1968

Disc Electrophoretic Studies of the Soluble Fraction of Normal and Cryptorchid Rat Testes

Robert P. N. Shearin
Loyola University Chicago

DISC ELECTROPHORETIC STUDIES OF THE SOLUBLE FRACTION OF NORMAL AND CRYPTORCHID RAT TESTES

by

ROBERT P. N. SHEARIN

A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Master of Science

May
1968

LIFE

Robert P. N. Shearin was born in Chicago, Illinois on December 25, 1939. He attended Quigley Preparatory Seminary, Chicago, Illinois. In June, 1964 he received the degree of Bachelor of Science from Loyola University, Chicago, Illinois.

In September, 1964 the author began his medical studies at the Loyola University Stritch School of Medicine and in June, 1965 he was accepted into that school's combined medical and graduate studies program.

In May, 1967 he was married to the former Mary Ann Sheehan of Chicago, Illinois.

ACKNOWLEDGEMENTS

The author wishes to take this opportunity to express his gratitude to those who have inspired and stimulated him to achieve this present goal. A special feeling of admiration and appreciation must be directed to Dr. Joseph R. Davis, major advisor, a peerless teacher, investigator, and mentor. His fascination with science has been truly inspiring. Appreciation must also be here expressed to Dr. Alexander G. Karczmar, Chairman of the Department of Pharmacology, a man of generous spirit. The author cannot adequately express his deep debt of gratitude to his parents who have, after all, been helping him for a time longer than any else. Lastly, the author is compelled to here mention his deepest love for his wife, Mary, who puts up with him.

STATEMENT OF PURPOSE

In the white rat the cryptorchid state produces in the testis gross atrophy, spermatogenic arrest, an increase in the protein content of the 15,000 x gravity supernatant, and increases in the activities of lactate dehydrogenase and malate dehydrogenase. In the human cryptorchid testis, in addition to atrophy and spermatogenic arrest, an increased tendency toward the development of malignancy has long been noted. The present thesis is a report on efforts to define by disc electrophoresis the general protein, lactate dehydrogenase, and soluble malate dehydrogenase profiles of the 15,000 x gravity supernatants of normal pre-pubertal and mature Sprague-Dawley rats and to detect changes in these profiles as induced by the cryptorchid state.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
	A. Disc Electrophoresis	1
	1. The origins of disc electrophoresis	1
	2. Mechanisms involved in disc electrophoresis	1
	3. The polyacrylamide system	5
	4. Uses of disc electrophoresis	6
	B. Cryptorchidism	6
	1. Cryptorchidism in man	6
	2. Cryptorchidism in rats	8
	a. Method	8
	b. Abdominal and scrotal temperatures	9
	c. Effects of the duration of abdominal fixation	9
	d. Histological comparisons between pre-pubertal and adult cryptorchid rat testes	10
	e. Some biochemical differences between scrotal and cryptorchid rat testes	11
	1.) Carbohydrate metabolism	11
	2.) Protein metabolism	12
	3.) Lipid metabolism	13
	C. Dehydrogenases	14
	1. Lactate dehydrogenase	14
	a. General characteristics	14
	b. Localization in the testis	14
	c. LDH isoenzymes	15
	d. LDH-X	16
	2. Malate dehydrogenase	17
II.	MATERIALS AND METHODS	19
	A. Animals	19
	1. Immature cryptorchid rats	19

2. Mature cryptorchid rats 19
B. Sample preparation 19
C. Gel preparation 20
1. Reagents 20
2. Stock solutions 20
3. Working solutions 21
4. Column preparation 21
5. Polymerizations 22
D. Electrophoresis 22
1. Reagents 22
2. Procedure 22
E. Staining 23
1. Lactic acid dehydrogenase 23
2. Malic acid dehydrogenase 24
3. General protein 24
F. Densitometry 24
G. Photography 24
III. RESULTS 25
A. Lactic Acid Dehydrogenase Isoenzyme Patterns of the Testes of Normal and Artificially Cryptorchid Rats . . 25
1. Normal rats 25
2. Pre-pubertal cryptorchid rats 25
3. Mature cryptorchid rats 26
4. Planimetry of lactic acid dehydrogenase profiles . 26
B. Malic Acid Dehydrogenase Patterns of the Testes of Unilaterally Cryptorchid Rats 26
C. General Protein Patterns of the Soluble Fraction of The Testes of Normal and Artificially Cryptorchid Rats 27
1. Normal rats 27
2. Pre-pubertal cryptorchid rats 27
3. Mature cryptorchid rats 28

IV. DISCUSSION 29
V. SUMMARY 35
VI. FIGURES 36
Figure I Disc Electrophoresis Apparatus 37
Figure II LDH Isoenzymes of Pre-Pubertal and Adult Rat Testes 38
Figure III LDH Isoenzymes of Pre-pubertal Scrotal and Cryptorchid Rat Testes 39
Figure IV LDH Isoenzymes of Mature Scrotal and Cryptorchid Rat Testes. 40
Figure V Malic Acid Dehydrogenase Patterns in Scrotal and Cryptorchid Rat Testes 41
Figure VI General Protein Patterns of Pre-pubertal and Adult Rat Testes 42
Figure VII General Protein Patterns of Pre-pubertal Scrotal and Cryptorchid Rat Testes 43
Figure VIII General Protein Patterns of Mature Scrotal and Cryptorchid Testes 44
VII. TABLES 45
Table I . . . Percent of Total LDH Activity in the Scrotal and Cryptorchid Rat Testis by Isoenzyme . . . 46
BIBLIOGRAPHY 47

CHAPTER I

INTRODUCTION

A. DISC ELECTROPHORESIS

Disc electrophoresis is the term applied to zone electrophoresis through small columns of polyacrylamide gel while employing a discontinuous buffer system for conduction of the applied current. The term "disc" is derived primarily from the method's dependence on discontinuities in the electrophoretic matrix and secondarily from the discoid shapes of the separated zones (58).

1. The origins of disc electrophoresis.

In 1955 Smithies added a new dimension to zone electrophoresis, namely a sieving action through molecular-size pores, with his report on starch gel electrophoresis (67). Resolution of serum proteins was further increased by the introduction of discontinuous buffer systems to the procedure by Poulik in 1957 (61). Having searched for a more manageable medium for zone electrophoresis than Smithies' friable potato starch gel, Raymond reported on his work with flat slabs of cross-linked polyacrylamide gel in 1959 (62). Simultaneous with this Davis and Ornstein, using cylindrical polyacrylamide columns and a discontinuous Tris-glycine buffer system, developed the prototype procedure for contemporary disc electrophoresis (12).

2. Mechanisms involved in disc electrophoresis.

Ornstein (58) has rigorously investigated the mechanisms of electrophoresis through long chain polymer gels in discontinuous buffer systems. The basic theoretical advantage inherent in disc electrophoresis is described by the Kohlrausch Regulating Function:

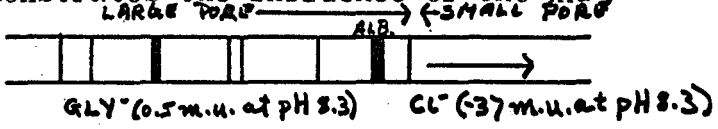
$$\frac{(A)}{(\Gamma)} = \frac{m_\alpha z_\gamma (m_\gamma - m_\beta)}{m_\gamma z_\alpha (m_\alpha - m_\beta)}$$

where A = protein
Γ = chloride ion
z_α = charge on protein
z_γ = charge on chloride
m_α = mobility of protein
m_β = mobility of potassium ion
m_γ = mobility of chloride ion

A practical result of the Kohlrausch Regulating Function (KRF) is the production, under specified electrophoretic conditions and using a soluble protein mixture as sample, of a stack of thin discs of protein electrophoretically purified, concentrated, and serially arranged according to their charge and mobility. The purity and arrangement of these laminae are related to the charge and mobility of each protein and to the differences in these quantities, one protein species from another. The concentration of each protein in its lamina tends to be maintained by the relation (A)/(Γ). The width of each lamina is directly proportional to the amount of its protein in the sample, i. e. :

Arrangement	$\propto$	charge and mobility
Purity	$\propto$	differences of charge and mobility
Concentration	$\propto$	(A)/(Γ)
Width of disc	$\propto$	initial amount of protein in sample

In the following example the electrophoresis of a sample containing albumin demonstrates the influence of the KRF:



where $z_\alpha = -30$ (approximate charge on albumin at pH 8.3) (Z)
$z_\gamma = -1$
$m_\alpha = -6.0$ mobility units (1 m. u. = 10^{-5} cm^2/volt-sec)
$m_\beta = +37$ mobility units
$m_\gamma = -37$ mobility units
employing the KRF:

$$\frac{(\text{Albumin})}{(\text{Chloride})} = \frac{m_\alpha z_\gamma (m_\gamma - m_\beta)}{m_\gamma z_\alpha (m_\alpha - m_\beta)}$$

$$\frac{(\text{Albumin})}{(\text{Chloride})} = 9.3 \times 10^{-3}$$

if (Chloride) = 0.06 M
then (Albumin) = 5.6×10^{-4} M or 3.4% within its lamina

Assuming the use of a serum with 3.5 grams albumin per 100 ml. and the dilution of this serum fifty-fold, then: the concentration of albumin in 100 microliters applied to the gel is 0.07%; the KRF concentrates the albumin to 3.4% within its disc which has a volume of 0.018 cm^3; and the albumin disc width is 0.47 mm. at the point of its entrance into the small pore gel column with a diameter of 7 mm.

Ornstein's theoretical groundwork and Davis' application of it involved the use of three contiguous layers of polyacrylamide gel serially polymerized within one piece of glass tubing (13,58). The base of the gel column, as positioned for vertical electrophoresis, is composed of 7.5% acrylamide gel and is called the separation gel or the small pore gel. This gel is polymerized by the incorporation of 70 mg% of the catalyst ammonium persulfate into the sol. Above this is layered to a depth of one centimeter a 5% acrylamide gel called the spacer gel or the large pore gel. This gel is photopolymerized by the action of fluorescent light on its 0.5 mg% concentration of riboflavin. Above the large pore gel is layered 0.15 to 0.20 ml. of the large pore sol containing not more than 200 micrograms of sample protein. This last layer is called the sample gel and is also photopolymerized. For increased resolution of some sample components the sample gel may be replaced by an anticonvection medium of 40% sucrose in which the sample protein is dissolved. This method has been used by many, including Dietz and Lubrano (18).

A verbalized illustration of the mechanisms in disc electrophoresis is given by Davis (13):

"Electrophoretic separation is proportional to time 't' and diffusion to '$t^{\frac{1}{2}}$', at first glance indicating that electrophoretic resolution is enhanced by increasing the length of the run. Practically, however, resolution is the detection of separation, not separation per se, and present methods of detection are positively related to the concentration of the protein. Therefore widely separated but dilute protein species are less easily resolved than closer discrete concentrated protein bands.

"Since diffusion during electrophoresis continuously dilutes the separating fractions and blurs their boundaries, it is desirable to reduce the running time to the minimum necessary to achieve a desired separation of the constituent ions. For this reason the thickness of the starting zone critically affects the resolution of a mixture of ions; the thinner the starting zone, the shorter the running time

necessary for a given separation of constituents. Thus an additional gain in electrophoretic resolution can be achieved by means of a step that reduces the thickness of the starting zone. In the technique we have named disc electrophoresis, this step is based on simple electrochemical laws, as formulated in the Kohlrausch Regulating Function (40), and the rationale of the procedure, although only implicit in a report by Kendall et al. (39), is virtually identical to that of their technique for the purification and concentration of mesothorium present in small amounts from a solution of barium chloride.

"The concentration step is achieved by introducing the mixture of sample ions into an electrophoretic column near the boundary of two ions whose sign is like that of the sample ions at a given pH. One ion is faster, the other (an ion of a weak acid or base) slower than all of the sample ions at this pH. Electrical polarity is set so that the fast ion is situated ahead, i.e., in the direction of migration, of the sample and the slow ions. Application of a voltage results in the segregation from one another and stacking of the constituent ions of the sample into contiguous zones in order of their relative mobilities, the entire sample sandwiched between the slow and fast ions. The final concentration of each constituent in the stack is independent of its original concentration in the mixture but is proportional to the concentration of the fast ion. Thus a dilute sample can be concentrated into a zone the thickness of which is fixed by the amount of ionic material present in the sample and by the chosen concentration of the fast ion.

"Shortly after the sample ions have reached fixed high concentrations, they are arranged to migrate into a region of the electrophoretic column where a new set of conditions of pH or pore size or both obtains so that the mobility of the ions of the weak acid or base now exceeds that of, for example, the fastest protein. The ions of the weak acid or base now continuously overtake and pass through the sample species, establishing a comparatively uniform voltage gradient in which electrophoretic separation of the sample occurs" (13).

Raymond, who prefers to use flat slabs of polyacrylamide gel with continuous buffer systems, has attacked various features of Ornstein's method (63). He says, in fact, that "disc electrophoresis... does not in itself make use of the special features of gel electrophoresis, which are the molecular filtration effects." His reasoning on this point seems skewed, for, as indicated by both Davis and Ornstein (13,58), the use of a separation gel of different (smaller) pore size (different molecular filtration characteristics and increased viscosity) is essential to disc electrophoresis.

Raymond rejects the use of a sample gel and a spacer gel in the following terms: "The thinness of the sample zone depends on the ratio of migration velocity in the sample gel to migration velocity in the supporting gel. The lower the concentration of the sample gel, the higher this migration ratio will be. Therefore the maximum degree of thinness in the starting zone will be achieved when the sample gel is omitted entirely." (63) No proof of this is offered.

In the system of Ornstein and Davis the thinness of the sample zone, using a discontinuous buffer system to which the KRF is applicable, is primarily determined by:

1.) the concentration of the fast buffer ion of like sign as that of the sample at the running pH
2.) the initial amount of the sample
3.) the cross sectional area of the column (58).

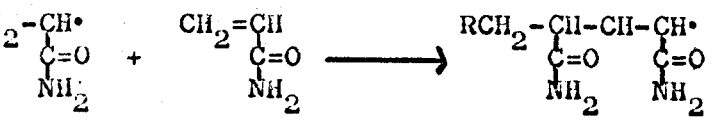
Raymond rejects discontinuous buffer systems because they "introduce nonlinearities" into electrophoretic pattern which obscure interpretation (63). It may be, therefore, that he is comparing incomparable systems in his critique.

3. The polyacrylamide system.

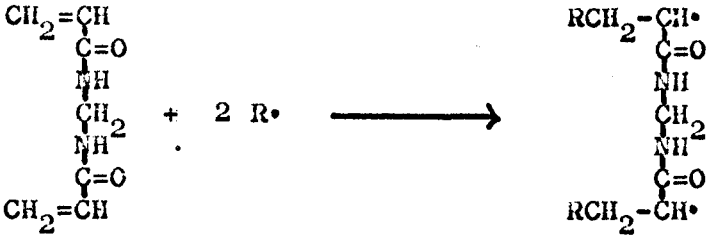
The acrylamide monomer $CH_2=CH-\overset{O}{\overset{\|}{C}}-NH_2$ is polymerized by a free radical (R•) produced by an initiator system in the following manner:

$$CH_2=CH-C(=O)-NH_2 + R\bullet \longrightarrow RCH_2-CH\bullet-C(=O)-NH_2$$

to which is added the

monomer  in a rapid reaction without residual products to slow the rate of polymerization.

Cross-linking of these polymers occurs through N,N'- Methylene-bisacrylamide interposition after activation by R• as:



Usual mixtures are 95% monomer and 5% bisacrylamide. Although various initiator systems may be used, usual systems are ammonium persulfate, $(NH_4)_2S_2O_8$, as initiator for the separation gel and riboflavin, $C_{17}H_{20}O_6N_4$, in the presence of fluorescent light for the spacer gel (59).

White (77) found that permeability decreases with increasing acrylamide concentration over the range from 5% to 30% and that it is independent of cross-linking. He, therefore, assumes a "brush heap" type of gel structure. Ornstein (58) has computed that in a 7% separation gel proteins with a minimum diameter less than 200 Å and a maximum diameter less than 400 Å will be separated.

4. Uses of disc electrophoresis.

Because of the workable nature of polyacrylamide gel and its ready application to histochemical techniques, disc electrophoresis has found many applications, e.g., separation of protein solutions (53, 70); resolution of various enzymes and their isoenzymes (18,36,72,24); isolation of proteolytic enzymes (75); identification of DNA-ase (3); and analysis of C^{14}-labeled proteins (20).

B. CRYPTORCHIDISM

1. Cryptorchidism in man.

Cryptorchidism, the failure of one or both testes to descend into the scrotum, is found in 10% of newborn infants, 2% of one year olds, 1% of boys at puberty, and 0.3% of adult men (5). United

States Army statistics derived from a study of 10,000 consecutive inductees place the incidence of cryptorchidism at 0.75% (2). Unilateral cryptorchidism is five times as common as the bilateral condition (57).

The specific cause of cryptorchidism is unknown but theories of etiology include mechanical interferences with descent; endocrinopathies; and defects in testicular response to stimuli for descent (5).

Pathological changes are generally not seen in the cryptorchid testis before age 10, although in 20% of the cases seen by Wolgin and Charny (5) occasional testicular degeneration was noted. After age 10 progressive atrophy ensues with decreasing size and increasing consistence due to fibrosis (64). Histological changes include hyaline thickening of the basement membranes and an increase in the interstitial connective stroma concomitant with decreasing spermatogenic activity. The germinal epithelium then atrophies, leaving but a few persistent spermatogonia and Sertoli cells. Hyalinization of the basement membranes proceeds to fill the tubular structures with dense hyaline cords. The usual picture also includes Leydig cell hyperplasia which may be absent in the event of underlying pituitary insufficiency (64).

Explanation of degenerative changes seen in the extra-scrotal cryptorchid testis has been sought in theories of inherent congenital deficiency, first proposed by Hunter in 1786 (34); developmental disturbance (30); premature sclerosis of the testicular artery (68); and the effect of high environmental temperature, first proposed by Moore in 1924 (51).

It is interesting to note that although the cryptorchid testis almost always becomes aspermatogenic due to tubular degeneration as noted above, urinary excretion of 17 - ketosteroids is usually normal as is the development of secondary sex characteristics, even in the bilaterally cryptorchid male without generalized endocrinopathy (5). Lest it be assumed that the functional capacity of the Leydig cells is not deleteriously affected by the cryptorchid state it should be noted that

gonadotrophin excretion in cryptorchid males is uniformly elevated (5).

The significance of cryptorchidism in man, itself not life-threatening or even detrimental to health, is seen in the relation of this condition to fertility, to the development of testicular neoplasms, and to psychopathology.

The usual finding in the bilaterally cryptorchid man is infertility, although preservation of fertility has been reported (57). In uncomplicated unilateral cryptorchidism fertility is the rule (5).

The risk of malignant changes developing in a cryptorchid testis is a matter of some controversy. Robbins (64) discounts an increased vulnerability of the cryptorchid testis to neoplasia as being "probably small," citing the experience of Gross and Jewett in their series of 1222 surgical cases (28). Campbell, however, in his classical study of 9,741,097 military recruits, makes a convincing case for a 33-fold increase in tumors seen in cryptorchid as compared to scrotal testes (4). Of malignant tumors arising in cryptorchid testes there is a marked tendency to seminoma formation (19).

The relationship of cryptorchidism to psychic disturbance is problematical. Pediatricians may note emotional changes in the maturing cryptorchid youth who becomes increasingly aware of his defect (5). Although the absence of scrotal testes would seem a logical cause for abnormal feelings of depression, anxiety, and inferiority, Davidoff (11) concludes that there is no psychic entity associated with cryptorchidism. Charny and Wolgin (5) have not observed emotional disorders in cryptorchid boys without hypogonadism who had not been frequently reminded of their defect. They concluded that the average adolescent with cryptorchidism and normal sexual maturation is unaware of or unconcerned with his defect.

2. Cryptorchidism in rats.

a. Method

Experimental cryptorchidism in rats involves the anchoring, by various means, of one or both testes in the abdominal cavity with the intent of producing, by this re-positioning alone, testicular chan-

ges characteristic of congenital cryptorchidism.

Clegg (8) and Niemi and Kormano (56) accomplished this by retracting both testes into the abdomen and then closing the inguinal canals. Nelson (55) anchored both testes to the abdominal wall with a silk suture through the epididymal fat pad. Clegg (8) has made rats unilaterally cryptorchid, using the scrotal testis as a control in his studies. Davis and Firlit (40) have refined the unilateral fixation of the right testis of an etherized rat through an abdominal incision. They fixed the testis to the anterior abdominal wall by placing a 6-0 silk suture through the avascular tunica albuginea, avoiding the testicular artery. They noted no testicular ischemia during the thirty day abdominal retention in their study.

b. Abdominal and scrotal temperatures

Assuming that the testicular vasculature is not impaired and that the influence of peritoneal fluid is innocuous, the primary cause of the anatomical and biochemical changes seen in the experimentally cryptorchid rat testis would seem to be the elevated temperature of the abdomen relative to the scrotum. In the rat the abdominal temperature varied from 4.0° C. to 7.4° C. higher than the scrotal temperature in varying ambient temperatures (50). Hollinger (31), using a thermometric probe, found the mean peritoneal temperature to be 37.5° C. in the male Sprague-Dawley rat while the mean scrotal temperature approximated 34.5° C. at an ambient temperature of 25° C.

c. Effects of the duration of abdominal fixation

In the abdominally transplanted adult and pre-pubertal rat testes Davis and Firlit (16) found marked differences in wet weight when compared to the scrotal control testes. These differences were marked at 10 days after cryptorchiopexy and apparently stabilized at 20 days. At 30 days the abdominal testis averaged about 25% of the wet weight of the scrotal testis. Clegg (8), working with unilaterally cryptorchid rats found a general but slight reduction in the length and breadth of abdominal testes relative to the scrotal controls over a 33 day period of abdominal retention. The mean breadth/length ra-

tios for the two groups of testes, however, were not statistically different. In 1963 Clegg (9) noted a significant increase in the number of Sertoli cells in the cryptorchid tubule between 10 and 21 days postoperatively. He postulated the cause of this increase as being the amitotic division of Sertoli cells and suggested that they phagocytosed spermatogonia and spermatocytes during this period. Later in 1963 he described a broader theory of the mechanism of cryptorchid changes based on his observation that degeneration of the germinal epithelium was maximal at the 15th postoperative day and showed significant recovery by day 35, with the reappearance of spermatids at this time. He postulated a temperature-invoked block of gonadotrophin action on the testis coupled with thermal degenerative effects and suggested that the recovery was due to the over-riding of this block by increased levels of pituitary gonadotrophins.(10).

d. Histological comparisons between prepubertal and adult cryptorchid rat testes

Davis and Firlit (16) noted that experimental cryptorchidism in the rat, as studied by various investigators, has invariably been produced by anchoring the adult, sexually mature, testis in the abdomen, a condition not exactly duplicating natural cryptorchidism. In an effort to more closely duplicate nature they performed unilateral cryptorchiopexy on 20 day old Sprague-Dawley rats prior to testicular descent which normally occurs at about 35 days of age. These animals were sacrificed at intervals of up to 30 days and histological differences were surveyed in the abdominal versus the scrotal testes. The testes of these animals were in turn compared to those of animals made unilaterally cryptorchid at age 60 days, after spontaneous testicular descent and spermatogenic maturity. These animals were also sacrificed at intervals of up to 30 days post-operatively. Their results indicated that spermatogenesis in both the pre-pubertal and the adult cryptorchid testis was arrested at the early pachyneme stage of meiosis. After 30 days in the abdomen the histological pictures of the pre-pubertal and adult cryptorchid testes were quite similar, consisting of seminiferous tubules containing much Sertoli cell

cytoplasm, some Type B spermatogonia, and an occasional early pachyneme spermatocyte. This similarity suggests that transplanting the adult testis to the abdomen probably closely approximates the natural failure of testicular descent. They suggest that this similarity may be due to the absence of a regulator contained within the spermatid which may act on hypophyseal gonadotrophin secretion, as supposed by Johnsen (37), or directly as a trigger mechanism on the testis itself (21,65).

e. Some biochemical differences between scrotal and cryptorchid rat testes.

1.) Carbohydrate metabolism

Tepperman, et al. (73)found that, in the absence of added substrate, the QO_2 of the normal rat testis decreased with age. The QO_2 of the cryptorchid testis was significantly higher, presumably due to the absence of more mature cell types with less active carbohydrate metabolism. The cryptorchid testis has been shown, however, unable to oxidize glucose (46) and has a QO_2 glucose of 0.5 while the normal testis has a QO_2 glucose of 0.93 (73). One suggestion consonant with these findings is that the cryptorchid testis utilizes increased amounts of substrates other than glucose in its relatively increased metabolic state. Hollinger and Davis (32), incubating slices of normal testes and testes cryptorchid for 30 days with radioactively labeled glucose for one hour found a five-fold increase in labeled protein in the cryptorchid testis compared to the control. Also noted was a generalized decrease in glucose metabolism, 65% of total in the cryptorchid versus 88% of total in the normal. Concomitantly they found a diminution of labeling of aspartate in the cryptorchid testis out of proportion to the generalized decrease in labeling of glucose metabolites in the cryptorchid tissue. The normal testis yielded an aspartate peak labeled with 23,000 dpm/100 mg wet weight tissue compared to the cryptorchid aspartate peak of 3,840 dpm/100 mg wet weight tissue.

In considering these and other biochemical variables it is essential to remember that the cryptorchid and the scrotal testes are in

great measure two separate tissues, with some distinct differences in cell type and number.

2.) Protein metabolism

Hollinger (31) measured the protein content of whole homogenates and 15,000 x gravity supernatants of scrotal and cryptorchid testes from the same animal. The whole homogenates contained 102.8 mg protein/gram wet weight scrotal testis and 108.3 mg protein/gram wet weight cryptorchid testis. The 15,000 x gravity supernatants contained 36.3 mg protein/gram wet weight scrotal testis and 53.5 mg protein/gram wet weight cryptorchid testis. He had previously determined that the water content was 87.1% and 83.7% for the scrotal and cryptorchid testes respectively. It is interesting to note that the "atrophic" cryptorchid testis, after 30 days of abdominal confinement and weighing about 25% of the scrotal control testis, contains the same relative amount of protein as its control, with a greater fraction of this protein being present in the soluble fraction of the cell.

Morris (52) has advocated the use of radioactively labeled lysine in the study of testicular protein metabolism because it appears to be a dietary requirement for maintaining testicular integrity, because only a small fraction of it is catabolized, and because these catabolites are not incorporated into protein.

Davis, Firlit, and Hollinger (14), studying the incorporation of L-lysine-U-C^{14} into slices of scrotal rat testes, found that maximal incorporation occurs at 32° C. and decreases by almost 50% at 37.5° C., the abdominal temperature of the rat, although glucose metabolism at the latter temperature was accelerated. In an attempt to clarify these acute studies (one hour incubation) Hollinger (31) studied L-lysine-U-C^{14} incorporation into scrotal and cryptorchid rat testis slices at various ages and after varying periods of abdominal confinement. He found that the scrotal testis incorporates about 3800 cpm/mg protein at age 15 days and that this decreases to less than 500 cpm/mg protein at age 60 days. On the other hand, the cryptorchid testis incorporates about 500 cpm/mg protein after 5 days of abdominal confinement and

this increases after 20 days of confinement to about 1800 cpm/mg protein. In direct contrast to the scrotal testis, he found that protein labeling in the testis cryptorchid for 20 days increases over the temperature range from 32°C. to 38°C.

Various enzymes have been studied in the normal and cryptorchid testis . Ford and Huggins (23) found malate and lactate dehydrogenases increased in the cryptorchid rat testis beginning on the fourth post-operative day. Kormano (41) found an increase in succinic dehydrogenase in cryptorchid testes and a decrease in beta-hydroxybutyrate and steroid dehydrogenases. Hayashi et al. (29) found increases in the level of beta-glucuronidase, esterase, and lipase activities in the cryptorchid testis. Steinberger and Nelson (69) found a decrease to zero in hyaluronidase activity in the cryptorchid testis.

3.) Lipid metabolism

Lacy (44) pointed out that as spermatogenesis progresses in the rat, there is a regular and gradual increase in the intratubular lipids and a shift of lipid toward the center of the tubular lumen. George and Ambadkar (25) point out that, in the rat testis, senescence and cryptorchidism are associated with lipid accumulation in the interstitium. They confirmed Lacy's observations and demonstrated four phases of lipase migration from the periphery of the seminiferous tubule to a central band around the heads of the spermatozoa. They also found a correlative migration of neutral lipids toward the central lumen and identified the interstitial lipids as being mainly phospholipids. They concluded that lipids have a definitive role in spermatogenesis and may also serve as a major energy fuel for spermatozoa movement.

Terner and Korsh (74), working with washed bull spermatozoa, demonstrated active labeling of sperm lipids, especially the diglyceride fraction, when incubated with either glycerol-1-C^{14} or randomly labeled glucose-C^{14}. Fatty acid labeling in the presence of acetate-1-C^{14} was markedly less than the diglyceride labeling by labeled glucose or glycerol. They concluded that in bull spermatozoa diglycer-

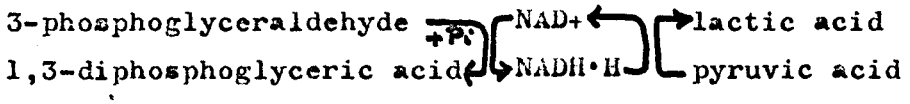
ides may be a ready source of fatty acids to be utilized in support of endogenous respiration. Mann (47) has stated that phospholipids, especially plasmalogen, are the prominent sources of aerobic metabolism in mammalian sperm.

C. DEHYDROGENASES

1. Lactate dehydrogenase.

a. General characteristics

Lactate dehydrogenase (LDH) is found in at least five molecular sub-types or isoenzymes which, in a given species, appear to have identical molecular weights (about 135,000) as measured by light scattering and ultracentrifugation (80). LDH reversibly catalyzes the reduction of pyruvic acid by reduced nicotine adenine dinucleotide (NADH·H) producing lactate in the coupled reactions:



The reaction favors lactate production as can be seen by comparing the Michaelis-Menten constants (Km) reported by Wilkinson (80):

Human heart LDH (primarily LDH_1), substrate pyruvate 1.18×10^{-4} M

Human heart LDH (primarily LDH_1), substrate lactate 0.44×10^{-2} M

Human liver LDH (primarily LDH_5), substrate lactate 2.56×10^{-2} M

Also noted by Wilkinson is the greater activity of the electrophoretically fast LDH isoenzymes (LDH_1 and LDH_2) than the slow (LDH_4 and LDH_5)

An interesting sidelight to the activity of LDH is the observation that the enzyme offers no binding site for pyruvate until NADH·H is fixed in place. This indicates that LDH undergoes a conformational change while functioning analogous to similar conformational changes seen in hemoglobin and cytochrome c (76).

b. Localization in the testis

Ambadkar and George (1), testing their hypothesis that lipids are an important source of energy in spermatogenesis, sought to localize oxidative enzymes in the rat testis. They found that LDH was present in both the seminiferous tubules and the interstitium and that the pattern of its distribution was parallel to the distribution of

lipids and lipase which were previously determined to go through four phases of migration in relation to spermatogenic activity (25).

c. LDH isoenzymes

Physical heterogeneity of LDH was established by Meister in 1950 when he separated crystalline beef heart LDH into 2 electrophoretic zones (49). He attributed all enzymatic activity to the larger faster component. Neilands in 1952 found enzymatic activity in the smaller, slower component (54). Wieland and Pfleiderer in 1957 separated up to 5 bands of enzymatic activity from various LDH preparations by paper and starch block electrophoresis (78). Hunter and Markert, also in 1957, first applied histochemical techniques to starch gel electrophoresis and produce the first zymograms of LDH.(35).

There has been some confusion in the numbering of the five commonly found LDH isoenzymes. Wieland and Pfleiderer numbered the most mobile (anodic) isoenzyme LDH-1 and the least mobile LDH-5 (78). Most American workers, however, employ a numbering system directly opposite, i.e., the most mobile (anodic) isoenzyme is called LDH-5, the least mobile is numbered LDH-1 (68). Although there has been some obvious confusion, the most recent ruling of the Standing Committee on Enzymes of the International Union of Biochemistry was in favor of the prior European numbering system (80). This system will be used throughout this thesis with prior interpretation by the author when necessary to equate American and European work.

In addition to differences in electrophoretic mobility and Michaelis-Menten constants, the isoenzymes of LDH differ in regard to their optimum temperatures. The optimum temperatures for the LDH isoenzymes as determined by Kreutzer are: LDH-1 62.5°C.; LDH-2 60°C.; LDH-3 52.5°C.; LDH-5 42°C.;(42). Clausen and Øvlisen (7) determined the optimum pH's for pyruvate reduction to be pH 8.00 for LDH-1 and pH 7.25 for LDH5.

The amino acid content of the isoenzymes of LDH has been worked out and, not surprisingly, indicates that the fast isoenzymes, e.g. LDH-1 and LDH-2 have more of the acidic amino acid aspartic acid and

less of the more basic acids histidine and arginine than do the slower LDH-4 and LDH-5 isoenzymes (79).

The structure of the five common LDH isoenzymes has been investigated by Markert (48) who proposed that each isoenzyme is a tetramer composed of different ratios of two distinct monomers, A and B. LDH-1, therefore, is composed of A^0B^4; LDH-2 is A^1B^3; LDH-3 is A^2B^2; LDH-4 is A^3B^1; and LDH-5 is A^4B^0. In a critical test of this hypothesis he separated pure LDH-1 and pure LDH-5 from beef tissues. These two isoenzymes were mixed in equal proportions and subjected to dissociation in 1 M sodium chloride. This mixture, when subsequently electrophoresed, produced five bands of LDH activity in the relative proportions 1:4:6:, 4:1 for LDH-1 through LDH-5 respectively. These are the predicted results if random association of monomers into tetramers occurs.

The synthesis of each monomer, A and B, is apparently controlled by two distinct genes, A and B (48). The repression and/or induction of these two genetic sites varies from tissue to tissue. For example, adult heart muscle of man contains more B than A, while in adult skeletal muscle the reverse is true. Another variation in the expression of these genes is seen in the ontogeny of specific tissues, e.g., the fetal rat heart and brain exhibit much more A activity than B while in the adult tissues the opposite is true (45).

d. LDH-X

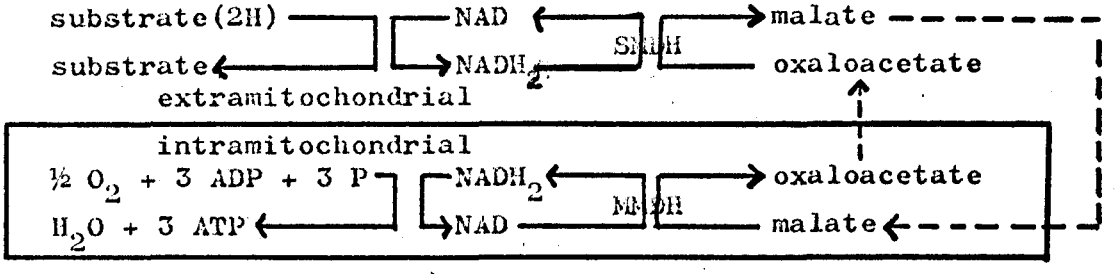
Zinkham, et al., using starch gel electrophoresis, have detected a number of bands of LDH activity which are apparently found only in the mature testes and semen of some species. The testes of man, mouse, rabbit, and dog contained one additional band of LDH activity, labeled LDH-X. The testes of the guinea pig, rat, and bull showed more than one band of LDH-X. In the rat testis two bands of LDH-X were present, one between LDH-3 and LDH-4, the other between LDH-4 and LDH-5. LDH-X was found to be the predominant isoenzyme found in washed spermatozoa of rabbits and men. Zinkham and his co-workers found that LDH-X was not present in pre-pubertal testes nor in testes immunologically rendered aspermatogenic (82).

Following Markert's lead they subjected human testicular isoenzymes to gentle dissociation and recombination and demonstrated a subsequent decrease in activity of LDH-1,2,4,5, and X with the appearance of three new bands between LDH-2 and LDH-3, LDH-3 and LDH-X, and LDH-4 and LDH-5. They proposed that, in animals with a single "band X", the subunits are identical and genetically independent of Markert's A and B genes. They designate the single LDH-X as C^4 (82).

2. Malate dehydrogenase.

Malate dehydrogenase (MDH), I.U.B. Number 1.1.1.37, systematic name L-malate:NAD oxidoreductase, catalyzes the reversible oxidation of malic acid to oxaloacetic acid. Although LDH is a cytoplasmic enzyme (60), MDH is found in two distinct types and locales, the soluble or cell sap enzyme (SMDH) and the mitochondrial enzyme (MMDH) (80). Grimm and Doherty (27) have determined the Km and the maximal turnover number for the soluble and mitochondrial types: Km (malate) MMDH = 9,9 x 10^{-4}M and SMDH 5.4 x 10^{-4}M; Km (oxaloacetate) MMDH = 4.0 x 10^{-5} and SMDH = 5.1 x 10^{-5}M. The maximal turnover number (malate and NAD) expressed as moles of NAD reduced per minute per mole of enzyme is 35,000 for MMDH and 20,000 for SMDH. The maximal turnover number (oxaloacetate and $NADH_2$) expressed as moles $NADH_2$ oxidized per minute per mole of enzyme is 59,000 for MMDH and 72,000 for SMDH.

Kaplan (38) has proposed the following scheme to account for these differences in catalytic efficiency:



The net reaction is:

Substrate(2H) + ½ O_2 + 3 ADP + 3 P ⟶ Substrate + 3 ATP + H_2O

The isoenzyme complement of MDH is relatively unclear but various investigators have reported from two to six electrophoretically distinct isoenzymes (80).

Goldberg (26) used disc electrophoresis to separate MDH isoenzymes from the 10,000 x gravity supernatant of human sperm sonicates and detected two bands of activity which he considered to be the SMDH and the MMDH.

Ambadkar and George (1), in attempting to histochemically localize the oxidative enzymes of the rat testis, noted that sites of MDH activity generally paralleled those of LDH, succinic dehydrogenase and beta-hydroxybutyrate dehydrogenase activities with the exception that MDH is relatively more active in the testicular interstitium than in the luminal portion of the seminiferous tubule.

The preceding presentation regarding the mechanisms of disc electrophoresis, some changes seen in the cryptorchid testis, and the heterogeneity of LDH and MDH is essential to the understanding of the attempts at establishing electrophoretic profiles of the soluble fraction of the normal and cryptorchid rat testis.

CHAPTER II

MATERIALS AND METHODS

A. ANIMALS

1. Immature cryptorchid rats.

Twenty day old Sprague-Dawley male rats, litter mates, were fed Rockland Mouse/Rat diet pellets ad libitum. At age 25 days they were surgically made unilaterally cryptorchid. The surgical technique consisted of opening the abdominal wall and peritoneum with a one inch midline incision under ether anesthesia. The right testis was manually expressed through the incision and a single 6-0 silk suture was placed beneath the capsule of the testis just lateral to the spiral artery and anchored on a short tether to the abdominal musculature just to the right of the midline. Operative mortality approached 20% and with the exception of a single inadvertent bowel perforation was due to overdose of ether. These litter mates were sacrificed from 21 to 58 days postoperatively and at weights ranging from 195 g to 336 g.

2. Mature cryptorchid rats.

Sixty day old Sprague-Dawley male rats, not litter mates, were fed Rockland Mouse/Rat diet pellets ad lib. At age 66 days they were made unilaterally cryptorchid as outlined above. They were sacrificed from 31 to 43 days postoperatively at weights ranging from 246 g to 314 g.

All rats were obtained from the Abrams Small Stock Breeders of Chicago, Illinois and were housed at 72° F. All animals resumed their pre-operative ad lib. diet within a matter of hours after surgery.

B. SAMPLE PREPARATION

All rats were sacrificed by decapitation and taken into a 4°C cold room. The right cryptorchid and left scrotal testes were removed. The testicular capsule and visible arteries were dissected free and approximately 250 mg of tissue was weighed from each testis on a Roller-Smith balance.

Each 250 mg was homogenized in 0.75 ml of 0.25 M sucrose, pH 7.3 at 4°C., in a small Potter-Elvehjem tissue grinder. The homogenates were centrifuged at 15,000 x gravity for twenty minutes at 4°C. in an International Centrifuge, Model HR-1. The central, relatively lipid-free zones of each supernatant were aspirated via a 17 gauge needle and a syringe into chilled 10 mm x 75 mm test tubes.

Hollinger (31) found that the protein content of the 15,000 x gravity supernatant of the scrotal testis homogenized in pH 7.5 Tris buffer was 36.3 mg protein / gram wet weight tissue while the similarly prepared supernatant of the cryptorchid testis contained 53.5 mg protein / gram wet weight of tissue. The percentage of water in each tissue was found to be 87.1% and 83.7% respectively. To more closely equilibrate the protein contents of the scrotal and cryptorchid samples, therefore, the scrotal supernatant was diluted in four volumes of 40% sucrose while the cryptorchid supernatant was diluted in six and one-half volumes of 40% sucrose. These two protein-sucrose solutions were used as samples for disc electrophoresis.

C. GEL PREPARATION

Gels were prepared for electrophoresis generally following the methods of Davis (13) and Clarke (6).

1. Reagents.

2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) as Trizma Base obtained from Sigma Chemical Co. St. Louis, Missouri

N, N, N', N'-tetramethylethylenediamine (TEMED) as Eastman 8178

Acrylamide as Eastman 5521

N, N'-methylenebisacrylamide (BIS) as Eastman 8383

Riboflavin as Eastman 5181

Hydrochloric Acid (HCl) 1N reagent grade

Ammonium persulfate

Sucrose reagent grade

2. Stock solutions

The following were prepared, filtered, and stored in brown bottles at 4° C.

(A.)	1N HCl	48.00 ml	
	Tris	36.60 g	
	TEMED	0.23 ml	
	H_2O	q.s. 100.00 ml	(pH 8.9)
(B.)	1N HCl approx.	48.00 ml	(titrate to pH 6.7)
	Tris	5.98 g	
	TEMED	0.46 ml	
	H_2O	q.s. 100.00 ml	
(C.)	Acrylamide	28.00 g	
	BIS	0.735 g	
	H_2O	q.s. 100.00 ml	
(D.)	Acrylamide	10.00 g	
	BIS	2.50 g	
	H_2O	q.s. 100.00 ml	
(E.)	Riboflavin	4.00 mg	
	H_2O	q.s. 100.00 ml	
(F.)	Sucrose	40.00 g	
	H_2O	q.s. 100.00 ml	

3. Working solutions.

The following were prepared from stock solutions and used immediately:

Small pore solution 1:	2.0 ml A	
	4.0 ml C	
	2.0 ml H_2O	(pH 8.8-9.0)
Small pore solution 2:	8.0 ml ammonium persulfate 140 mg%	
Large pore solution:	1.0 ml B	
	2.0 ml D	
	1.0 ml E	
	4.0 ml F	(pH 6.6-6.8)

4. Column preparation.

Twelve 100 mm lengths of 7 mm outside diameter Flint glass tubing are cut from the same piece and marked at 10 mm, 20 mm, and 95 mm from one end. The tubes are boiled in Alconox solution, rinsed in an acid

bath, rinsed thrice in distilled water, and finally rinsed in a 1:250 dilution of Kodak Photo-Flo Solution. The tubes are then oven-dried.

5. Polymerizations.

The twelve lengths of tubing are placed, 10 and 20 mm marked end uppermost, in a suitable stand, e. g., 12 rubber grommets cemented to a Plexiglass base. Small pore solutions 1 and 2 are mixed in equal proportions in a small beaker and used to fill the tubes, via a syringe and small diameter plastic tubing, to the mark 20 mm from the top. Atop the small pore sol distilled water is carefully layered to a depth of about 5 mm using a syringe and 26 gauge needle.

After allowing one hour for the small pore gel to polymerize, the water layer is poured off and the gel surface is washed with a few drops of the large pore solution. This wash is drained off and the tube is filled to a depth of 10 mm with the large pore solution and water-layered as above. A fluorescent lamp is placed just over the uncovered tube tops and photopolymerization is allowed to proceed for one-half hour.

D. ELECTROPHORESIS

1. Reagents.

Stock buffer solution for reservoirs (diluted with water 9:1 prior to use):

	Tris	6.0 g
	Glycine (ammonia free)	28.8 g
	H_2O	q.s. 1.0 l
Marking dye:	Bromphenol blue	0.001%

2. Procedure.

The gel columns are carefully removed from the stand and the top water layer is drained off. They are then inserted, from the bottom, into the electrophoresis apparatus shown in Figure I to a depth of about one centimeter. The top and bottom reservoirs are filled with a 9:1 dilution of stock buffer solution at 4° C. Care is taken to exclude air bubbles from the top and bottom of the gel columns. The filled apparatus is taken into the cold room and one ml of the marking dye is added to the upper buffer reservoir. The top electrode (cathode) and the

bottom electrode (anode) are connected to the respective terminals of a Constant Current DC Power Supply made by Buchler Instruments, Fort Lee, New Jersey.

With 50 volts applied to the electrophoresis apparatus 0.1 ml of sample is applied to each gel column, using a tuberculin syringe and a 21 gauge needle. The power supply is adjusted to provide a constant current and electrophoresis is carried out a 3 ma/tube in the 4° C. cold room. When the marker dye in a given tube reaches the mark 95 mm from the top of the tube, that tube is pushed from below high enough to break the surface of the upper buffer reservoir, thereby stopping electrophoresis in that tube. This is done to standardize the migration in all tubes. Total running time is about one and one-half hours.

When electrophoresis is completed all the columns are removed from the apparatus. The gels are removed from the tubes by introducing a two inch 27 gauge needle on a syringe filled with the cold electrophoresis buffer between the gel and the tube. The needle rims the gel, the while expressing cold buffer. Removed gels are placed in 10 mm x 75 mm test tubes at room temperature and are ready for staining.

E. STAINING

1. Lactic acid dehydrogenase.

Reagents: Tris, hydrochloric acid, DL lactic acid (sodium salt) 60% syrup; Nicotine adenine dinucleotide (NAD); Phenazine methosulfate (PMS); and Nitro blue tetrazolium (NBT); all obtained from the Sigma Chemical Company, St. Louis, Missouri.

Procedure: The method used was a modification of that used by Dewey and Conklin (17). For 33 ml of a substrate solution add 4.5 ml of 0.67 M DL lactate (3/4 ml stock 60% sodium DL lactate syrup plus 5 ¼ ml water) to 28.5 ml of 0.2 M Tris-HCl buffer, pH 8.0. Add to this 10 mg NAD, 3 mg NBT, and 4 mg PMS. This is stored in the dark until ready for use the same day. The stain-substrate solution is poured into the test tubes containing the gels. The tubes are then corked and incubated for thirty minutes at room temperature in the dark. When incubation is completed the reaction is stopped by emptying the stain-substrate

solution from the tubes and filling them with 7% acetic acid. One hour later the gels are rinsed in tap water and then stored in the same tube in fresh 7% acetic acid.

2. Malic acid dehydrogenase.

Stained using the procedure outlined above with the following exception: use 0.7 ml of 50% sodium malate (4.7 M) instead of the 4.5 ml of 0.67 M sodium lactate.

4. General protein.

Reagents: Buffalo Black NBR (Amido Black); 7% acetic acid and 3% acetic acid.

Procedure: Gels are stained for one-half hour in 0.1% Amido Black in 7% acetic acid, then leached overnight in 7% acetic acid. The gels are then inserted, sample side uppermost, in the glass tubing of medicine droppers. Care is taken to exclude air bubbles from top, bottom, and sides of each column. A wet cotton pledget is inserted above each gel. The tubes are placed in the electrophoresis apparatus and the chambers are filled with 3% acetic acid. The anode and cathode are reversed, relative to their electrophoresis position, for de-staining. 10 ma/tube are applied to the circuit and de-staining is completed in about one hour. Following removal from the dropper tubes the gels are stored in 7% acetic acid in 10 x 75 mm test tubes.

F. DENSITOMETRY

Densitometry of the stained gels was carried out on a Photovolt Densicord Densitometer employing a starch gel carriage and a 445 mu filter.

G. PHOTOGRAPHY

The tubes containing the gels were placed on ground glass supported seven inches above the frame level of a Polaroid 208 Copymaker and photographed by transmitted fluorescent light. Type 42 Polaroid film was exposed for 1/15 second with camera settings of f 22 and three feet, employing Polaroid close-up lenses +2 and +4. Copymaker height was set at 7 and 5/8 inches.

CHAPTER III

RESULTS

A. LACTIC ACID DEHYDROGENASE ISOENZYME PATTERNS OF THE TESTES OF NORMAL AND ARTIFICIALLY CRYPTORCHID RATS.

1. Normal rats.

Figure II displays the photographic and densitometric records of the lactic acid dehydrogenase isoenzymes, as separated by disc electrophoresis from testis of a normal pre-pubertal Sprague-Dawley rat (left) and from a testis of a normal mature Sprague-Dawley rat (right). The pre-pubertal testis contains isoenzymes LDH-1 through LDH-4 with the latter band exhibiting an indistinct broadening. The mature rat testis contains at least eight bands of LDH activity corresponding to the labels LDH-1 through LDH-5 and LDH-X_1, LDH-X_2, and LDH-X_3. The greatest activity as measured by this histochemical system, can be seen to rest in LDH-1 and LDH-2 in both of the tissues here represented. This figure essentially confirms, in disc electrophoresis, the findings of Zinkham et al. (82) in starch gel electrophoresis which indicated that the testis engaged in active spermatogenesis produced electrophoretically unusual isoenzymes apparently distinct from the more usual LDH-1 through LDH-5 types.

2. Pre-pubertal cryptorchid rats.

The LDH isoenzymes of the scrotal testis and the abdominal testis from a rat made unilaterally cryptorchid prior to the normal testicular descent and the onset of sexual maturity are seen in Figure III. The scrotal testis contains the isoenzyme profile characteristic of the spermatogenic testis, i. e., the usual five LDH bands and three LDH-X bands. The abdominal testis in this type animal contains an LDH isoenzyme profile similar to, yet distinct from, the normal pre-pubertal testis. The similarity is apparent in the presence of the same number of bands, four, as the pre-pubertal tissue. The distinction is the visual and densitometric sharpness of LDH-4 relative to the consistently

broader and indistinct LDH-4 of the pre-pubertal testis as seen in Figure II. In Figure III, as in Figure II, the highest activity is apparent in isoenzymes LDH-1 and LDH-2 in both abdominal and scrotal testes. One plausible hypothesis from a comparison of Figures II and III is that the maintenance of abdominal position of the pre-pubertal testis in the mature rat partially simulates the pre-pubertal condition yet either directly deletes variant molecular forms responsible for the broadening of pre-pubertal LDH-4 or enhances the molecular uniformity of this isoenzyme.

3. Mature cryptorchid rats.

The effect of returning a mature spermatogenetic testis to the abdomen and maintaining its position there until typical cryptorchid changes are induced is shown in Figure IV. The abdominal testis here displays the effect of regression to a more pre-pubertal state with respect to the LDH isoenzyme profile. The scrotal testis contains the usual five LDH isoenzymes together with the three LDH-X types. The abdominal testis contains the LDH isoenzyme pattern similar to that seen in Figure III with a sharp and distinct LDH-4 and without the LDH-5 and LDH-X complex seen in the spermatogenic testis.

4. Planimetry of lactic acid dehydrogenase profiles.

Figure V indicates the typical percentage of total LDH activity possessed by each isoenzyme peak and measured by planimetry. The figures for LDH-4 in the pre-pubertal testis, 29.62%, the abdominally retained testis, 15.23% and the abdominally re-positioned testis, 16.40% seems to indicate that the cryptorchid condition produces, with respect to LDH-4, an effect greater than mere regression to the pre-pubertal state.

B. MALIC ACID DEHYDROGENASE PATTERNS OF THE TESTES OF UNILATERALLY CRYPTORCHID RATS

Having noted differences in LDH isoenzyme patterns between scrotal and abdominal testes in artificially cryptorchid rats, a similar study of possible variation in malic acid dehydrogenase in the soluble fraction was carried out. Figure VI indicates that the SMDH pattern of ab-

dominally retained and abdominally re-positioned testes did not indicate the presence of isoenzymes in this tissue and that both were grossly similar to their scrotal controls.

C. GENERAL PROTEIN PATTERNS OF THE SOLUBLE FRACTION OF THE TESTES OF NORMAL AND ARTIFICIALLY CRYPTORCHID RATS

1. Normal rats

A comparison of the Amido-Black-stained soluble proteins, as separated by disc electrophoresis, from the pre-pubertal testis (left) and the mature testis (right) of Sprague-Dawley rats is seen in Figure VII. The patterns are visibly similar and bear close relation to the patterns obtained from human serum by Ornstein (58) and described in the terminology of Smithies and Poulik by Davis (13). According to the densitometric record the prepubertal testis contains at least 17 bands and the mature testis 18. It is here noted that all numbering of this and subsequent figures begins with the mobile dense band electrophoretically resembling albumin and therefore deletes from the record the constant three faint "prealbumin fractions". Visual and densitometric similarities are noted between bands I, IX, and X, left and right. Relating these patterns to the description of Davis of the electropherogram of human serum proteins the peak labeled X resembles Transferrin C and the labeled bands between I and X may, by like analogy, be called "post albumins". Due to the gross nature of the present method only the following general similarities can be pointed out with regard to Figure VII:

a. In both pre-pubertal and mature testes there are a number of dark-staining bands in the mid-"post albumin" region

b. In both testes there are a number of dark bands just proximal to band X (Transferrin C)

c. In both testes there are a number of dark bands just distal to the origin ("haptoglobulins").

2. Pre-pubertal cryptorchid rats.

Figure VIII compares the soluble proteins of the abdominally retained pre-pubertal testis with those of the scrotal control testis of the same animal. Eighteen densitometric and photographic bands are la-

beled in each electropherogram and the three regions of general similarity seen in Figure VII are also here visible. In addition, scrotal bands labeled I, VI, IX, X, XIII, and XVII correspond to abdominal bands labeled I, V, IX, X, XII, and XVI, respectively. In this figure "Transferrin C" corresponds to band IX in both patterns. Turning from points of similarity, a subtle but constant difference is evident between the two patterns. The band labeled VI in the cryptorchid abdominal testis seen in the proximal third of the "post albumins" does not appear in the scrotal pattern at the left. The position of this unusual abdominal band VI corresponds to the space between scrotal bands VII and VIII. The ratio of migration of this cryptorchid protein to the migration of "albumin" in this experiment is 0.59.

3. Mature cryptorchid rats.

The soluble proteins of the abdominal and scrotal testes of a rat made unilaterally cryptorchid after normal testicular descent and the onset of sexual maturity are compared in Figure IX. From each tissue fourteen bands have been separated and labeled. With the exception of bands II and III, all labeled bands in one electropherogram correspond to their numerical counterparts. The unusual cryptorchid protein seen in Figure VIII is here seen as band VI in the abdominal testis which corresponds to the very faint band VI in the pattern of the scrotal testis at the left. The ratio of the migration of this protein to "albumin" in this experiment is 0.58.

CHAPTER IV

DISCUSSION

In view of the histological, histochemical, and metabolic changes consistently seen in the cryptorchid testis, specifically Hollinger's (31) findings that the cryptorchid testis contained a greater concentration of protein in its soluble fraction than did the scrotal testis; that the cryptorchid testis was relatively more active in labeling protein with L-lysine-U-C^{14}; and that this labeling was distinct from that found in the scrotal testis in respect to its response to temperature changes in the range 32° C. to 38° C., it became of interest to investigate the soluble fraction of the scrotal and cryptorchid testis as such. Indeed, these findings and others indicated that the cryptorchid testis was an "atrophic" tissue by criteria of size and weight, which gave evidence of an increased protein metabolism and, in the human, a marked tendency toward malignant change.

One goal of this study as originally conceived was the definition of the soluble general protein profile and certain enzyme profiles of the normal rat testis in the hope of establishing markers indicative of biochemical and genetic changes induced not only by the cryptorchid condition but also by possibly deleterious drugs.

Accordingly, work was begun toward the definition of the general Amido Black-staining protein profile of the soluble fraction (15,000 x gravity supernatant of a 2:1 normal saline:testis homogenate) of the scrotal and cryptorchid testes of the rat. This work was carried out by electrophoresis of the soluble fraction in potato starch gel according to the method of Smithies (67). These experiments led to the separation of 9 bands from the scrotal testis and 7-10 bands from the cryptorchid testis. Inconsistency of results, the relatively heavy background staining of the starch gel, and the friable nature of the gel led to a search for a new electrophoretic medium.

The medium finally used was polyacrylamide gel as part of the

disc electrophoresis method of Ornstein and Davis (12) as modified by Dietz and Lubrano (18). Disc electrophoresis permitted the use of a more dilute sample and, thereby, allowed the use of a 3:1 homogenate 15,000 x gravity supernatant without the need of dialytic concentration.

An investigation was carried out at this time into the effects produced by various homogenization media, including 0.154 M sodium chloride; 0.25 M sucrose; 0.154 M sodium chloride in 0.25 M sucrose; and distilled water. The clearest and most reproducible results were obtained with 0.25 M sucrose which was, therefore, used throughout. Inasmuch as a good deal of protein-staining material remained at the spacer gel-separation gel interface after electrophoresis it was thought that either these proteins were too large to enter the intermolecular pores of the gel or that particulate matter of microsomal size was obstructing these pores. Accordingly, the 15,000 x gravity supernatant was ultracentrifuged at 105,000 x gravity at 4° C. for one hour. No substantial difference in pattern or residue resulted.

The interpretation of the general protein profiles of the pre-pubertal scrotal and cryptorchid testes and the mature scrotal and cryptorchid testes are here described in terms used by Davis (13) in his description of serum electropherograms. In the system reported here at least 14 to 18 bands of protein were separated and stained. One of these bands consistently appeared in the proximal "post albumins" only in the cryptorchid testis and in both pre-pubertal and mature cryptorchid testes. The ratio of its migration to that of the albumin-like band was 0.58.

Possible explanations of its presence include fragmentation of or coalescence of other proteins, modification of electrophoretic mobility of another protein by a non-protein moiety and the induction of or increased synthesis of a soluble protein not normally present in such quantity in the scrotal testis. All these possibilities are untested and await further investigation of this unusual cryptorchid protein. It is interesting to note, however, that this protein is seen in both

pre- and post-pubertal cryptorchid testes, each of which show spermatogenesis arrested at the pachyneme stage of meiosis with a cell population of only four types: "crust" spermatogonia 31%, pachyneme primary spermatocytes 3%, normal-appearing Sertoli cells 28%, and atrophic-appearing Sertoli cells 38% (16,22). Clegg (10) proposed that this spermatogenic arrest was due to a temperature-invoked blockade of gonadotrophin action. A similar histological picture is seen, however, in testes rendered aspermatogenic by immunological (82) and chemical, i.e., nitrofurazone, means (33). Davis and Firlit (16) postulate that the spermatogenic arrest is due to the loss of a spermatid-produced regulator. Speculation, in the plain absence of confirmatory evidence, entices one to consider the induction or de-repression of a proteinaceous inhibitor of meiosis by the inactivation of its repressor which is vulnerable to multi-phase injury.

The finding, by Zinkham et al. (82), of several bands of LDH-X activity in starch gel electrophoresis of the mature rat testis indicated that there was yet another variable, perhaps with direct genetic relationship, in the soluble fraction of the scrotal and cryptorchid testes. Goldberg (26), using disc electrophoresis of washed sperm sonicates from many species also detected LDH-X activity. Both Zinkham and Goldberg relate the appearance of LDH-X activity to the presence of spermatids and other mature forms in the spermatogenic cycle. Zinkham et al., however, have detected LDH-X in the pubescent male rabbit prior to the development of secondary spermatocytes (82). They maintain that this early appearance of an isoenzyme thought to be unique to the mature sperm is a necessary concomitant of the relative inability of the mature sperm to synthesize protein and is analogous to the early appearance of the enzymes of the erythrocyte. They deleted LDH-X from the LDH isoenzyme complement of the mature guinea pig testis by immunologically rendering it aspermic. The effect of temperature as such on early spermatogonia, Sertoli cells, Leydig cells, and fibroblasts was, therefore, not present in their experiments.

Although Markert's A and B tetramer theory of LDH structure appears consistent with most of the present knowledge (48), it is not a complete explanation of LDH isoenzyme composition. Shaw (66) has reported 15 LDH bands in many tissues of the deer mouse, Peromyscus, apparently due to genetic variation in the B subunit. Kreutzer et al. (43) have reported on five cases of altered serum LDH isoenzyme mobility seemingly not of genetic origin. They were able to restore regular mobility by the addition of NAD to the serum and propose that alteration is caused by a thermostable serum factor which reversibly competes with NAD for the latter's binding site and, when bound, influences LDH isoenzyme mobility, specifically accelerating LDH-5 and retarding LDH-4.

In the experiments here reported, LDH-1, LDH-2, and LDH-3 show little variation in the pre-pubertal, mature, and cryptorchid rat testes. These isoenzymes are predominantly composed of the B monomer (48), have higher optima for pH (7), and temperature (42), and lower Michaelis-Menten constants than the predominantly A isoenzymes (80).

In the pre-pubertal rat there are four LDH bands present, LDH-1 through LDH-4, the latter showing considerable broadening or smearing. The cryptorchid testis in all cases showed only LDH-1 through LDH-4 without smearing of the latter band. If the Markert model for LDH-4 is accepted as A^3B^1 it is difficult to accept the apparent absence of A^4B^0 or LDH-5. This same point is made in all tissues which show an LDH isoenzyme pattern of more than one and less than five isoenzymes.

The mature rat testis displays more or less clearly LDH-1 through LDH-5 and three bands of LDH-X activity clustered about LDH-4 and LDH-5. The slow isoenzymes, LDH-4 and LDH-5, predominantly composed of the A monomer (48), with lower optima of pH (7) and temperature (42) and higher Michaelis-Menten constants than the B isoenzymes (80) are related by Latner and Skillen (45) to increased facility for anaerobic glycolysis.

LDH-X, electrophoretically similar to the A isoenzymes, has been related to the A^4 isoenzyme in terms of pH optima and lactate concen-

tration required to saturate the enzyme (7). LDH-X shows similar substrate specificities to other LDH isoenzymes with the apparently exceptional ability to utilize the 3-acetylpyridine analogue of NAD (47).

In considering the question of why LDH-X is the predominant LDH in the mature sperm of some species, Zinkham et al. (82) point out that mammalian spermatozoa are able to carry out aerobic and anaerobic glycolysis and are also capable of carrying on oxidative respiration. They feel that LDH-X is but another manifestation of metabolic diversity which may enable spermatozoa to survive and function in the variety of micro-environments present in the female genital tract.

Although the number of LDH isoenzymes in the cryptorchid rat testis is reduced, LDH activity is markedly increased (23), probably due in part to the deletion of the less active LDH-5. Also increased in the cryptorchid testis are the labeling of protein with radioactive lysine and the soluble fraction of the total protein content. The findings of Davis et al. (15) that protein labeling was but slightly augmented in the cryptorchid rat testis by the administration of exogenous glucose in acute experiments would seem to dissociate LDH activity from the increased protein labeling seen in the cryptorchid testis.

Soluble fraction malate dehydrogenase was studied electrophoretically as a convenient and salient feature of the enzyme profile of the rat testis and as a possible marker of genetic change in the cryptorchid state. However, malate dehydrogenase in the soluble fraction of scrotal and cryptorchid testes did not show electrophoretic variation in this system.

In conclusion it appears that there is no difference in the general protein, LDH, or MDH profiles in the rat testis made cryptorchid prior to puberty relative to the rat testis made cryptorchid after puberty. Cryptorchidism in the rat does not appear to alter the soluble MDH profile as determined by disc electrophoresis. LDH-5 and three LDH-X isoenzymes are deleted from the LDH profile by the cryptorchid state. A cryptorchid protein, not apparent in the soluble fraction of

the scrotal testis, appears in the "post albumin" zone of the cryptorchid testis electropherogram.

Much further work can be done using the basic method employed in these experiments. The mitochondrial MDH could be tested for electrophoretic variation in the cryptorchid testis by comparing the MDH from disrupted mitochondria to that of the 15,000 x gravity supernatant. The unusual cryptorchid protein could be eluted and biochemically characterized, including assays for enzymatic activity.

Inasmuch as Firlit and Davis (22) have localized the increased labeling of protein by radioactive lysine to the Sertoli cells of the cryptorchid testis, it would be of interest to homogenize acutely labeled cryptorchid testis slices and use the 15,000 x gravity supernatant as a disc electrophoretic sample. After electrophoresis the gels could be sliced and the activity of the slices counted to test the hypothesis that the increase in labeling is due to the increase in the unusual cryptorchid. The appearance of the cryptorchid protein could be more accurately dated simply by sequential sampling of cryptorchid testis at various intervals after abdominal fixation.

Reference has been made to changes resembling those seen in experimental cryptorchidism produced by immunological sensitization (82), by 7,12-dimethylbenzanthracene (23), and by nitrofurazone (33). It may well prove practical to use the method outlined in this thesis to detect drug-induced changes in the mammalian testis heretofore unnoticed. Of particular interest would be the correlation, if present, between electrophoretic variations of testicular or seminal samples and spontaneous abortions or congenital defects.

CHAPTER V

SUMMARY

1. The technique of disc electrophoresis was applied to the separation of the soluble proteins of the rat testis and a protein not seen in the scrotal testis was noted in the cryptorchid testis.

2. LDH zymograms revealed three bands of LDH-X activity in the mature rat testis together with the five other LDH isoenzymes seen in other tissues.

3. The three bands of LDH-X activity and LDH-5 were deleted after twenty days of abdominal cryptorchidism, producing a pattern similar to than seen in the pre-pubertal testis.

4. Soluble fraction MDH showed no electrophoretic variation in the scrotal and cryptorchid rat testis.

CHAPTER VI

FIGURES

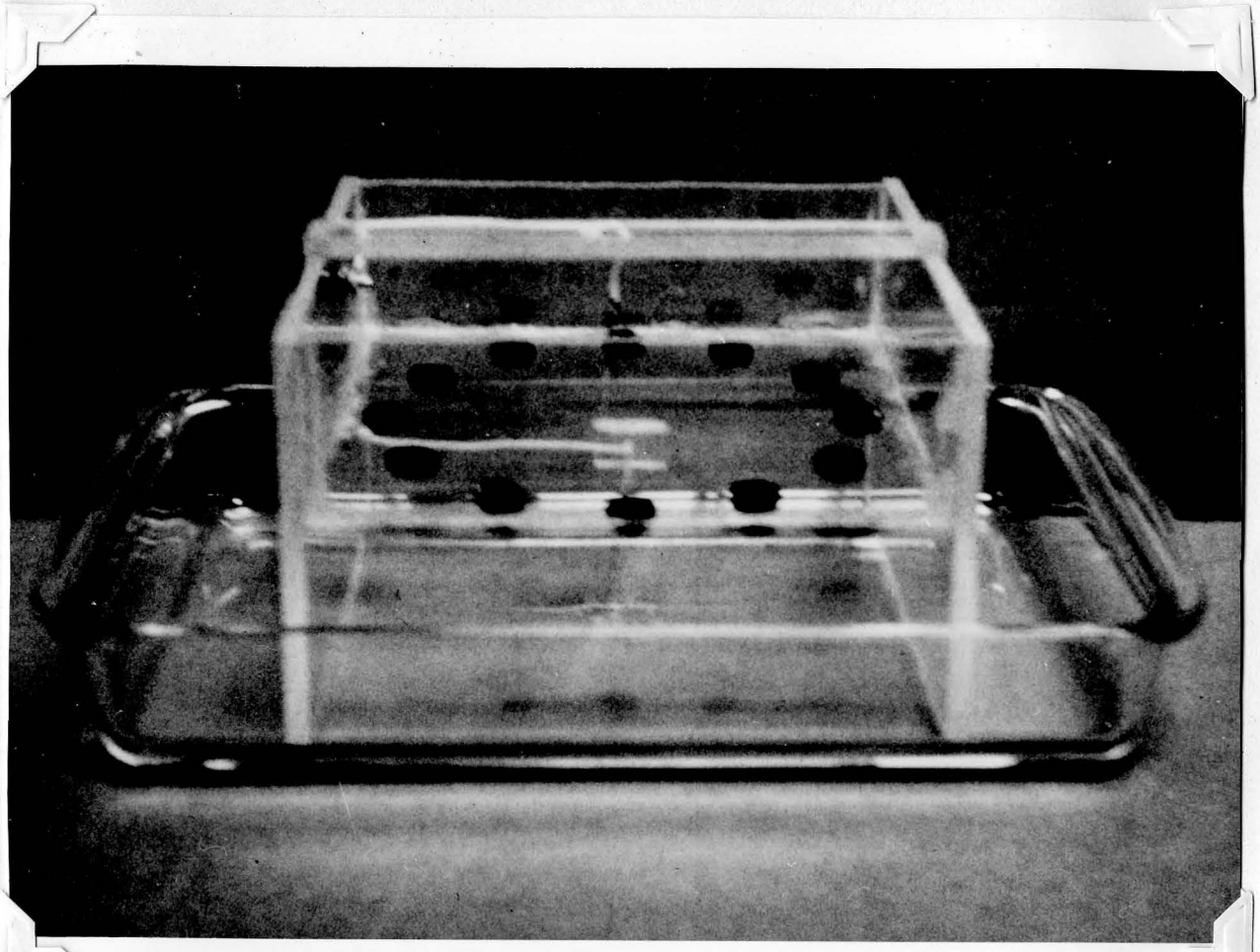


FIGURE I

The disc electrophoresis apparatus employed in these experiments is shown above. Its dimensions are 7" x 7 3/8" x 4 5/8". It is made of 1/4" and 1/8" Plexiglass. Electrodes are straight platinum wire. The grommets which hold the gel columns are 3/8" inside diameter.

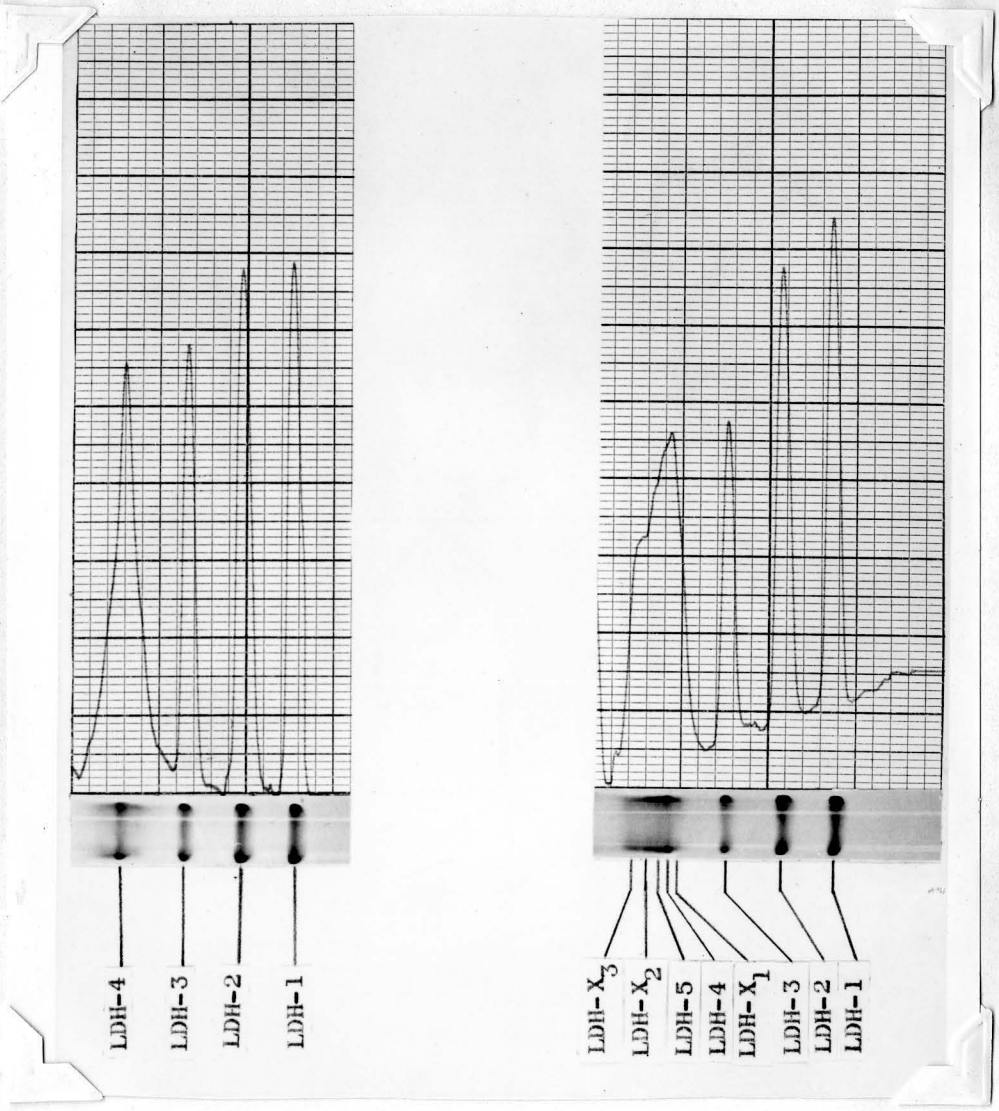


FIGURE II

Lactic acid dehydrogenase isoenzyme patterns of the 15,000 x gravity supernatants of the testes of two Sprague-Dawley rats. The pattern at left is from a rat sacrificed at age 25 days. The pattern at right is from a rat sacrificed at age 67 days.

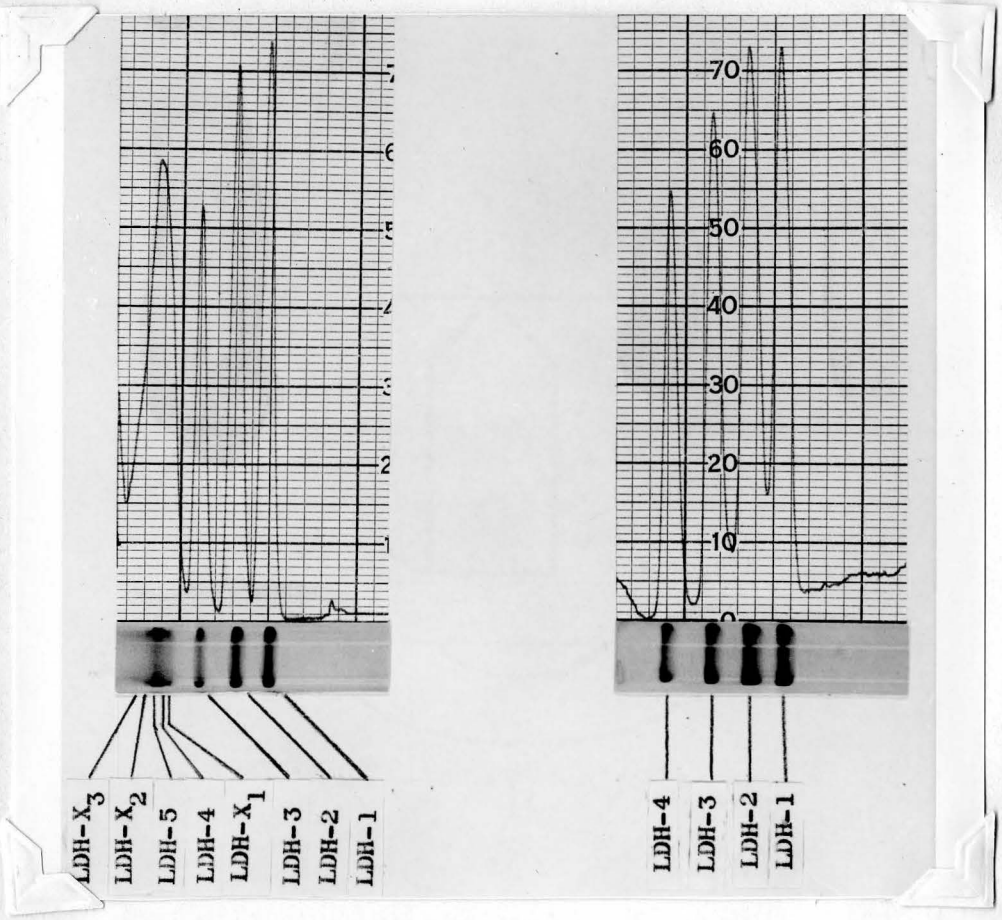


FIGURE III

Lactic acid dehydrogenase isoenzyme patterns of the 15,000 x gravity supernatants of the scrotal (left) and cryptorchid (right) testes from a Sprague-Dawley rat made unilaterally cryptorchid at age 24 days and sacrificed at age 66 days.

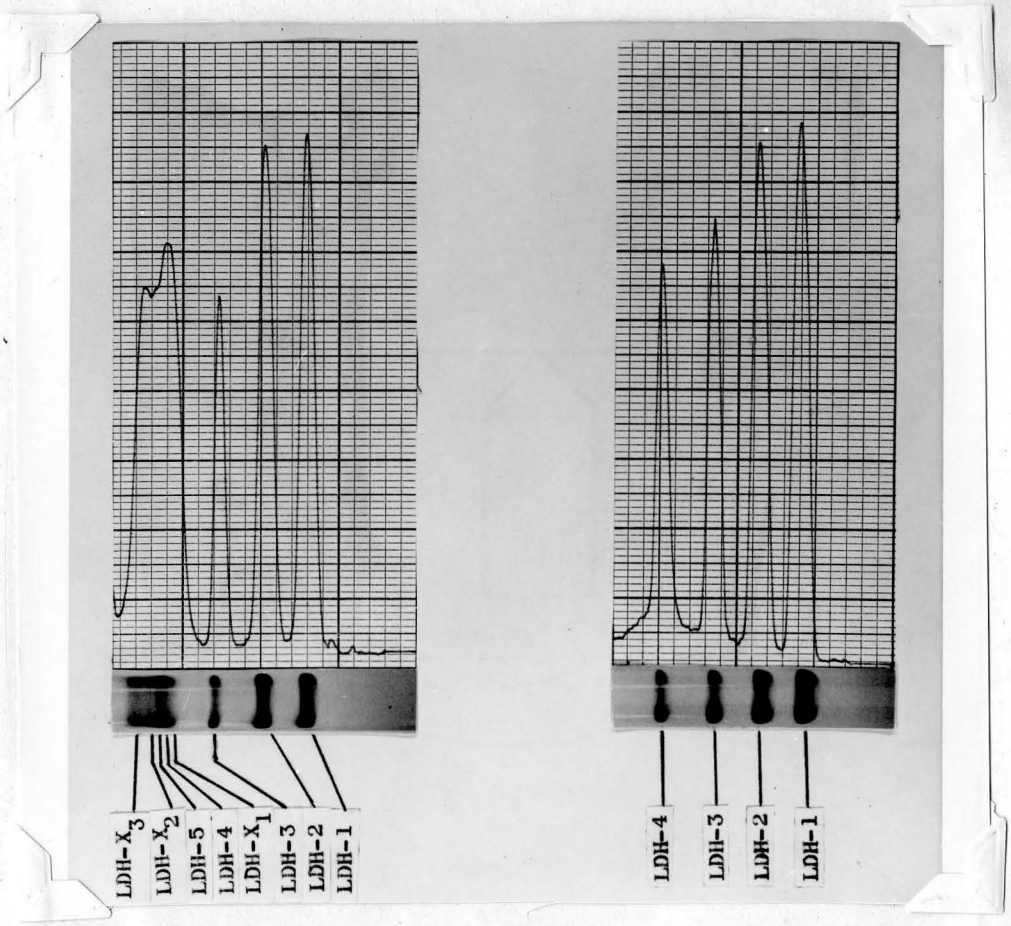


FIGURE IV

The lactic acid dehydrogenase isoenzyme patterns of the 15,000 x gravity supernatants of the scrotal (left) and cryptorchid (right) testes from a Sprague-Dawley rat made unilaterally cryptorchid at age 60 days and sacrificed at age 113 days.

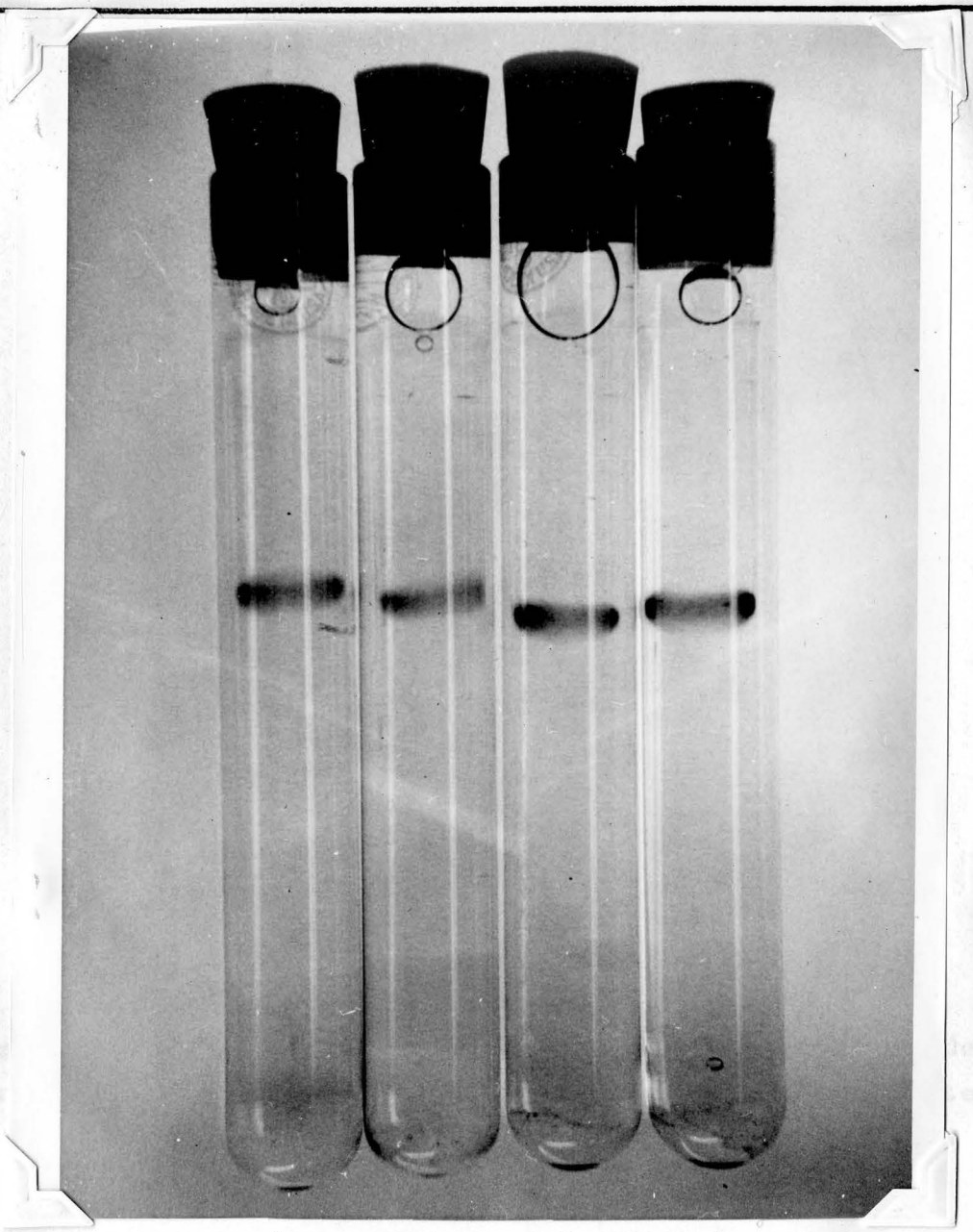


FIGURE V

The malic acid dehydrogenase patterns of the 15,000 x gravity supernatants from the testes of two Sprague-Dawley rats. 1c and 1s are the patterns, respectively, from the cryptorchid and scrotal testes of a rat made unilaterally cryptorchid at age 62 days and sacrificed at age 107 days. 2c and 2s are the patterns, respectively, from the cryptorchid and scrotal testes of a rat made unilaterally cryptorchid at age 25 days and sacrificed at age 68 days.

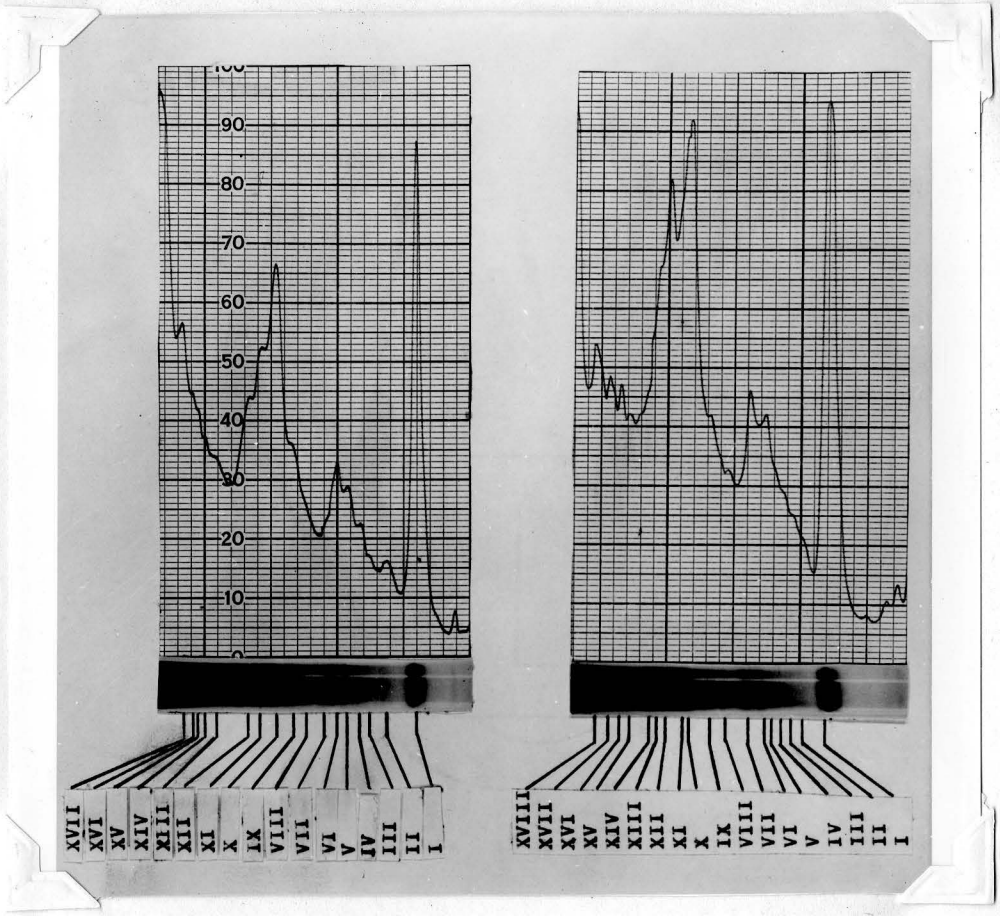


FIGURE VI

The general protein pattern, as stained by Amido Black, and densitometric recording of the 15,000 x gravity supernatants from the testes of a 25 day old Sprague-Dawley rat (left) and from the testes of a 67 day old Sprague-Dawley rat (right).

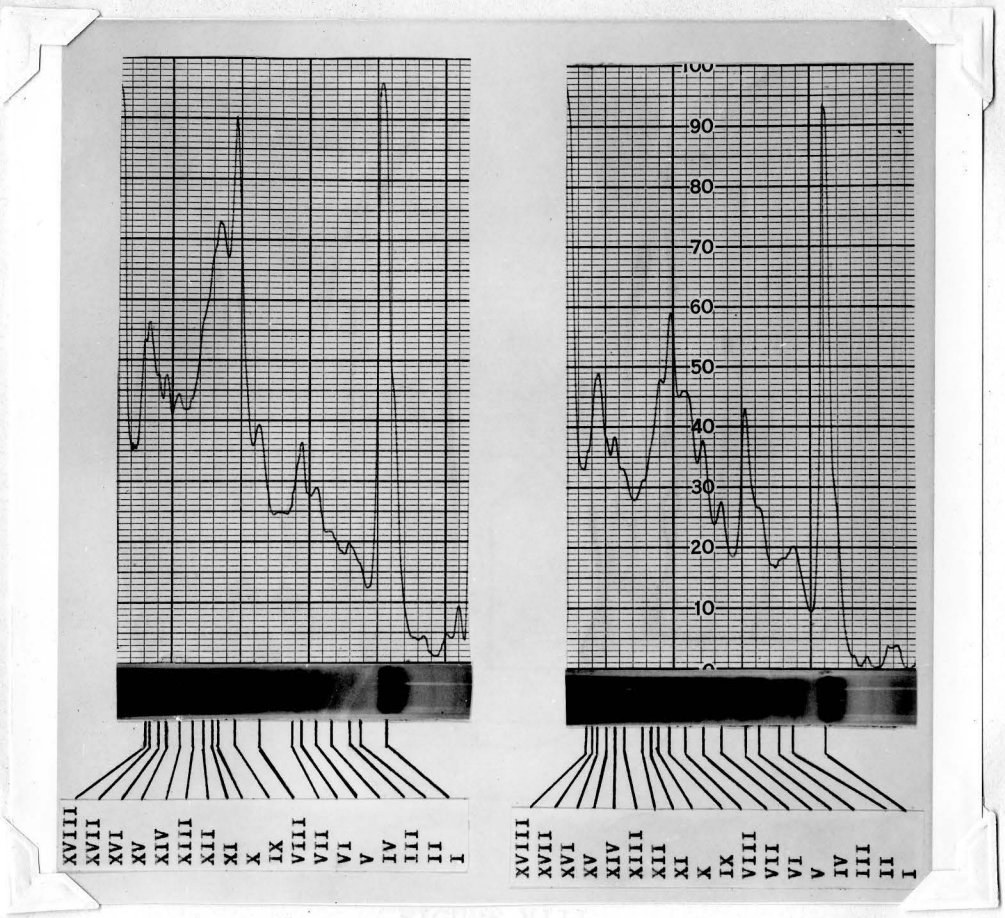


FIGURE VII

The general protein pattern and densitometric recording of the 15,000 x gravity supernatants from the scrotal (left) and the cryptorchid (right) testes of a Sprague-Dawley rat made unilaterally cryptorchid at age 24 days and sacrificed at age 87 days.

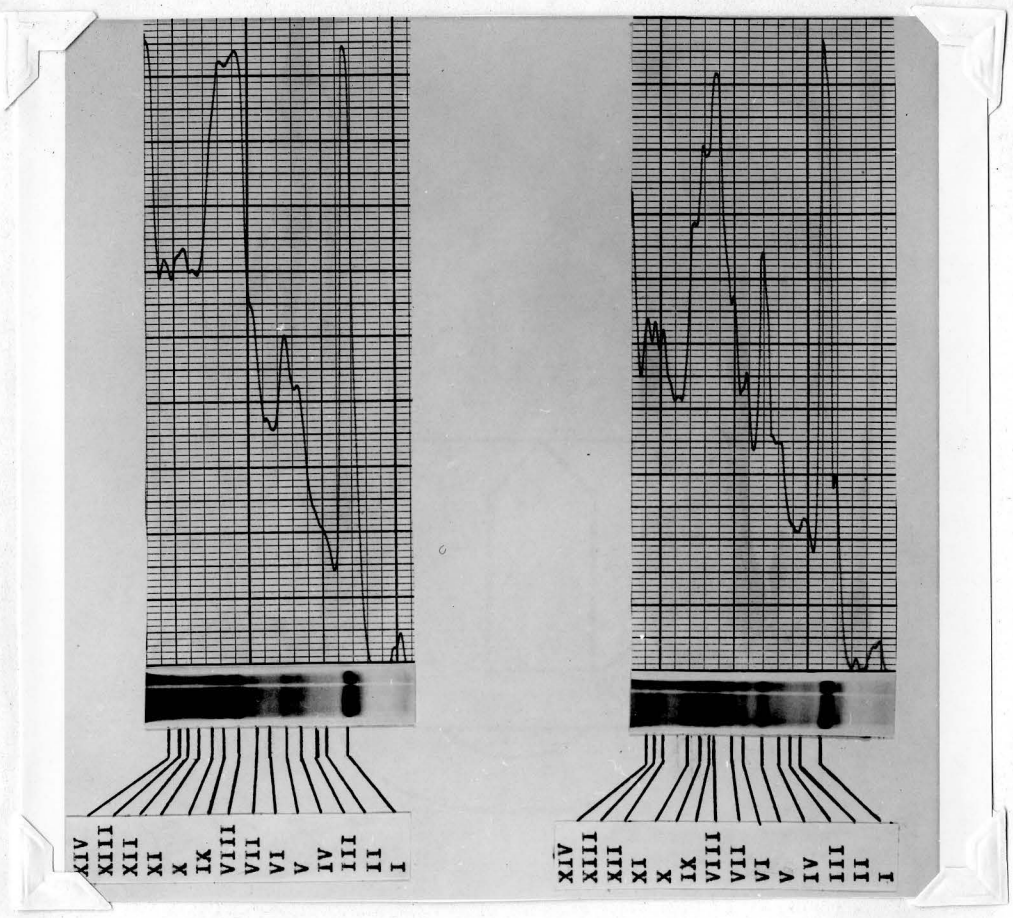


FIGURE VIII

The general protein pattern and densitometric recording of the 15,000 x gravity supernatants from the scrotal (left) and from the cryptorchid (right) testes of a Sprague-Dawley rat made unilaterally cryptorchid at age 60 days and sacrificed at age 113 days.

CHAPTER VII

TABLES

	Percent of total LDH activity				
	LDH-1	LDH-2	LDH-3	LDH-4	LDH-5
Immature testis	28.67	26.54	15.16	29.62	———
Mature testis	26.02	23.22	15.05	*35.69	
Immature cryptorchid	26.65	33.06	25.05	15.23	———
Immature control	22.51	22.29	14.71	*40.47	
Mature cryptorchid	32.13	31.01	20.44	16.40	———
Mature control	20.90	21.07	10.36	*47.65	

*complex of LDH-4, LDH-5, LDH-X_1, LDH-X_2, and LDH-X_3

TABLE I

The immature testis is that of a 25 day old rat. The mature testis is that of a 67 day old rat. The immature cryptorchid and its scrotal control are those of a rat operated on at age 24 days and sacrificed at age 66 days. The mature cryptorchid testis and its scrotal control are those of a rat operated on at age 60 days and sacrificed at age 113 days. All animals used were Sprague-Dawley rats. Percent of total LDH activity for each isoenzyme was determined by averaging three planimetric tracings of records obtained from a Photovolt Densicord Densitometer after scanning the disc electrophoretic gels stained for LDH.

BIBLIOGRAPHY

1. Ambadkar, P. M. and George, J. C. 1964 Histochemical localization of certain oxidative enzymes in the rat testis. J. Histochem and Cytochem., 12: 587-590.
2. Baumrucker, G. O. 1946 Incidence of testicular pathology. Bulletin of the U. S. Army Medical Department, 5: 312-314.
3. Boyd, J. B. and Mitchell, H. K. 1965 Identification of deoxyribonucleases in polyacrylamide gel following their separation by disc electrophoresis. Analytical Biochemistry, 13: 28-42.
4. Campbell, H. E. 1942 Incidence of malignant growth of the undescended testicle; a critical and statistical study. Arch. Surg., 44: 353-369.
5. Charny, C. W. and Wolgin, W. 1957 Cryptorchism. Hoeber-Harper, Philadelphia.
6. Clarke, J. T. 1964 Simplified "disc" (polyacrylamide gel) electrophoresis. Ann. N. Y. Acad. Sci., 121 Art. 2: 428-436.
7. Clausen, J. and Ovlisen, B 1965 Lactate dehydrogenase isoenzymes of human semen. Biochemical J., 97: 513-517.
8. Clegg, E. J. 1961 Further studies on artificial cryptorchidism: quantitative changes in the interstitial cells of the rat testis. J. Endo., 21: 433-441.
9. Clegg, E. J. 1963 Studies on artificial cryptorchidism: morphological and quantitative changes in the Sertoli cells of the rat testis. J. Endo., 26: 567-574.
10. Clegg, E. J. 1963 Studies on artificial cryptorchidism: degenerative and regenerative changes in the germinal epithelium of the rat testis. J. Endo., 27: 241-251.
11. Davidoff, E. 1940 Psychiatric aspects of the treatment of cryptorchidism. J. of Nerv. and Ment. Dis., 91: 724-741.
12. Davis, B. J. and Ornstein, L. March 24, 1959 A new high resolution electrophoresis method. Delivered at the Society for the Study of Blood at the New York Academy of Medicine.

13. Davis, B. J. 1964 Disc electrophoresis II: method and application to human serum proteins. Ann. N. Y. Acad. Sci., 121 Art. 2: 404-427.

14. Davis, J. R., Firlit, C. F., and Hollinger, M. A. 1963 Effect of temperature on incorporation of L-lysine-U-C^{14} into testicular protein. Am. J. Physio., 204: 696-698.

15. Davis, J. R., Morris, R. N., and Hollinger, M. A. 1964 Incorporation of L-lysine-U-C^{14} into proteins of cryptorchid testis slices. Am. J. Physio., 207: 50-54.

16. Davis, J. R., and Firlit, C. 1966 The germinal epithelium of cryptorchid testes experimentally induced in prepubertal and adult rats. Fertil. Steril., 17: 187-200.

19. Dewey, M. M. and Conklin, J. L. 1960 Starch gel electrophoresis of lactic dehydrogenase from rat kidney. Proc. of Soc. Exp. Biol. and Med., 105:492-494.

20. Fairbanks, G. Jr., Levinthal, C., and Reeder, R. H. 1965 Analysis of C^{14}-labeled proteins by disc electrophoresis. Biochem. and Biophys. Res. Comm., 20: 393-399.

21. Firlit, C. and Davis, J. R. 1965 Morphogenesis of the residual body of the mouse testis. Quart. J. Micr. Sci., 106:93-98.

22. Firlit, C. F. and Davis, J. R. 1966 Radio-autographic incorporation of L-lysine-^{3}H into protein of cells of the germinal epithelium in cryptorchidism. J. Reprod. Fert., 11:125-131.

23. Ford, E. and Huggins, C. 1963 Selective destruction in testis induced by 7, 12-dimethylbenzanthracene. J. Exp. Med., 118: 27-40.

24. Frederick, J. F. 1964 The origin of the multiple forms of phosphorylase in algae. Ann. N. Y. Acad. Sci., 121 Art. 2: 634-639.

25. George, J. C. and Ambadkar, P. M. 1963 Histochemical demonstration of lipids and lipase activity in rat testis. J. Histochem and Cytochem,, 11: 420-425.

26. Goldberg, E. 1964 Lactate dehydrogenases and malate dehydrogenases in sperm: studied by polyacrylamide gel electrophoresis. Ann. N. Y. Acad. Sci., 121 Art. 2: 560-570.

27. Grimm, F. C. and Doherty, D. G. 1961 Properties of two forms of malic dehydrogenase from beef heart. J. Biol. Chem., 236: 1980-1985.

28. Gross, R. E. and Jewett, T. C. 1956 Surgical experience from 1,222 operations for undescended testis. J. A. M. A., 160: 634-641.

29. Hayashi, M., Ogata, K., Shiraogawa, T., Murakami, Z., and Tuneishi, S. 1958 The effect of unilateral and bilateral cryptorchidism upon the activities of beta-glucuronidase, lipase, and esterase in the rat testis. Acta Path. Jap., 8: 113-126.

30. Heinicke, E. 1932 Zum Kryptorchismus Problem. Deutsche Ztschr. f. Chir., 235: 334-358.

31. Hollinger, M. A. 1965 Protein labeling of the cryptorchid testis of the rat. Thesis for Master of Science Degree. Loyola University, Chicago, Illinois.

32. Hollinger, M. A. and Davis, J. R. 1966 Metabolic fate of D-glucose U-C^{14} in slices of scrotal and cryptorchid rat testes. Fed. Proc., 25:313.

33. Hollinger, M. A. and Davis, J. R. 1966 Effect of nitrofurazone on the incorporation of L-lysine-U-^{14}C into protein of the rat testis. Biochemical Pharmacology, 15:1235-1237.

34. Hunter, J. 1940 A description of the situation of testis in fetus with its descent into scrotum. M. Classics, 4: 421-441.

35. Hunter, R. L. and Markert, C. I. 1957 Histochemical demonstration of enzymes separated by zone electrophoresis in starch gels. Science, 125: 1294-1295.

36. Hunter, R. L., Rocha, J. T., Pfreuder, A. R., and De Jong, D. C. 1964 The impact of gel electrophoresis upon our understanding of the esterases. Ann. N. Y. Acad. Sci., 121 Art. 2: 532-543.

37. Johnsen, S. G. 1964 Studies on the testicular-hypophyseal feedback mechanisms in man. Acta endocrinol. (suppl.), 90: 99-124.

38. Kaplan, N. O. 1961 in Mechanism of Action of Steroid Hormones, ed. Villee, C. A. and Engel, A. A. Oxford:Pergamon Press.

39. Kendall, J., Jette, E. R., and West, W. 1926 The separation of radium and mesothorium I from barium by the ionic migration method. J. Am. Chem. Soc., 48:3114-3117.

40. Kohlrausch, F. 1897 Ueber Concentrations-Verschiebungen durch Electrolyse im Innern von Lösungen und Lösungsgemischen. Ann. Physik, 62: 209-239.

41. Kormano, M., Harkonen, M., and Kontinen, E. 1964 Effect of experimental cryptorchidism on the histochemically demonstrable dehydrogenases of the rat testis. Endo., 74: 44-51.

42. Kreutzer, H. J. H. and Kreutzer, H. H. 1965 Lactic dehydrogenase isoenzyme determination: effect of incubation temperature. Clin. Chim. Acta, 11: 578-581.

43. Kreutzer, H. H., Jacobs, Ph., and Francke, C. 1965 Lactate dehydrogenase isoenzymes: irregularities in electrophoretic mobility. Clin. Chim. Acta, 11: 159-169.

44. Lacy, D. 1960 Light and electron microscopy and its uses in the study of factors influencing spermatogenesis in the rat. J. Roy. Micro. Soc., 79: 209-225.

45. Latner, A. L. and Skillen, A. W. 1964 Lactate dehydrogenase isoenzymes in foetal and neonatal tissues. Journal of Embryology and Experimental Morphology, 12: 501-509.

46. Mann, T. 1955. Male sex hormone and its rôle in reproduction. Recent Prog. Hor. Res., 12: 353-376.

47. Mann, T. 1964 The Biochemistry of Semen. John Wiley and Sons Inc., New York.

48. Markert, C. L. 1963 Lactate dehydrogenase isozymes: dissociation and recombination of subunits. Science, 140: 1329-1330.

49. Meister, A. 1950 Reduction of alpha, gamma-diketo and alpha-keto acids catalyzed by muscle preparations and by crystalline lactic dehydrogenase. J. Biol. Chem., 184: 117-129.

50. Moore, C. R. and Chase, H. D. 1923 Heat application and testicular degeneration. Anat. Record, 26: 344-345.

51. Moore, C. R. 1924 Properties of the gonads as controllers of somatic and physical characteristics VI testicular reactions in experimental cryptorchidism. Am. J. Anat,, 34: 269-316.

52. Morris, R. N. 1966 The protective effect of glucose against heat-induced inhibition of testicular protein labeling. Dissertation for Doctor of Philosophy Degree. Loyola University, Chicago, Illinois.

53. Nagai, Y., Gross, J., and Piez, K. A. 1964 Disc electrophoresis of collagen components. Ann. N. Y. Acad. Sci., 121 Art. 2: 494-500.

54. Neilands, J. B. 1952 Studies on lactic dehydrogenase of heart: I purity, kinetics, and equilibria. J. Biol. Chem., 199: 373-381.

55. Nelson, W. O. 1951 Mammalian spermatogenesis: effect of experimental cryptorchidism in the rat and non-descent of the testis in man. Recent Prog. Hor. Res., 6: 29-62..

56. Niemi, M. and Kormano, M. 1965 Response of the cycle of the seminiferous epithelium of the rat to artificial cryptorchidism. Fertil. and Steril., 16: 236-242.

57. Odiorne, W, B. and Simmons, C. C. 1904 Undescended testicle. Ann. Surgery, 40: 962-1004.

58. Ornstein, L. 1964 Disc electrophoresis-I background and theory. Ann. N. Y. Acad. Sci., 121 Art. 2: 321-349.

59. Pallmann, H. and Deuel, H. 1945 Ueber die Wasserdurchlassigkeit kon Hydrolegen. Experientia, 1: 325-326.

60. Plomteux, G., Beaumariage, M. L., Bacq, Z. M., and Heusghem, C. 1967 Variations enzymatique dans le plasma du rat après injection d'une dose radioprotectrice de cysteamine. Biochemical Pharmacology, 16: 1601-1607.

61. Poulik, M. D. 1957 Starch gel electrophoresis in a discontinuous system of buffers. Nature, 180: 1477-1479.

62. Raymond, S. and Weintraub, L. S. 1959 Acrylamide gel as a supporting medium for zone electrophoresis. Science, 130: 711.

63. Raymond, S. 1964 Acrylamide gel electrophoresis. Ann. N. Y. Acad. Sci., 121 Art. 2: 350-365.

64. Robbins, S. L. 1962 Textbook of Pathology. W. B. Saunders Company, Philadelphia and London.

65. Roosen-Runge, E. C. 1952 Kinetics of spermatogenesis in mammals. Ann. N. Y. Acad. Sci., 55: 574-584.

66. Shaw, C. R. 1965 Electrophoretic variation in enzymes. Science, 149: 936-943.

67. Smithies, O. 1955 Zone electrophoresis in starch gels. Biochem. J., 61: 629-641.

68. Staemmler, M. 1923 Uber Arterienveranderungen in Retinierten Hoden. Virchow's Arch. Path. Anat., 245: 304-321.

69. Steinberger, E. and Nelson, W. O. 1955 The effect of hypophysectomy, cryptorchidism, estrogen and androgen upon the level of hyaluronidase in the rat testis. Endo., 56: 429-444.

70. Steward, F. C. and Barber, J. T. 1964 The use of acrylamide gel electrophoresis in the investigation of the soluble proteins of plants. Ann. N. Y. Acad. of Sci., 121 Art. 2: 525-531.

71. Tanford, C. S., Swanson S., and Shore, W. J. 1955 H-ion equivalent of bovine serum albumin. J. Am. Chem. Soc., 77: 6414-6421.

72. Tappan, D. V., Jacey, M. J., and Boyden, H. M. 1964 Carbonic anhydrase isozymes of neonatal and adult human and some animal erythrocytes. Ann. N. Y. Acad. Sci., 121 Art. 2: 589-599.

73. Tepperman, J., Tepperman, H. M., and Dick, H. 1949 A study of the metabolism of rat testis in vitro. Endo., 45: 491-503.

74. Terner, C., and Korsh, G. 1962 The biosynthesis of C^{14}-labeled lipids by isolated bull spermatozoa. Biochemistry, 1: 367-372.

75. van Venrooij, W. J. W. 1965 A method for the detection of proteolytic enzymes in electropherograms. Clin. Chem. Acta, 11: 483-484.

76. White, A., Handler, P., and Smith, E. 1964 Principles of biochemistry. McGraw-Hill Book Co., New York, N. Y..

77. White, M. L. 1960 The permeability of an acrylamide polymer gel. J. Physical Chem., 64: 1563-1565.

78. Wieland, T. A., and Pfleiderer, G. 1957 Nachweis der Heterogen-

ität von Milchsäure dehydrogenasen verschiedenen Ursprungs durch Trägerelektrophorese. Biochem. Z., 329: 112-116.

79. Wieland, T. A., and Pfleiderer, G. 1961 Chemical differences between multiple forms of lactic acid dehydrogenases. Annals of the New York Academy of Sciences, 94: 691-700.

80. Wilkinson, J. H. 1966 Isoenzymes. J. B. Lipincott Co., Phil.

81. Wrobleski, F., and Gregory, K. F. 1961 Lactic dehydrogenase isoenzyme and their distribution in normal tissues and plasma and in disease states. Annals of the New York Academy of Sciences, 94: 912-932.

82. Zinkham, W. H., Blanco, A., and Clowry, L. J. Jr. 1964 An unusual isozyme of lactate dehydrogenase in mature testes: llocalization, ontogeny, and kinetic properties. Annals of the New York Academy of Sciences, Art. 2:571-588.

APPROVAL SHEET

The thesis submitted by Robert P. N. Shearin has been read and approved by three members of the faculty of Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 27, 1968
Date

Joseph R. Davis, M.D., Ph.D.
Signature of Advisor