothesized that the lysosomes may be involved in two cellular functions. The first was a cellular autolytic function, i.e. the lysosome may lysis the cell containing it. The term "suicide bag" was given to these particles because of this possible function (1). The second function was that lysosomes may play a role in intracellular digestion (1). Evidence for the function of the lysosomes came from the work

of Werner Strauss, who tested a theory proposed by Jean Oliver (4). Oliver had obtained evidence that droplets of kidney tubule cells were a site of storage and breakdown of reabsorbed proteins (1). However, he believed these droplets originated from the mitochrondria. His theory was based mainly on morphological observations.

Strauss believed that the droplets of kidney tubule cells were actually the lysosomes de Duve had isolated from rat liver tissue. He isolated the kidney tubule cells by differential centrifugation, and by 1954 he had succeeded in obtaining purified kidney droplets which were found to be rich in acid phosphatase and protease (4). After noting the appearence of de Duve's work on hepatic lysosomes, Strauss proceeded to look for other hydrolases. He found that the kidney droplets were indeed very similar to the lysosomal particles that de Duve had isolated from rat liver tissue (11). Strauss's work provided a clear link between lysosomal digestion and endocytic uptake of extracellular materials.

In view of Strauss's work, additional experiments were performed by de Duve. He then summarized both Strauss's experiments and his own, and presented the first schematic outline of the possible biological functions of lysosomes at a meeting of the Society of General Physiology at Woods Hole in June 1958 (12). The schematic proposed by de Duve is shown in Fig. I (13).

Lysosomes



Waring Blender
hypotonic media
isotonic NaCl or KCl
freezing and thawing
sonic vibrations

Intact particles ----------> Injured particles

Lecithinase
proteases
Enzymes bound and inactive — incubation at pH 5.37° — Enzymes soluble and active
detergents
CCl_4

Fig. I. Schematic Representation of the Lysosome Concept.

EXPERIMENTAL RATIONALE

The variety of hydrolytic enzymes associated with the lysosomes renders these particles centers for catalytic activity. Lysosomes have been shown to be involved in the digestion of extracellular material ingested during pinocytosis, and under certain conditions intracellular organelles, such as the mitochrondria. In view of these demonstrated functions, a more routine function of the particles might involve the degradation of individual molecules (e.g. enzymes) which are no longer needed for cellular function. Experiments to explore this possibility are difficult to design since exceedingly small amounts of endogenous material would be involved.

The present experiments were designed to test the degradation hypothesis. Tryptophan pyrrolase, an enzyme which can be induced 50-100 fold in rat liver tissue by the injection or feeding of tryptophan, has been shown to be related to an increase in enzyme protein, (14). If a radioactive amino acid is injected during the induction period, the induced enzyme should become labeled. Subsequently, if the enzyme is degraded in the lysosomes during the post-induction period, radioactivity should occur in the lysosomes. After initial induction of tryptophan pyrrolase, stabilization of the enzyme occurs upon additional administration of tryptophan (15). In this case entrance of the labeled enzyme into the lysosomes would be delayed.

Male three month old Holtzman albino rats were injected with tryptophan and radioactive ^{14}C-L-Leucine according to the injection schedule shown in Table V, in order to test the above hypothesis. The details of the experiments comprise the text of the thesis.

Fractions FI, FII, FIII and FIV were collected using an IEC International Refigurated Centrifuge (Model B-20). The size of the sucrose gradient: 10 ml of 0.7 M sucrose; 9 ml of 0.6 M sucrose; 8 ml of 0.45 M sucrose. The gradient was prepared just prior to use to avoid diffusion or disruption.

Homogenate (1:8 w/v) 0.25 M sucrose

1. Centrifuged at 750 x g for 10 min.
2. Centrifuged at 3300 x g for 10 min.

Pellet (discard) (1) — Supernatant

Centrifuged at 16,300 x g for 20 min.

Pellet — Light mitochrondrial fraction (FI) — Supernatant (discard) (2)

Resuspended carefully in 0.3 M sucrose and centrifuged at 9500 x g for 10 min.

Pellet — Light mitochrondrial fraction (FII) — Supernatant (Discard) (3)

Resuspended carefully in 0.45 M sucrose and layered over a discontinuous gradient of 0.7 M sucrose (bottom layer) and 0.6 M sucrose (middle layer), centrifuged at 9500 x g for 30 min.

Pellet (FIII) — Supernatant (discard) (FIII-S)

Resuspended in 0.7 M sucrose, centrifuged at 5900 x g for 30 min.

Pellet (discard) (P-5)

Supernatant carefully decanted centrifuged at 17,000 x g for 20 min. (S-5). Pellet washed again and centrifuged at 17,000 x g for 10 min. to yield purified lysosomes (FIV).

Fig. II. Scheme for the Isolation of Lysosomes.

EXPERIMENTAL

I. Tissue Preparation

The animals used were three month old Holtzman albino rats, weighing approximately 400 grams. These animals were sacrificed by cervical dislocation, the liver was removed quickly, washed free of blood with cold sucrose, and weighed. All further operations were carried out between 0-4°C. The livers were placed on glass plates, scraped free of connective tissue, then homogenized in a glass-teflon homogenizer. The homogenizer was driven by a 1/4" electric drill, and the speed was regulated by a variable autotransformer at 120 v and a dial setting of 40. The homogenates were filtered through four layers of cheese cloth to remove any other larger tissue particles, then transferred to 50 ml centrifuge tubes. The homogenate was fractionated centrifugally as described in Fig. II (16).

II. Purification of Rat Liver Homogenate

Purification of rat liver lysosomes followed a modification of the centrifugal technique described by A. Tappel et. al. (16). This procedure is shown in Fig. II.

III. Aryl-Sulfatase Enzyme Assay

Aryl-Sulfatase enzyme assay was used as a lysosomal "marker" enzyme to determine the activity of aryl-sulfatase in the lysosomal fraction (7). These solutions were analysed on a Beckman D. B. Spectrophotometer at 515 nm.

IV. Succinate-INT-Reductase Enzyme Assay

A succinate-INT-reductase enzyme assay was used as a mitochrondrial "marker" enzyme. This assay was performed only on the

lysosomal fractions (FIV) to determine mitochrondrial contamination. The final solutions were analysed on a Beckman D. B. Spectrophotometer at 490 nm (7).

To determine the amount of mitochrondrial contamination in the FIV lysosomal fractions, a homogenate standard was used. One gram of rat liver was homogenized with 15 ml of 0.25 M sucrose. This solution was diluted 1:10 with 0.25 M sucrose. The succinate-INT-reductase enzyme assay was performed (7).

V. Protein Determination

Protein was determined according to Lowry et. al., using bovine serum albumin as standard (19).

VI. Radioactivity Counting

a. Preparation of Solvent Fluor Solution.

Radioactivity was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3310). The solvent fluor solution used for the ^{14}C benzoic acid quench standards contained 15.53g or 15.00 ml of p-dioxane (Beckman and Eastman), 9.67 g of PPO (2,5-diphenyloxazole), 96.7 g scintillation grade naphthalene, 0.2 g of dimethyl POPOP (1,4-bis-2-4-mehtyl-5-phenyloxazolyl) benzene (21). The final solutions were mixed by swirling to avoid aeration.

b. Preparation of ^{14}C Benzoic Acid Quench Standards for Channels Ratio.

Into each of six vials was placed 1 ml of ^{14}C benzoic acid in solvent fluor solution which contained 11, 314 dpm/ml, 9.0 ml of solvent fluor solution, and 1.0 ml of distilled water. As the quenching agent the following volumes of carbon terachloride were added, 0.0 ml, 0.05 ml, 0.10 ml, 0.15 ml, 0.20 ml and 0.25 ml, to vials 1-6 respectively. These standard quench sam-

ples were analysed in the above mentioned liquid scintillation counter.

c. Preparation of the Radioactive Lysosomal Samples.

These samples were prepared by placing 1.0 ml of radioactive lysosomal tissue into a vial containing 10.0 ml of solvent fluor solution. These solutions were also analysed in the liquid scintillation counter.

VII. Gain and Window Settings for the Parkard Tri-Carb Liquid Scintillation Counter (Model 3310).

a. Settings for the ^{14}C Benzoic Acid Quench Standards.

The gain setting for both the red channel window and the green channel window was 13%. The red channel (A) window setting was 50, and the red channel (B) window setting was 1000. The green channel (B) window setting was 1000, and the green channel (A) window setting was varied in increments of 50 starting at an initial setting of 50. The ^{14}C quench standards were analysed at each different green channel (A) window setting. A set of quench curves were then plotted and the curve which exhibited the best linear plot was chosen in order to determine the percent efficiency and the disintegrations per minute of the radioactive lysosomal samples. This curve is presented in Fig. III.

b. Settings for the Radioactive Lysosomal Samples.

The gain settings are the same as the above mentioned. The red channel and the green channel window settings are as follows:

Red Channel		Green Channel	
A	B	A	B
50	1000	100	1000

VIII. Channels Ratio Method

This method was used in order to determine the percent efficiency

and the disintegrations per minute of the radioactive lysosomal samples. The graph that was used for these determinationa are presented in Fig. III. The channels ratio was obtained by:

$$\frac{\text{Counts per Minute Green Channel}}{\text{Counts per Minute Red Channel}}$$

The values for the percent efficiency were obtained also from the graph shown in Fig. III. The values for the disintegrations per minute were obtained as follows:

$$\% \text{ Eff.} = \frac{\text{cpms Red Channel}}{\text{dpms}}$$

IX. Reagents

1. The substrates, p-nitrocatechol sulfate and sodium succinate (disodium salt), and 2-(p-iodophenyl) - 3 - p - nitrophenyl) - 5 - phenyl-tetrazolium chloride (INT), were obtained from Sigma Chemical Company. All other chemicals used were reagent grade, and solutions were prepared using deionized water.

2. ^{14}C-L-Leucine was obtained from Cal-Biochem. and had a specific activity of 222 mC/mM.

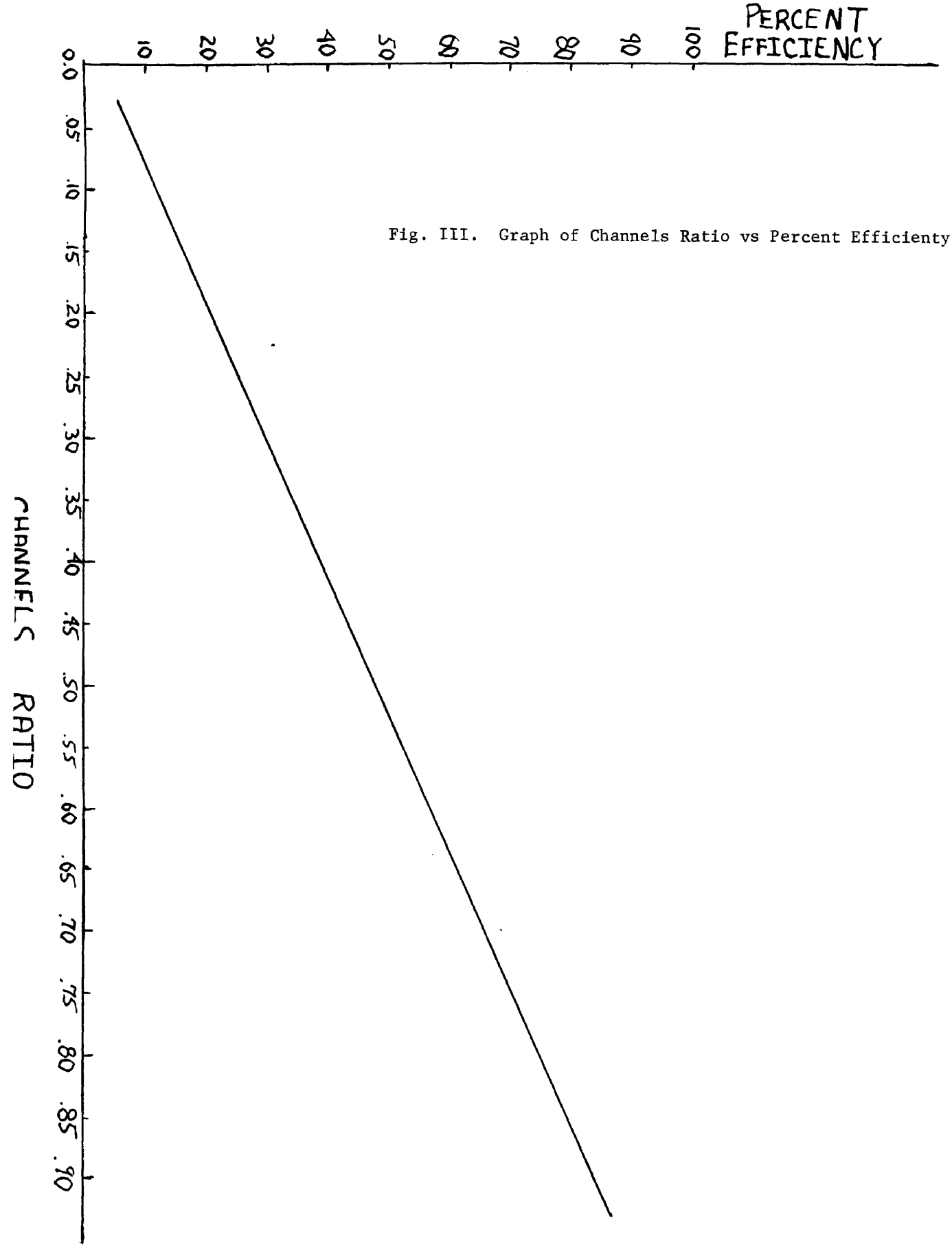


Fig. III. Graph of Channels Ratio vs Percent Efficienty

RESULTS

Two rat livers were fractionated according to Fig. II. The total aryl-sulfatase activity per fraction is shown in Table I. The lysosomal fraction (FIV) of rat 2 showed no aryl-sulfatase activity. In order to determine the location of aryl-sulfatase activity, all pellets and supernatants were examined from fraction FIII (Fig. II) on a third rat liver. The results are also shown in Table I. These results indicated that aryl-sulfatase activity was present in fraction FIV.

Four additional rat livers were fractionated. Two received no injections and two received saline injections. The results from this experiment are shown in Table II. The results of the succinate-INT-reductase enzyme assay performed on FIV fraction of rats 4,5,6 and 7, indicated that there was essentially no mitochrondrial contamination. The absorbance values ranged from 0.00 to 0.02.

The rats numbered 6 and 7 were injected with saline to determine if this solution would have any effect on the sedimentation properties of the lysosomal particles. The lack of aryl-sulfatase activity in the FIV fraction of rat 6 is an apparent anomally.

Another rat liver was fractionated to determine the amount of lysosomal protein from fraction FIV that would dissolve in 10.0 ml of solvent fluor solution. It was found that 1.0 ml of fraction FIV would dissolve in the 10.0 ml of solvent fluor solution.

Protein analysis and aryl-sulfatase activity of the five fractions (Fig. II) for rats 5 and 7 are shown in Table III. Radioactive rats were injected according to the schedule presented in Table IV. The results from these nine injected rats are found in Tables V, VI and VIII. A summary of the nine radioactive animals is presented in Table VII.

Table I. Total Aryl-Sulfatase Activity Per Fraction for Rats 1,2 and 3.

Rat	Fraction	Activity Per Fraction[a]
I	Homogenate	3.60
	FI	4.90
	FII	2.80
	FIII	1.80
	FIV	.09
2	Homogenate	1.00
	FI	.87
	FII	.77
	FIII	.25
	FIV	.00
3	FIII	.88
	FIII-s	.80
	P-5	.56
	S-5	.33
	FIV	.08
	FIV-S	.09

a--indicates the activity is expressed in absorbance units.

Table II. Total Aryl-Sulfatase Activity Per Fraction (expressed in absorbance units) of Rats 4, 5, 6 and 7.

Rat	Homogenate	Total Activity Per Fraction FI	FII	FIII	FIV
4*	.46	3.4	2.8	1.4	.45
5p*	5.3	3.9	2.6	1.1	.15
6*'	5.3	3.7	2.6	1.1	.00
7p*'	7.6	5.1	4.3	2.1	.27

* - Indicates succinate-INT-reductase enzyme assay performed

p - Indicates protein determination performed

' - Indicates the rats that were injected with 8.0 ml of 0.85% saline intraperitoneally

Table III. Protein Analysis of Rats 5 and 7, and Aryl Sulfatase Specific Activity.

Rat	Fraction	Milligrams Protein Per ml	Specific Activity (absorbance units per mg protein)
5	Homogenate	8.50	.620
	FI	6.85	.570
	FII	5.90	.432
	FIII	4.00	.285
	FIV	7.25	.207
7	Homogenate	4.62	.164
	FI	8.80	.574
	FII	6.50	.660
	FIII	4.25	.323
	FIV	4.25	.655

Table IV. Injection Schedule for the Induction of Tryptophan Pyrrolase and ^{14}C-L-Leucine Incorporation.

Time	Control*	Induced*	Induced and Stabilized*
0	8.0 ml of 0.85% saline (sterilized)	150 mg tryptophan dissolved in 8 ml of 0.85% saline solution	
1 hr.	1.0 ml ^{14}C-L-Leucine dissolved in saline, 12 mC/ml, specific activity of 222 mC/mM	SAME AS CONTROL	
4 hr.	.....		150 mg of tryptophan in 8.0 ml of .85% saline
12 hr.	All animals sacrificed and lysosomes isolated from rat liver tissue according to the scheme presented in Fig. II.		

* - Three rats were used for each separate group shown above.

Table V. Total Aryl-Sulfatase Activity Per Fraction, Milligrams Protein, and Specific Activity for the Three Control Rats.

Rat	Fraction	Total Enzyme Activity (absorbance units)	Milligrams Protein (per ml)	Specific Activity (absorbance units per mg protein)
C1	Homogenate	4.14	-------	-------
	FI	6.00	-------	-------
	FII	6.14	-------	-------
	FIII	3.52	-------	-------
	FIV	.176	1.33	.133
C2	Homogenate	4.47	-------	-------
	FI	5.85	-------	-------
	FII	3.75	-------	-------
	FIII	2.44	-------	-------
	FIV	.079	1.33	.595
C3	Homogenate	4.10	-------	-------
	FI	3.30	-------	-------
	FII	3.72	-------	-------
	FIII	1.19	-------	-------
	FIV	.20	.620	.327

Table VI. Total Aryl-Sulfatase Activity of the FIV Fraction for Induced and Induced and Stabilized Rats.

Rat	Total Enzyme Activity (absorbance units)	Milligrams Protein Per Ml	Specific Activity (absorbance units per mg Protein)
I1[a]	.68	1.67	.407
I2[a]	1.07	.84	.727
I3[a]	.70	2.03	.345
S1[b]	.37	1.45	.255
S2[b]	.22	.900	.244
S3[b]	.41	.725	.566

a - Indicates induced rats

b - Indicates induced and stabilized rats

Table VII. Liquid Scintillation Data

Rat	Red Channel CPMS	Green Channel CPMS	Channels Ratio	Efficiency	Disintegrations Per Minute	Disintegrations Per Minute Per MG (protein)	Disintegrations Per Minute Per S A[a]
C1	127.6	111.8	.88	.82	155.6	117	1170
C2	33.6	28.4	.84	.78	43.1	32.5	72.5
C3	18.5	16.1	.87	.81	22.8	36.8	70.0
I1*	62.6	47.7	.76	.71	88.2	52.8	215
I2	178.6	131.6	.74	.69	258.8	308	356
I3	199.4	152.6	.76	.71	280.8	112	815
S1	250.2	176.9	.71	.66	379.1	261	1485
S2	665.9	49.4	.75	.70	87.9	97.6	360
S3	155.8	110.0	.71	.66	236.1	339	416

* - Indicates an error in fractionation procedure.

a - S. A. is an abbreviation for specific activity.

The error in the fractionation procedure of rat 11 involved fraction FIII. This fraction was spun at 17,000 x g for twenty minutes before it was noticed that this fraction should have first been spun at 5,900 x g for thirty minutes. The pellet obtained was resuspended in 0.7 M sucrose and the isolation procedure proceeded from the 5900 x g spin according to Fig. II.

The results from the succinate-INT-reductase enzyme assay performed on 1.0 gram of rat liver tissue used as the homogenate standard, showed 0.67 mg of mitochrondrial homogenate contamination. The results from the succinate-INT-reductase enzyme assay on the radioactive FIV lysosomal fractions are shown in Table VIII.

Table VIII. Mitochrondrial Contamination in Radioactive Rats.

Rat	Total Enzyme Activity FIV Fraction	[a]Milligrams of Mitochrondrial Protein in the FIV Fractions
C1	0.00	0.00
C2	0.00	0.00
C3	0.00	0.00
I1[b]	0.48	1.11
I2	0.52	0.97
I3	0.43	0.96
S1	0.17	0.48
S2	0.10	0.27
S3	0.37	1.25

a - Indicates these values are in reference to homogenate standard.

b - Indicates error in fraction procedure.

DISCUSSION

Results shown in Table VII indicate that lysosomal fractions isolated from induced animals had significantly higher specific radioactivity than saline injected controls. The results are consistant with the original hypothesis, which suggested that the labeled tryptophan pyrrolase might enter the lysosomes in the post induction period. However, there are other reasons which can explain the results equally well. One explanation is that the lysosomal proteins (lysosomal enzymes and lysosomal membranes) may undergo an increased rate of synthesis during the induction period, thus directly increasing the radioactivity of the lysosomes. Too, the increase in mitochrondrial contamination may be significant in the increase in radioactivity in the lysosomes isolated from induced animals.

Animals which received a second injection of tryptophan should have had a prolonged induction period and a stabilization of the existing tryptophan pyrrolase molecules. According to the hypothesis, this should have resulted in delayed entrance of the enzyme into the lysosomes. Accordingly lower specific radioactivity would be expected in lysosomes isolated from these animals. However, results in Table VII indicate that lysosomal specific radioactivity is approximately the same in lysosomes isolated from induced and induced and stabilized animals. These results would not appear to support the contention that radioactive tryptophan pyrrolase is delayed in its entrance into the lysosomes.

Although the present results are somewhat promising, they do not definitly answer the questions concerning the intracellular function of lysosomes. Further experiments would include the isolation of extremely pure lysosomes and the positive identification of the enzyme tryptophan pyrrolase from these pure preparations. The latter could be accomplished by specific antibody precipitation.

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