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PURIFICATION OF 17β-HYDROXYSTEROID DEHYDROGENASE OF RABBIT LIVER AND IDENTIFICATION OF A PARTIAL cDNA CLONE

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master of Science.

University of Ottawa
Ottawa, Ontario, Canada
May, 1989

This is dedicated to the loving memory of Norma B. Dohlen who contributed more than she knew to its completion.

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Abbreviations used in the text

ATP : adenosine 5'-triphosphate

Androstenedione : Δ^4-androstene-3,17-dione

BSA : bovine serum albumin

CTP : cytidine 5'-triphosphate

DTT : dithiothreotol

dATP : deoxyadenosine 5'-triphosphate

dCTP : deoxycytosine 5'-triphosphate

dGTP : deoxyribose guanosine triphosphate

5α-DHT : 5α-dihydrotestosterone

DNA : deoxyribonucleic acid

EDTA : ethylenediaminetetracetic acid

FCA : Freund's Complete Adjuvant

FIA : Freund's Incomplete Adjuvant

FSH : follicle stimulating hormone

GnRH : gonadotropin releasing hormone

GTP : guanosine 5'-triphosphate

IAA : iodoacetic acid

IPTG : isopropyl ß-D-thiogalactopyranoside

hCG : human chorionic gonadotropin

3α-HSD : 3α-hydroxysteriod dehydrogenase

17ß-HSD : 17ß-hydroxysteroid dehydrogenase

LH : leutinizing hormone

MRF : Müllerian Duct Regression Factor

NAD^+ : nicotinamide adenine dinucleotide, oxidized form

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NADH : nicotinamide adenine dinucleotide, reduced form

NADH : nicotinamide adenine dinucleotide, reduced form

$NADP^+$: nicotinamide adenine dinucleotide phosphate, oxidized form

NADPH : nicotinamide adenine dinucleotide phosphate, reduced form

PCR : polymerase chain reaction

pfu : plaque forming units

PPO : 2,5-diphenyloxazole

SDS : sodium dodecylsulphate

SDS-PAGE : SDS-polyacrylamide gel electrophoresis

TEMED : N,N,N',N'-tetramethylethylenediamine

Testosterone : Δ^4-androstene-3-one-17ß-ol

TFA : trifluoroacetic acid

Tris : tris[hydroxymethyl]aminomethane

TTP : thymidine 5'-triphosphate

X-gal: 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

SUMMARY

The biosynthesis and degradation of androgens and estrogens is dependent on the oxidoreductive catalysis of 17β-hydroxysteroid dehydrogenase. Antoun et al. (1985a) identified multiple forms of this enzyme from rabbit liver cytosol and purified four of the isoenzymes forms. The procedure established for their isolation by Antoun et al. (1985a) has been modified in this study to reduce both the steps involved and the time required. The initial size fractionation of the total 17β-HSD enzyme solution by Sephadex G-75 was omitted from the previous procedure used and protein purification by affinity chromatography of the liver cytosol preparation was carried out using a stepwise increase in the NaCl concentration of the eluting buffers in place of the salt gradients previously used. In this study the 17β-HSD isoenzymes were then separated by chromatofocusing on a pH gradient from 6.15-5.15 . Use of this modified procedure permitted the isolation of the two most prominent isoforms of the enzyme, 17β-HSD-I and 17β-HSD-III, in pure form as determined by isoelectricfocusing gel analysis and in amounts sufficient for subsequent sequencing.

Peptides established through sequencing of form I of the enzyme were translated into the equivalent nucleic sequences and used for screening a cDNA λgt10 library which was prepared from rabbit liver poly A$^+$ RNA. No positive clones were identified in this library.

A preparation of pure 17β-HSD-III was further utilized for immunization of adult white leghorn roosters. Production of anti-sera cross-reacting with both forms I and III was successful as determined by Western analysis. Screening of a λgt11 expression library also prepared from rabbit liver poly A$^+$ RNA with antisera produced in rooster against 17β-HSD resulted in the isolation of a cDNA clone of approximately 0.2 kbp.

INTRODUCTION

The biosynthesis of androgens and estrogens involves a series of enzymatically catalyzed reactions culminating with the conversion of Δ^4-androstene-3,17-dione (androstenedione) to testosterone, or estrone to estradiol. These two reactions, which produce the potent androgen and estrogen respectively, are both dependent upon the activity of 17β-hydroxysteroid dehydrogenase (17β-HSD, 17β-ketosteroid reductase or 17β-hydroxysteroid oxidoreductase). Although the biosynthesis of these steroids requires 17β-HSD for reductive catalysis, the enzyme also catalyses of the reverse reaction required for the catabolism of these substances.

The Role of 17β-HSD for Androgen and Estrogen Synthesis in Fetal and Pubertal Development.

17β-HSD converts androstenedione to testosterone and also catalyses the reverse reaction. It is the sole enzyme in steroid biosynthesis which catalyses a reversible reaction. This reverse catalysis is utilized by the enzyme for degradation of the potent androgen testosterone, to the less potent forms such as androsterone, epiandrosterone, and etiocholanolone for excretion. The enzyme is essential in humans for fetal development and at puberty for proper differentiation and development of male gonads, internal sex organs and genitalia and later for virilization and masculinization of secondary sex characteristics, and in the female for fertility and development of the female sex characteristics.

At four to five weeks of age the human embryo possesses indifferent, or bipotential, gonads. Despite an XX or XY karyotype the gonad is capable of developing with either male or female characteristics during this stage. The indifferent gonad is composed of a genital ridge containing primitive sex cords and coelomic (or germinal) epithelium. At the time of the indifferent gonad both the Müllerian and Wolffian ducts are present. In the male fetus the Wolffian ducts differentiate into the epididymis and accessory reproductive structures of the urogenital system. In

normal female fetuses the majority of these ducts atrophy leaving only some structures required for the urinary system. Müllerian ducts in the female embryo differentiate to form fallopian tubes, uterus and a portion of the vagina. The male fetus secretes a substance promoting regression of the Müllerian ducts. In female fetuses the unnecessary Wolffian structures atrophy due to the absence of androgen stimulation. This does not occur until about the eleventh week of gestation, at a point when in male fetuses the Wolffian ducts have already differentiated. At this stage the ovary, which has only begun morphological differentiation, is producing estrogens.

A genotypic XY makeup is required for the differentiation of the bipotential gonad into testes barring any Y chromosomal mutations affecting the differentiation. The H-Y antigen produced only by male fetuses for growth of the male gonads is associated with the Y chromosome. The undifferentiated gonads of both sexes are susceptible to the H-Y antigen. In the absence of the H-Y antigen the gonad will develop as an ovary-like structure as observed in the development of normal females. Gonocytes of germinal origin develop into spermatogonia in the male fetus and do not become mature, mobile sperm until puberty. Oocytes develop in the female fetus from the gonocytes but, as in the male, these do not become mature until the hormonal influences of puberty.

Testosterone production occurs as early as the 8th week of gestation in the male and it is formed by the interstitial cells of Leydig which differentiate from the gonadal tissue of mesenchymal origin. Other mesenchymal cells develop to form connective tissue required for normal invasion of blood and lymphatic vessels and nerve endings into the testes. The cortical regions of the gonads, also mesenchymal, develops into the tunica albuginea and tunica vasculosa of the testes.

By the 7th or 8th week of gestation of the XY fetus the Sertoli cells secrete a glycoprotein called Müllerian Duct Regression Factor (MRF) (Müllerian Duct Inhibitor, Müllerian Inhibitory

Substance, or anti-Müllerian Substance) which promotes degeneration of the Müllerian Duct structures. As the Müllerian structures begin to regress, the Sertoli cells begin to secrete testosterone to promote virilization of the Wolffian structures. In the absence of MRF, the Müllerian structures develop at almost 3 months of gestation to form the upper vagina, the oviducts and the uterus in XX fetuses. This female differentiation occurs without hormonal influence. Genotypic XX fetuses are also susceptible to Wolffian virilization should abnormally high levels of androgens be present at the early period of development, approximately 8-11 weeks of gestation.

Testosterone secreted by the gonads of the XY fetuses promotes development of the vas deferens, epididymis, seminal vesicle, ejaculatory ducts and prostate from the Wolffian duct structures. Masculinization of external genitalia is also dependent on testosterone and its conversion by 5α-reductase to 5α-dihydrotestosterone (5α-DHT). Under this more potent androgen the genital tissues differentiate. Undeveloped genital tissues anterior to and at the base of undifferentiated Müllerian and Wolffian ducts are composed of the genital tubercle, the genital swelling and the urogenital sinus and fold. The genital tubercle forms the glans penis, the genital folds fuse to form the body of the penis and the urogenital swellings form the scrotum. The urogenital sinus develops into parts of the prostate and bulbourethral glands and a portion of the urethra. In the absence of androgens these structures develop as female structures. The fetus is visibly identifiable as male as early as 12 weeks post-conception but continued production of androgens is required for continued growth of the external genitalia and successful descent of the testes into the scrotum which is not complete until the time of birth or soon after.

Hormonal stimuli have not been shown to specifically direct the differentiation of the Müllerian ducts into the female accessory structures as in the male. The external genitalia of normal XX fetuses start to develop into the female structures at about the same time as male fetuses begin to produce and respond to 5α-DHT in the formation of the penis and scrotum. The differ-

entiation in females is slower than in the male and continues over a longer period of time. In the female the genital tubercle differentiates with slight growth to form the clitoris. The genital folds form the labia minora, and the urogenital swellings fuse posteriorly and enlarge anteriorly to form the labia majora. Unlike the differentiation of these structures in the male fetus which is dependent upon adequate production of testosterone and its conversion to 5α-DHT, the development of the female external genitalia is not dependent on the 'female hormones', the estrogens. The female structures develop as a result of the absence of testosterone and 5α-DHT due to the innate potential of tissues to become female.

During prepubertal development of the human male testosterone levels decrease dramatically with concomitant decrease in the population of interstitial cells. Prepubertal human males testes secrete a peptide substance (inhibin) which inhibits the secretion of FSH by pituitary and secretion of gonadotropin releasing hormone (GnRH) by the hypothalamus. The low FSH levels prevents sequestering of testosterone in the seminiferous tubules and epididymis and causes degeneration or quiescence of interstitial cells. At the onset of puberty surges in LH secretion cause increases in testosterone production and adult levels of the hormone can be measured. This increase causes proliferation of the interstitial cells of Leydig. Prior to LH increase there is a transient increase of follicle stimulating hormone (FSH) which produces growth, proliferation and specialization of the seminiferous tubules. Spermatogenesis can then be initiated and maintained. There is also gradual loss of the negative feedback of steroids on the pituitary which begins before puberty.

Other marked traits of masculinization noted at puberty result from the increased levels of circulating testosterone. The notable increase in body muscle mass reflects the action of androgens on skeletal muscle. Other effects of androgen action include enlargement of the cartilage of the larynx, sebaceous gland activity, growth of body hair and recession of the hair line.

The production of estrogens by the female becomes crucial at puberty. Most oocytes are contained within primordial follicles by the time of puberty onset. Estrogen synthesis is not significant until the follicle has matured almost to the point of ovulation. Just prior to ovulation estrogen production falls and the level of progesterone increases. Androgens accumulate in those follicles not attaining maturity and they atrophy. Those females with a deficiency in estrogen production show masculinization of external features and experience primary or secondary amenorrhea.

As testosterone has been shown to be essential for proper development of the male phenotype all enzymes involved in the biosynthesis of the androgens are necessary for normal differentiation. Similarly these same enzymes with the addition of aromatase are essential for the establishment of fertility in females. The biosynthesis of the androgens and estrogens occurs initially in the developing gonads of the fetus. Although the testes and ovaries remain the major sites of steroid hormone synthesis, these are also synthesized in the adrenal cortex and the liver. Biosynthesis of testosterone in the testes occurs mostly in the interstitial cells of Leydig with cholesterol as precursor. The Sertoli cells may synthesize a very small amount of testosterone from other androgens supplied to them. The biosynthetic pathway of androgen production in Leydig cells is shown in figure 1. The cholesterol precursors are derived mostly from cholesterol of plasma lipoproteins however cholesterol may be synthesized de novo in Sertoli cells and passed to the mitochondria of the interstitial cells for conversion to pregnenolone. The Δ^5 pathway, indicated by the bold arrows in figure 1, is the major pathway in humans. In the female, plasma cholesterol is taken up by the thecal cells of the ovarian follicles for conversion to androgens. These can in turn be converted to estrogens or passed to the granulosa cells for conversion to estrogens. The conversion of androgens to estrogens is shown in figure 2. Again, the principal pathway used is indicated with bold arrows. The 17β-HSD required for the final catalytic reaction of testosterone

production is required also for estrogen production as 17β-estradiol and estrone are produced from testosterone and more abundantly from androstenedione. Its deficiency has been documented in a rare form of male pseudohermaphroditism (Saez et al., 1971a, Rosler and Kahn, 1983, Wilson et al., 1987), and one case of a female with the deficiency (Pang, et al., 1988) has been reported. These deficiencies reflect the absolute requirement of 17β-HSD for normal development.

Degradation of potent androgens occurs largely in the liver and kidney and involves 17β-HSD for the formation of 17-ketosteroids. Those androgen metabolites formed for excretion are shown in figure 3 and are found excreted both in urine and feces. Estrogens taken up by hepatocytes are largely converted to C2, C4, C6 and C16 hydroxylated forms or to C3 and C17 glucuronated metabolites. Estrogens reaching the kidney become sulphated at C3 or C17. The involvement of 17β-HSD in estrogen degradation is mainly associated with the estrone/17β-estradiol pair. This enzyme therefore has physiological importance in both the oxidative and reductive directions. Shown in figure 4 are the different substrate/product combinations for which 17β-HSD has been found to have the highest activity.

Properties and Characteristics of 17β-HSD

The biosynthesis of the steroid hormones occurs not only in gonadal tissue but also in other steroid synthesizing, target and metabolizing tissues, 17β-HSD can therefore be found in all tissues involved in biosynthesis or degradation of the androgens and estrogens. 17β-HSD has been studied not only in testes of several animals (Musto et al., 1972, Oshima et al., 1972, Inano and Tamaoki, 1974) but also in the liver of rabbit (Thaler-Dao et al., 1972, Hasnain and Williamson, 1974, Antoun et al., 1985) and of rat (Thaler-Dao, 1974), guinea pig kidney (Liu and Kochakian, 1971), ovary of both sheep (Michel et al., 1975) and rabbit (Rodway and Rahman, 1978) as well as in canine pancreas (Mendoza-Hernandez et al., 1988). The enzyme has also been studied from a variety of human sources, including the endometrium (Pollow et al., 1976), blood cells (Milewich

TESTOSTERONE BIOSYNTHESIS

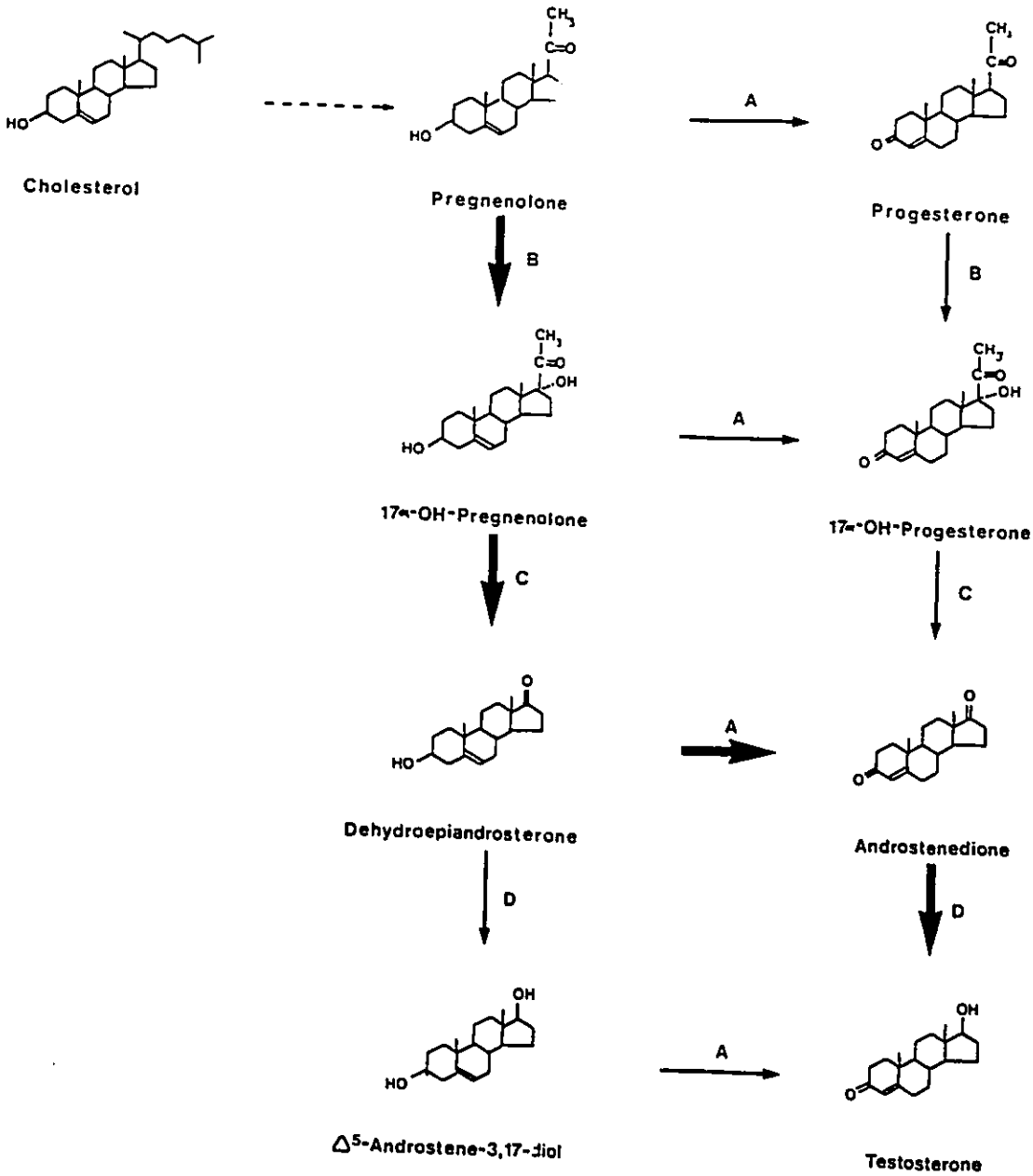


FIGURE 1

ANDROGEN CONVERSION TO ESTROGENS

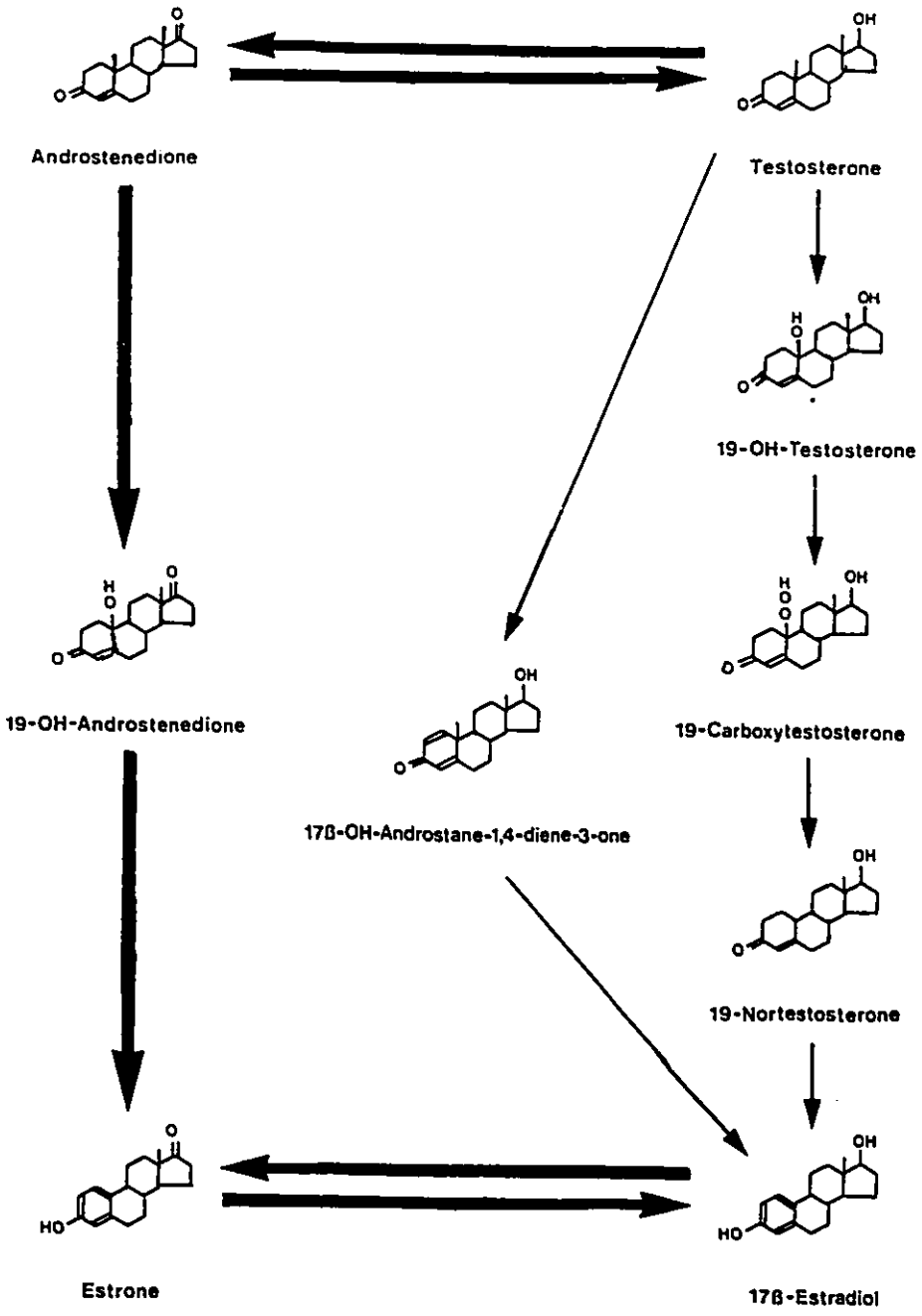
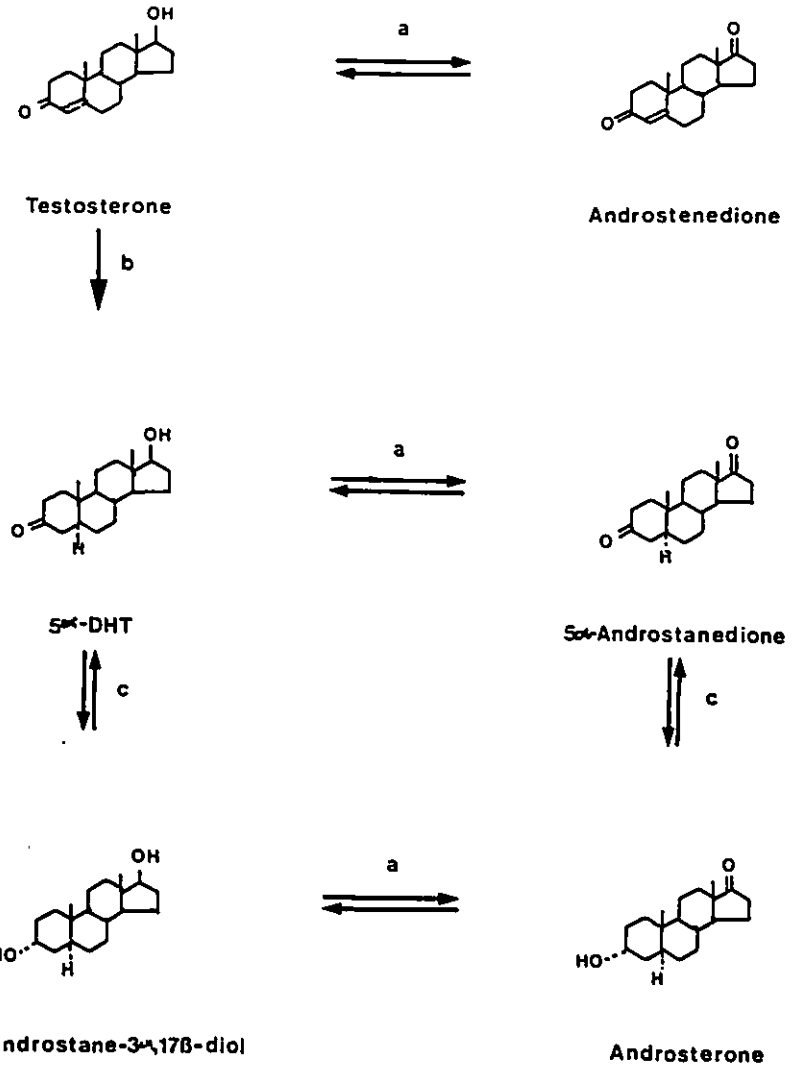


FIGURE 2

17β-HSD
in
ANDROGEN DEGRADATION



a; 17β-HSD

b; 5α-reductase

c; 3α-HSD

FIGURE 3

SUBSTRATES/PRODUCTS OF 17β-HSD

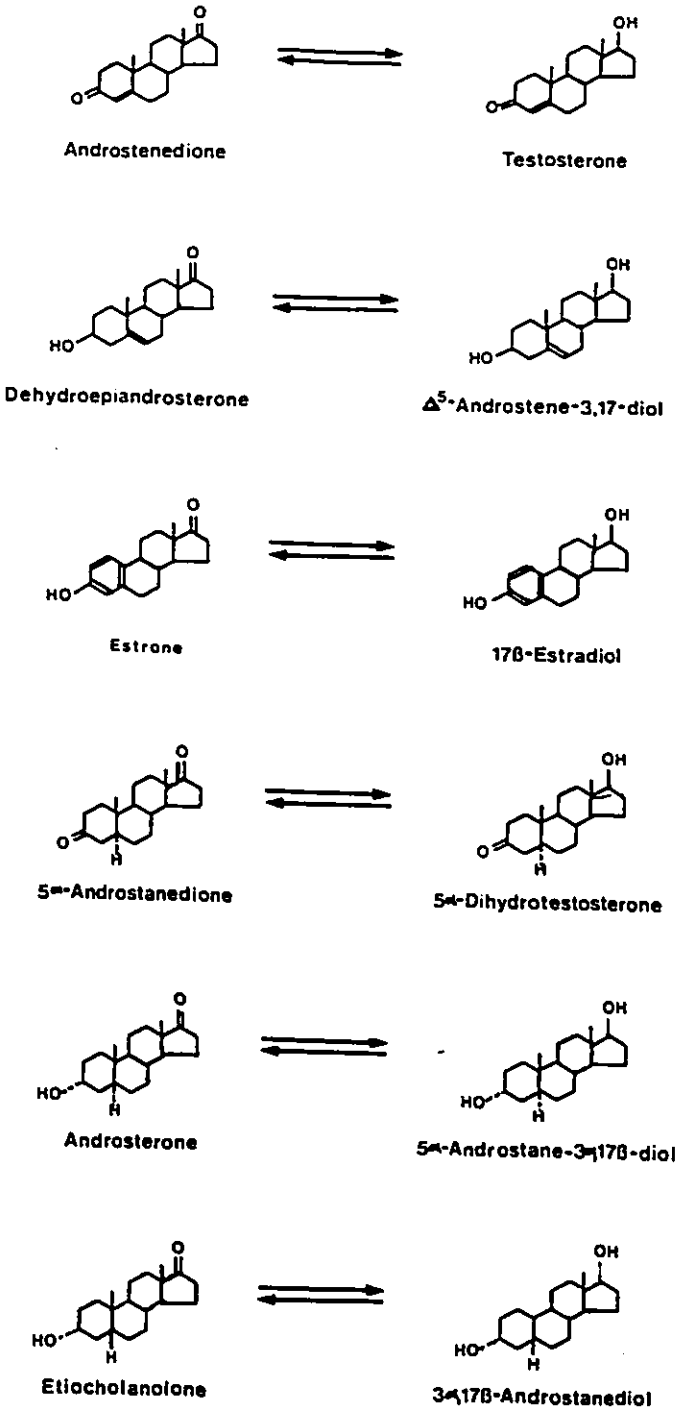


FIGURE 4

et al., 1982, Ranelletti et al., 1978, Mulder et al., 1972) lung tissue (Milewich et al., 1978) breast tissue (M*Indoe and Woods, 1981) and most extensively in human placenta (Peltoketo et al., 1988). Isozymic forms of the enzyme, first speculated to exist in canine pancreas (Liu and Kochakian, 1971b), have since been purified in all tissues from which the enzyme has been isolated (Antoun et al., 1985a, Inano et al., 1981, Liu and Kochakian, 1971b, Pollow et al., 1976).

The enzyme activity has been characterized primarily by kinetic analysis and substrate binding studies. Research has revealed that either little similarity exists amongst the enzymes of different organs and animals or that the enzymes are extremely sensitive to variations within experimental procedures.

The 17β-HSD of rabbit liver cytosol has previously been studied in this laboratory (Hasnain and Williamson, 1974, Antoun et al., 1985a, Antoun et al., 1985b). 17β-HSD is quantitatively measured by the conversion of testosterone to androstenedione as indicated in figure 4 with the addition of $NADP^+$ as cofactor. The oxidative direction of the reaction has a pH optimum of 9.5 at 37°C (Antoun, PhD thesis, 1983). The pH optimum of the reverse reaction is 6.5 - 7.5 at the same temperature. The Km of the enzyme for testosterone is 3.0 - 3.2 μM (Antoun et al., 1985a). The mechanism of catalysis has been found to involve a 1:1 ratio of NADPH to androstenedione and involves transfer of the hydrogen from the 4-pro-S position of NADPH to the 17α position of androstenedione (Inano and Tamaoki, 1975).

17β-HSD purified from rabbit liver cytosol has been found to show marked heterogeneity. A preparation of 17β-HSD was shown to be separable into one form preferentially utilizing NAD and NADH as cofactors and at least four other isoenzymes forms which catalyze the oxidoreduction reaction using either $NADP^+$ or NADPH as cofactors (Antoun et al., 1985a). These $NADP^+$-dependent isoenzymes are all acidic and vary in pI from 5.4 - 5.7 with molecular weights 37,000 -

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40,000. An age dependence is also apparent when comparing the relative amounts of the different isoforms in enzyme preparations of immature and mature rabbits. Total 17β-HSD activity is shown also to increase with age of the rabbit (Antoun et al., 1985b)

To date the amino acid sequences of the 17β-HSD isoenzyme forms of rabbit liver have not been established. Low abundance and instability of the 17β-HSD hinders isolation of homogenous samples of the different charge isomers. Recently the amino acid sequence of the placental enzyme as deduced from its cDNA sequence has been published (Peltoketo et al., 1988). This enzyme's notably higher affinity for estrone compared to androstenedione affords it the name estradiol-17β-dehydrogenase, and not 17β-HSD. The placental form is also quite different from the liver enzymes in molecular weight and has been characterized as a dimer of molecular weight 65,000 - 70,000 (Jaraback and Street, 1971, Nicolas et al., 1972, Burns et al., 1972, Peltoketo et al., 1988). Comparison of the amino acid compositions of placental and liver enzymes also reveals a large difference between the two (Table 1).

The origin of the diversity in the amino acid sequences of the isoenzymes has not been established. The heterogeneity of the enzyme may be attributed to post-translational modifications of a nascent 17β-HSD, possibly cleavage of a terminal sequence, to differential splicing of a single gene to produce unique mRNA species for each isoenzyme or to the existence of multiple genes coding for 17β-HSD. Extensive screening of a cDNA library prepared from the total mRNA of rabbit liver with probes prepared from the enzymes would eventually lead to isolation of all messages coding for these isoforms. Specifically, complete sequencing of the peptides for each of the 17β-HSD species would allow identification of variations between sequences. Use of the nucleic translations of peptides found to be unique to only one of the isoenzymes as probes for the library would facilitate isolation of different messages.

Table 1

Amino acid residue	Placental number	%	17β-HSD-I number	%	17β-HSD-III number	%
Cys	6	1.8	8	2.3	9	2.8
Asx	22	6.7	37	10.7	38	11.8
Thr	16	4.9	17	4.9	16	5.0
Ser	19	5.8	24	6.9	17	5.3
Glx	31	9.4	40	11.5	40	12.4
Pro	20	6.1	8	2.3	8	2.5
Gly	32	9.8	28	8.1	20	6.2
Ala	38	11.6	29	8.4	25	7.7
Val	32	9.8	18	5.2	16	5.0
Met	6	1.8	12	3.4	10	3.1
Ile	4	1.2	16	4.6	17	5.2
Leu	42	12.8	39	11.2	38	11.7
Tyr	6	1.8	12	3.4	15	4.6
Phe	13	4.0	13	3.7	14	4.3
His	7	2.1	10	2.9	10	3.1
Lys	9	2.8	24	6.9	23	7.1
Arg	23	7.0	12	3.4	7	2.2
Trp	1	0.3	N.D.		N.D.	

Amino acid compositions of human placental 17β-estradiol dehydrogenase and rabbit liver 17β-hydroxysteroid dehydrogenase. The differences apparent between the compositions of the enzymes of the two tissues supports their existence as distinct proteins. Compositions are as determined by Peltoketo et al. (1988) and G.R. Antoun, PhD thesis (1983).

Isolation of specific mRNA sequences can also be accomplished through the use of an expression library. Insertion of cDNA sequences into phage DNA within an inducible gene provides a method of expressing the recombinant proteins. Identification of the desired protein requires screening with antibody obtained to that protein. For this purpose polyclonal antibody was raised in rooster against 17β-HSD-III and was found to react also with 17β-HSD-I.

This study was undertaken to pursue the molecular basis of the different forms of 17β-HSD of rabbit liver. The two most prominent isoenzyme forms present in the liver of mature female rabbits, 17β-HSD-I and 17β-HSD-III would be purified to homogeneity. An isoform labelled IIIY is present only in immature rabbit liver (Antoun et al., 1985a) and 17β-HSD-II occurs in very low amounts.The rationale of the experiments originally designed to elucidate this objective was that the isoenzymes could be purified to homogeneity and sequenced. This accomplishment would permit the comparison of the sequences obtained and thereby identify all dissimilarities between the structures. Screening of a cDNA library synthesized from rabbit liver RNA with oligonucleotides prepared from unique sequences within the different isoenzymes could then be carried out to isolate the mRNA species encoding the proteins. Alternatively, an expression library also synthesized from rabbit liver RNA could be screened with antibody raised against the 17β-HSD to isolate the mRNA.

MATERIALS AND METHODS

A. Materials:

Radioactively labelled compounds;

[1α, 2α^{3}H]-Testosterone of specific activity 45-60 Ci/mmol, [α^{32}P]-dATP, [α^{32}P]-dCTP and [τ^{32}P]-ATP all of specific activity 3000Ci/mmol were purchased from Amersham Canada Limited.

Unlabelled Steroids;

Testosterone and androstenedione were obtained in recrystallized form from Sigma Chemical Company.

Animal tissue, affinity chromatography and chromatofocusing;

Female New Zealand white rabbits were purchased from Riemans Fur Ranch. Procion Red HE3B Matrix gel A for affinity columns was obtained from Amicon Corporation, chromatofocusing was carried out on a MONO P column from Pharmacia (Canada) Inc. Buffers for chromatography were prepared from reagent grade salts except for chromatofocusing elution which required polybuffer 74 purchased from Pharmacia (Canada) Inc. Buffers for the purification included Tris[hydroxymethyl]aminomethane (Tris) purchased from Sigma Chemical Company, and dithiothreitol (DTT) and histidine which were purchased from BDH Chemicals Ltd.

Enzyme assays and quantitation;

Ethylacetate, toluene and PPO were purchased from BDH Inc. Activated zinc silicate and Silica Gel G were obtained from J. T. Baker Chemical Co. $NADP^+$ was obtained from Boehringer Mannheim Canada Ltd.

SDS-PAGE and IEF;

Gels for electrophoresis were prepared with acrylamide from Serva (Terochem Laboratories Inc.), N,N'-methylene bisacrylamide purchased from Eastman Kodak, ammonium persulfate and TEMED from Bio-Rad Laboratories, Inc. LKB Ampholine pH 5-7 for IEF was obtained from Pharmacia (Canada) Inc. Sodium dodecylsulfate (SDS) and reagents for staining were purchased from Sigma Chemical Company. Buffers and solvents for staining were either of electrophoretic or reagent grade.

Antibody production and western analysis;

Adult white leghorn roosters were obtained from Agriculture Canada. FCA and FIA were purchased from ICN Biomedicals, Ltd. Goat anti-chicken IgG linked to horse radish peroxidase was obtained from Bio-Rad laboratories, Inc. Skim milk powder was obtained from Difco Laboratories, Tween 20 from Sigma Chemical Co., and thiomersol from BDH Chemicals Ltd. Peroxidase colour reaction required H_2O_2 from Fisher and 4-chloro-1-napthol obtained from Sigma Chemical Co. Nitrocellulose membranes were purchased from Bio-Rad Laboratories, Inc, and were stained with Ponceau S from BDH Chemicals Ltd.

RNA extraction and cDNA synthesis;

All extraction and synthesis buffers were of reagent grade. Oligo(dT) cellulose as well as non-radioactive nucleotides were purchased from Boehringer Mannheim Canada Ltd. All enzymes were purchased from Amersham Canada Ltd.

Synthesis of libraries and growth of E. Coli cultures;

Kits for the synthesis of both λgt10 and λgt11 libraries were purchased from Amersham Canada Ltd. Additional buffers, salts and solvents required were prepared with reagent grade

products. Bacto-agar and bacto-tryptone were obtained from Difco Laboratories, yeast extract was obtained from Oxoid Limited. Maltose for NM514 and L87 cells was purchased from BDH Chemicals Ltd. Additional reagents for Y1090 cultures were purchased form Boehringer Mannheim Canada Ltd.

Library probing, DNA extraction and removal of inserts:

Solutions for probing and DNA extraction were prepared with reagent grade products. Nylon filters for plaque lifting were purchased from Amersham Canada Limited. All enzymes for DNA extraction and cleavage of inserts were obtained from Amersham Canada Limited.

DNA Labelling and amplification of cDNA inserts (PCR);

T_4 polynucleotide kinase and Klenow fragment of DNA polymerase 1 for ^{32}P labelling and their buffers were purchased from Amersham Canada Limited. The kit for the polymerase chain reaction (PCR) was obtained from Perkin Elmer Cetus and contained all solutions required, with the exception of the DNA primers which were synthesized at the University of Ottawa Biotechnology Research Institute.

Elfo-Tris and glyoxal agarose gel electrophoresis;

All buffers and salts used were of reagent or electrophoretic grade. Staining of gels required ethidium bromide from Sigma Chemical Co. and bromophenol blue from BDH Chemicals Ltd. Agarose was purchased from International Biotechnologies, Inc.

Autoradiography and photography;

X-ray film was obtained from Dupont and photography film was purchased from Ilford.

B. Methods:

PURIFICATION OF 17β-HYDROXYSTEROID DEHYDROGENASE

Crude Enzyme Preparation

All procedures followed in the purification of 17β-HSD were carried out at 0-4°C. An adult female New Zealand white rabbit was sacrificed by cervical dislocation, the liver was removed and placed in ice cold 10 mM Tris/0.5 mM DTT buffer, pH 8.0. The liver was weighed after removal of the gall bladder and minced into 3-4 volumes (v/w) of buffer. The minced tissue was homogenized in a Waring blender at maximum speed for two minutes. The homogenate was centrifuged for 30 minutes at 10,000 g in a Sorval RC2-B centrifuge. The supernatant was centrifuged for 90 minutes at 105,000 g in a Beckman ultracentrifuge. The cytosolic supernatant obtained was concentrated under N_2 in an Amicon apparatus with a YM-10 membrane to a final volume of ca. 60 mL/100 g liver tissue. The concentrated liquid was centrifuged for 30 minutes at 10,000 g in a Sorval RC2-B centrifuge to remove any remaining precipitable matter.

Affinity Chromatography

An agarose immobilized Procion Red HE3B matrix gel A column (1.5 x 40 cm) (Watson et al., 1978) was equilibrated with eight bed volumes of 10 mM Tris/0.5 mM DTT pH 8.0 buffer. The crude enzyme preparation (concentrated cytosolic fraction) was applied to this column at a rate of 10-12 mL/h. The column was washed first with 200 mL of 10 mM Tris/0.5 mM DTT/5 mM $MgCl_2$ at a flow rate of 13-15 mL/h, then with 500 mL of 10 mM Tris/0.5 mM DTT/5 mM $MgCl_2$/0.3 M NaCl and finally with 500 mL of 10 mM Tris/0.5 mM DTT/10 mM $MgCl_2$/2.5 M NaCl. Fractions (5 ml) were collected and assayed for 17β-HSD activity. Those fractions showing high 17β-HSD activity were pooled and concentrated by ultrafiltration to a volume of 6-10 mL.

Chromatofocusing Chromatography

The concentrated enzyme solution obtained after affinity chromatography was dialyzed into 25 mM Histidine/0.5 mM DTT pH 6.15. This change in pH resulted in the formation of a precipitate which was removed by centrifugation for 20 minutes at 16,000 g in a Sorval RC2-B centrifuge. The supernatant was titrated down to pH 5.5 with 0.3 N HCl and the precipitate which formed was removed by centrifugation as before. The enzyme solution was then brought back to a pH of 6.0 with 0.3 N NaOH. Precipitation observed following this titration was removed by centrifugation as described.

Chromatofocusing (A. Æ.Sluyterman, 1982) was next carried out using a Pharmacia MONO P column. A maximum of 25 mg protein was applied to the column which was equilibrated with 25 mM Histidine/0.5 mM DTT pH 6.15. The separation of iso-enzymes was carried out by eluting the column with diluted polybuffer 74 (1:10) containing 0.5 mM DTT, pH 5.15. The elution profile was monitored by enzyme activity, pH gradient, and OD_{280}. Each fraction collected was brought to pH 8.0 by the addition of 0.4mL 50 mM Tris (pH 8.0)/0.5 mM DTT/20% glycerol to each. Those fractions having UV absorbance were assayed for 17β-HSD activity. Two peaks representing enzymes I and III were distinguished from the activity profile and the fractions of each peak were pooled and concentrated down to less than 6 mL if necessary. The fractions I and III were separately rechromatofocused through the MONO P column as before. The fractions having 17β-HSD activity were pooled as either purified enzyme I or enzyme III and analyzed further to establish purity.

PROTEIN ANALYSIS

Isoelectricfocusing Polyacrylamide Electrophoresis (IEF)

Following chromatofocusing, the purity of the 17β-HSD fractions was analyzed by IEF in

8.0% (w/v) rod gels as described by Hayes & Wellner, (1969). The pH gradient was established in the gels with a pre-run which involved a sequential increase of voltage through the gels, carried out at 4°C from 200 V for 15 minutes to 300 V and finally 400 V, both for 30 minutes. After rinsing of the gel top surfaces with distilled water and application of the protein samples (15 - 25 μg) prepared in 10% glycerol, electrophoresis was carried out for 30 minutes at 400 V followed by 500 V for 3 hours. The buffer of the lower anode compartment was 0.01 M H_3PO_4, while 0.02 M NaOH was in the cathode compartment.

Protein staining

The gels were heated at 60°C in 12.5% trichloroacetic acid for 30 minutes to fix the proteins. Protein staining was then carried out for 30 minutes at 60°C in a Coumassie staining solution (0.2% w/v coumassie blue, 45% v/v EtOH, 10% v/v acetic acid). Protein bands were then revealed following destaining of the gels in 10% v/v acetic acid/25% EtOH v/v. After complete destaining the gels were stored in 7.5% acetic acid.

ASSAY FOR 17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY

17β-HSD activity was assayed in the oxidative direction by measuring the conversion of testosterone to androstenedione. A small aliquot of protein solution to be assayed (0.5-10 μl) was mixed with one ml of incubation medium which contained 70 mM glycine/NaOH buffer pH 9.5, 45 mM KCl, 0.54 mM $NADP^+$, and 3.0 nmol ^{3}H-testosterone in 30 μl ethanol (specific activity 4.54 μCi/μmol). All reaction tubes were then incubated at 37°C in a shaking water bath for 30 minutes. A blank reaction which excluded enzyme sample from the incubation mixture, was also incubated. The reactions were stopped by the addition of 2 ml ethylacetate to each 1 ml reaction volume to extract the steroids into the organic phase. The organic phase was removed from each and dried under N_2. The dried samples containing substrate (testosterone) and product (androstenedione) were then redissolved in a minimum volume of MeOH containing sufficient unlabelled

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androstenedione and testosterone to visually locate the steroids on TLC plates.

The steroid samples were then separated on TLC plates coated with 0.25 mm silica gel G and zinc silicate (50:1 w/w) in a solvent system of ethylacetate:methylene chloride (7:3, v/v). The non-fluorescent areas on the plates were identified as the separated steroids and individual spots were scraped into scintillation vials containing 0.4 ml MeOH to extact the steroid from the silica, and mixed with 8.0 ml toluene/PPO (0.4% w/v). Radioactivity was then quantified by liquid scintillation counting. Enzyme activity of the fraction measured was initially quantitated as a percent conversion of the substrate (testosterone) to product (androstenedione) and expressed as munits/ml of enzyme solution. One unit of enzyme activity is that amount which catalyzes the conversion of 1 μmol of steroid per minute under the defined conditions of the reaction.

DIGESTION OF 17β-HSD AND SEQUENCING OF THE PEPTIDES

Sequencing procedures required that the protein samples be free of all other substances including salts and buffers as these would interfere in the analysis. The purified enzyme preparation obtained by chromatofocusing also contained polybuffer 74, histidine buffer, 10% glycerol (v/v), 25 mM Tris pH 8.0, and 0.5 mM DTT. Separation of the enzyme from these components was carried out by ammonium sulphate precipitation. The enzyme solutions were brought to 80% saturation with the addition of solid $(NH_4)_2SO_4$ (diJeso 1968) and the after allowing for precipitation at 4°C the pellets were collected by centrifugation. The pellets were each washed twice with saturated ammonium and the final 17β-HSD pellets were dissolved in 1.5 ml 10 mM Tris pH 8.0 and dialyzed 3 times against 3000 volumes of distilled water. Precipitation of the enzyme occurred during dialysis. The resulting enzyme suspensions were carefully removed from the dialysis membrane, rinsing the tubing with distilled water to minimize the loss and were lyophilized.

The dried enzyme samples (enzymes I and III, 24-30 nmol) were dissolved in 1.5 ml of

carboxymethylation buffer (6 M guanidine, 0.1 M Tris, 1 mM EDTA, 2 mM DTT) and left at room temperature under a stream of N_2 for 10 minutes and incubated for one hour at 37°C. The samples were then brought to 5 mM in iodoacetic acid (IAA), left under N_2 for another 10 minutes, and again incubated for one hour at 37°C while protected from the light. An amount of DTT equal to 50% of the initial addition was added to the samples and they were then left under nitrogen for a further 10 minutes. An amount of IAA equivalent to the first addition was combined with the reaction mixtures and incubation was carried out as before. The carboxymethylation was stopped by the addition of 0.1% (v/v) β-mercaptoethanol. (This procedure is as described through personal communication, Dr. G. Flynn, Queen's University.)

The carboxymethylated enzyme samples were dialyzed into 0.1 M $(NH_4)HCO_3$ at 4°C and then at 37°C before adding 1/100 (w/w) of endoproteinase Lys-C. After a 2h incubation at 37°C a second aliquot of endoproteinase Lys-C equal to the first was added and the digestion continued overnight. The samples were cooled on ice and dialyzed (membrane cut off 1000 daltons) against 4.0 l dH_2O at 4°C and finally against 1.0 l dH_2O containing 0.1% trifluoroacetic acid (TFA). The dialyzed samples were each filtered 2-3 times through an HPLC nylon MSI cameo filter and the enzyme digests were then resolved on a C18 reverse phase HPLC column by elution with a linear gradient of acetonitrile (15 - 80%) in 0.1% TFA.

Each peak collected from the column was lyophilized to dryness and was purified by rechromatography as above. The final pure peptide samples were lyophilized for the sequencing procedure carried out on Model 470A gas phase sequencer from Applied Biosystems.

EXTRACTION OF TOTAL RNA FROM RABBIT LIVER

A female New Zealand white rabbit was killed by cervical dislocation and the liver was removed and immediately cut into 5-10 g pieces which were placed into separate vials and frozen

in liquid nitrogen. Total RNA was extracted from rabbit liver essentially as described by Auffrey and Rougeon (1979), and Storey et al., (1984). The RNA pellets were dissolved in sterile water (9.3 ml). Purity and concentration were verified by OD 260/280 and denaturing glyoxal agarose gel electrophoresis (Maniatis et al., 1982).

PURIFICATION OF POLY A$^+$ RNA FROM TOTAL RNA

Poly A$^+$ RNA was isolated from total RNA using oligo(dT) cellulose columns essentially as described by Aviv and Leder (1972). The RNA pellets were recovered by centrifugation following ethanol precipitation, and washed with 70% EtOH/30% NaAcetate. The poly A$^-$ RNA pellet was dissolved in the starting volume of sterile dH_2O (4.3 ml) while the poly A$^+$ RNA was dissolved in 100 μl of sterile dH_2O. Absorbance readings for both poly A$^-$ and poly A$^+$ RNA were taken and purity was analyzed by glyoxal gel electrophoresis. The gels were stained with ethidium bromide and photographed by UV transillumination using Ilford FP4 film.

SYNTHESIS OF DOUBLE STRANDED cDNA FROM POLY A$^+$ RNA

The synthesis of double stranded cDNA (ds-cDNA) was carried out essentially as described by Gubler and Hoffman (1983) with some modifications (Rutledge et al., 1988). The synthesis reaction used a total of 10 μg of the purified poly A$^+$ RNA and was done both with and without random primers.

Single stranded cDNA synthesis:

A solution (50 μl) containing 5 μg oligo$(dT)_{12\text{-}18}$, 50 nmol of each dNTP, 50 μCi of [α^{32}P]-dATP, 30 μg of random primers (or dH_2O) prepared in reverse transcriptase buffer (0.1 M Tris, pH 8.3, 0.14 M KCl, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol) was heated to 60°C and then cooled on ice. 50 units of reverse transcriptase was added and the mixture and was incubated at 42°C for 30 minutes. The single strand cDNA in a hybrid with the RNA was ligated by addition of 800 units

T_4 DNA ligase and 1/10 volume 10X ligase buffer and incubation at 15°C for one hour. The enzyme was then inactivated by heating the reaction mixture to 65°C for 5 minutes.

Double stranded cDNA synthesis:

The RNA was removed from the newly synthesized DNA and the second strand of the DNA was produced by adding to the reaction tubes one unit of RNase H and 3 units of DNA polymerase 1 in nick translation buffer (50 mM Tris, pH 7.2, 10 mM $MgSO_4$, 1 mM DTT, 50 μg/ml bovine serum albumin) and incubating for 2 hours at 15°C.

The aqueous solution of ds-cDNA was extracted sequentially with 100 μl phenol/$CHCl_3$/isoamyl alcohol (25:24:1), and then with $CHCl_3$/isoamyl alcohol (SEVAG) (24:1). The organic phase was back extracted with 50 μl TE buffer (10 mM Tris, 1 mM EDTA) pH 8.0. The two aqueous layers were combined and unicorporated dNTP's were then removed by passing the samples through 1 ml G-50 columns (Maniatis et al., 1982). To calculate the amount of cDNA obtained 1 μl of the G-50 column eluant was counted.

Size fractionation of cDNA:

The samples of cDNA were passed down sepharose 4B columns prepared in 1 ml graduated glass pipettes equilibrated with STE buffer (100 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA). The eluant was collected dropwise into individual eppendorf tubes. Each fraction was then counted and the peak fractions corresponding to larger DNA fragments which eluted first from the column were pooled. The amount of cDNA obtained was calculated from the counts determined in 1 μl aliquots.

The ds-cDNA solutions were precipitated at -70°C for 30 minutes, or at -20°C for 12 hours after mixing with 1/10 volume 3 M NaAcetate and 2.5 volumes cold 95% EtOH. The precipitate

was collected by centrifugation for 30 minutes at 4°C. The pellet was washed with 70% cold EtOH and the final DNA pellet was redissolved in sterile dH_2O or TE buffer (10 mM Tris pH 7.5, 1 mM EDTA) to the desired concentration.

λgt10 LIBRARY SYNTHESIS

The λgt10 library was constructed from a kit supplied by Amersham Canada Limited which included all solutions required. The methods used in its construction were essentially those described in the kit instructions.

The procedures for the synthesis of the library are summarized in the table below.

Reaction/procedure	Solutions required	Incubation
Methylation of cDNA (protect EcoR 1 sites)	M buffer, SAM, EcoR 1 methylase	37°C 1 hour
Addition of linkers	EcoR 1 linkers, L buffer, ligase	15°C 16-20h
Production of cohesive ends	EcoR 1 enzyme, E buffer	37°C 5 hours
Removal of excess linkers	Columns, STE buffer	ppt eluant, dissolve in STE 50 ng/μl
Ligation of phage arms	Phage arms, ligase, L buffer	15°C 16-20h
Phage packaging	Extracts A and B	20°C 2 hours

The packaged phage was then diluted in 0.5 ml SM buffer (0.1 M NaCl, 10 mM $MgSO_4$, 50 mM Tris, 0.1% gelatin w/v, pH 7.5) and 10 μl $CHCl_3$. The final library ready for infection of competent cells was stored at 4°C.

Preparation of E. coli Cells:

Two strains of E. coli cells are supplied with the λgt10 library kit, L87 and NM514. These were both used to determine the recombinant frequency of the colonies.

L-agar plates were streaked with either of the E. coli stocks supplied and were incubated overnight at 37°C. A few colonies were then lifted from the plates to inoculate 10 ml of L-broth (1.0% w/v tryptone, 0.5% w/v yeast extract, 1.0% w/v NaCl) containing 0.4% (w/v) maltose. These cultures were incubated with shaking overnight at 37°C. Finally 1 ml of the overnight cultures was added to 50 ml of prewarmed L-broth containing 0.4% maltose. These were incubated (with shaking) at 37°C until reaching an OD_{600} of 0.5. For the LS7 strain this required about 1 hour and for the NM514 strain it required from 2-3 hours.

Once the cells were grown they were cooled to stop further growth and were centrifuged in a swinging bucket rotor for 15 minutes at 3,000 rpm. The supernatant was poured off and the pellet of cells was resuspended in 15 ml of cold 10 mM $MgSO_4$. These cells were stored at 4°C for up to one week.

L-B agar plates were prepared by pouring 20 ml/82 mm plate of a sterilized solution cooled to 60°C containing 1.0% w/v tryptone, 0.5% w/v yeast extact, 1.0% w/v NaCl and 1.5% w/v agar. Once plates had set they were stored at 4°C.

Infection of Competent Cells with Phage:

100 μl aliquots of competent cells were prepared in separate eppendorf tubes and 100 μl of an appropriate dilution of phage in SM buffer was added to each. The mixtures were incubated for 15 minutes at 37°C prior to plating.

Plating the Infected Cells:

The 200 μl solution of infected cells was added to 4 ml of top agar (1.0% w/v tryptone, 0.5% yeast extract, 0.5% NaCl, 0.25% w/v $MgSO_4$ and 1.0% w/v agar, sterilized) melted and maintained at 45-47°C and the mixture was poured onto prewarmed L-B plates. After the agar had set the plates were incubated upside down at 37°C overnight.

Replica Plating for Probing of Inserts:

Plates were grown up with infected NM514 cells and were allowed to grow to lysis before leaving them at 4°C for at least 1 hour to stop further growth. Nylon filters for lifting phage DNA from the lysed cells were prepared as described by Amersham. Each filter was placed on top of the agar for 30 seconds. Subsequent lifts from the same plate required that the filter be left an additional 30 seconds for each addition lift. The filters were oriented by alignment with marks on the petri dish. They were then lifted off and placed plaque side up for 5 minutes onto Whatman filter paper soaked with denaturing solution (1.5 M NaCl, 0.5 M NaOH). The filters were transferred into neutralizing solution (0.5 M Tris pH 7.0, 1.5 M NaCl) for 5 minutes, and finally rinsed in 2X SSC (1X SSC is 0.15 M NaCl, 16.7 mM tri-sodium citrate). The filters were air dried and were placed on sheets of Whatman paper, clamped between glass plates and dried under vacuum for 2 hours at 80°C. The filters were stored at room temperature until use.

Probing:

Hybridization:

All filters were incubated with prehybridization solution (6X SSC, 0.5% SDS, 5X Denhardt's and 100 μg/ml denatured calf thymus DNA, 25 ml/3 filters), in sealed plastic bags, for 4 hours at 65°C with shaking. To 15 ml of each 25 ml prehybridization solution was added ^{32}P-cDNA (see labelling of probes). This hybridization solution was placed in sealed plastic bags with the filters

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and the incubation continued for at least 12 hours at 65°C.

Washing:

All filters were washed at room temperature twice for 15 minutes in 2X SSC, once for 30 minutes in 2X SSC containing 0.1% (w/v) SDS and finally once for 10 minutes in 0.1X SSC. The filters were transferred onto a sheet of dry filter paper for a few minutes and placed onto a filter paper soaked with 2X SSC, covered in plastic and exposed to X-ray film. Any hybridization to the nucleic probe was then noted on the developed film. Positive colonies were picked and placed in 0.5 ml SM buffer with 10 μl $CHCl_3$.

DNA Purification from Phage:

Extraction of phage DNA proved to be most effective when the DNA was purified by the plate lysate method. The DNA obtained from liquid cultures appeared to have some impurities which prevented successful cleavage by EcoR 1.

Competent cells were infected with phage suspected to contain positive cDNA inserts at a dilution sufficient to ensure confluent lysis. Three 132 mm agarose plates per phage provided sufficient DNA for extraction. The phage were extracted from the plates by adding 10 ml SM buffer to each plate and gently shaking at 4°C for at least 2 hours. The buffer was then poured off and was combined in separate 50 ml conical tubes. The plates were rinsed with additional SM buffer, such that the total volume of buffer per phage sample was 30 ml.

To each lysate sample was added 10 μl of DNase (10 mg/ml) and 40 μl of RNase A (10 mg/ml) and this mixture was incubated with shaking at 37°C for one hour and centrifuged. NaCl and PEG 8000 was added to each of the supernatant samples to give a final concentration of 1 M and 10% (w/v) respectively. The solutions were placed on ice for one hour and then at 4°C

overnight with shaking. The samples were centrifuged and the pellets resuspended in 3 ml SM buffer. Aliquots (0.5 ml) of each 3 ml sample were placed in eppendorf tubes for subsequent extractions. All aliquots were made 0.1% in SDS, proteinase K (final concentration 0.3 $\mu g/\mu l$) was added and the mixture incubated at 65°C for one hour.

For the final extraction of the DNA, all tubes were sequentially extracted with 0.5 ml of each phenol, phenol:$CHCl_3$ and SEVAG. The DNA fraction was then EtOH precipitated and washed as described earlier. The washed pellets were each dissolved in 50 μl of TE buffer and stored at 4°C until further characterization.

λgt11 LIBRARY SYNTHESIS

A λgt11 library was constructed for probing with antibody prepared against 17β-HSD using a kit obtained from Amersham Canada Limited. All procedures involved were as described for the λgt10 library with the modifications summarized below:

1) **Cell strains.** Once the cloned DNA was packaged into λgt11 the phage was infected into the Y1090 strain of E. Coli. The Y1090 strain is grown at 43°C which allows lytic growth.

2) **Conditions for growth.** This cell type contains the plasmid pMC9 which has the ampicillin resistance gene, therefore all incubations included ampicillin at 50 $\mu g/ml$. The cDNA which has been synthesized is inserted into λgt11 within the β-galactosidase gene downstream from the lac operator. The pMC9 plasmid of the host cells used expresses a repressor of this operator which prevents expression of the fusion proteins unless the incubation includes the inducer, isopropyl β-D-thiogalactopyranoside (IPTG). IPTG forms a complex with the lac repressor such that this will not bind the operator and prevent transcription. Recombinants can be visually distinguished from non-recombinants when 0.02% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) is included in the

incubation. Expression of an intact β-galactosidase gene will result in reaction of this enzyme with its substrate X-gal producing a blue product. Thus the non-recombinant blue colonies are distinct from the recombinant white plaques.

3) **Preparation of filters for screening.** Y1090 cells infected with phage were plated and allowed to incubate without the IPTG inducer for only 3 hours at 43°C before lysis has occured. Nylon filters soaked in 10 mM IPTG and air dried were then placed on the plates and incubation continued overnight at 37°C for induction of protein synthesis into the filter paper. The filters were then removed and air dried before hybridizing.

4) **Probing.** All filters were blocked in 2% BSA for 1 hour. They were incubated for two hours in 0.1% BSA containing the primary antibody as a 1/100 dilution of rooster serum. The filters were then washed twice for ten minutes in TBST (0.05% v/v Tween 20 in TBS) and rinsed twice with TBS (0.9% w/v NaCl, 10 mM Tris pH 7.4). Finally the filters were left for 1 hour in 0.1% BSA containing a 1/1000 dilution of peroxidase-linked anti-chicken IgG. The filters were washed as before and then were subjected to the peroxidase reaction. 50 mg 4-chloronapthol dissolved in 20 ml MeOH and 40 μl H_2O_2 was added to 100 ml of TBS immediately prior to adding the filters. The filters were incubated in this solution for 10-30 minutes and were removed once background colour began to show or once positive blue spots could be noted. After rinsing thoroughly in water the filters were left to dry on filter paper and were stored in the dark.

RESTRICTION ENZYME DIGESTS OF PHAGE DNA

BamH 1 or EcoR 1

For digestion of phage DNA by either of these restriction enzymes the procedure was the same. BamH 1 was used to confirm digestibility of the DNA preparation and EcoR 1 digestion released the insert from within the lambda arms. In either case the digestion was performed by

mixing together in eppendorf tubes the phage DNA (2 μg), and enzyme (6-12 units) in its buffer. Digestion was carried out for 1.5-2 hours at 37°C and was stopped by heating the reaction tubes to 70°C for 10 minutes.

Sac 1/Kpn 1

The double digestion was performed on EcoR 1 undigestible DNA as this combination releases a fragment containing the insert DNA which can be distinguished on an agarose gel from non-recombinant phage DNA. The method is similar to the single digestion with the exception that the inactivation step is eliminated at the end of the first digestion. The Sac 1 digestion was performed first since its activity is optimal in a buffer containing no salt. After incubating this digestion in the 20 μl volume the reaction was continued for a further 1.5 hours after adding Kpn 1 (6-12 units) and 1/10 volume of its 10X buffer. The reaction was stopped as described above.

KLENOW LABELLING OF EcoR 1 DIGESTS

Radioactive labelling of EcoR 1 DNA digests was carried out with the Klenow fragment of DNA polymerase 1. 0.1 μl [α^{32}P]-dATP (1 μCi), DNA polymerase 1 Klenow fragment (4 units) was added to the 20 μl digest. The total volume was adjusted to 22.5 μl with dH_2O. Labelling proceeded at 30°C for 30 minutes and the digests were then analyzed by Elfo-Tris agarose gel electrophoresis as described in Maniatis et al. (1982).

END LABELLING OF PROBES

Oligonucleotides used for screening the λgt10 library were radioactively labelled by incubating 75 ng of the nucleic probe, 50 pmol of [γ^{32}P]-ATP and 10 units of T_4 polynucleotide kinase in kinase buffer at 37°C for 30 minutes. The reaction was stopped by bringing the mixture to 20 mM in EDTA and unincorporated ATP was removed by Sephadex G-50 chromatography.

1 μl of the eluant was counted to determine the amount of incorporated radioactivity.

AMPLIFICATION OF λgt11 INSERTS BY THE POLYMERASE CHAIN REACTION

Small positive inserts obtained from the λgt11 library were amplified using the polymerase chain reaction (PCR) from Perkin Elmer Cetus. This reaction was used to produce larger quantities of the inserts labelled with ^{32}P used subsequently analysis by agarose gel electrophoresis.

All the reagents required for this reaction were supplied in the kit purchased from Perkin Elmer Cetus with the exception of the primers which were synthesized at the University of Ottawa Biotechnology Research Institute. Four reaction mixtures were prepared, one for each of the four positive clones obtained from λgt11 screening. Each mixture contained dATP, dCTP, dGTP and TTP (0.2 mM each), reverse primer (50 mM), forward primer (50 mM), and λgt11 DNA (1 ng) in a total of 100 μl made up in the reaction buffer (50 mM KCl, 10 mM Tris, pH 8.3, 15 mM $MgCl_2$, 0.01% [w/v] gelatin). The DNA was denatured by heating the mixture to 94°C for 9 minutes before adding 0.5 μl (5 units/μl) Taq Pol 1. The tubes were mixed and a thin layer of parafin oil was laid overtop of the solutions.

The reaction mixtures were put through 9 cycles of the following 3 incubations: 1: 50°C for 2.5 minutes to anneal primers to DNA, 2: 72°C for 4.0 minutes for elongation 3: 94°C for 1.5 minutes to denature the hybridized DNA strands. One further annealing and elongation step was carried out before adding 33.5 μl of a cocktail containing 100 μCi each of [$\alpha^{32}P$]-dATP and [$\alpha^{32}P$]-dCTP, both the reverse and forward primers (50 mM each) and 2.5 units Taq Pol 1 in reaction buffer to each reaction mixture. The mixtures were then denatured at 94°C for 1.5 minutes before proceeding for 15 more cycles as described above. The reaction was terminated by annealing at 50°C for 2.5 minutes and elongating at 72°C for 13 minutes. The tubes were then cooled to room temperature before removing the oil on the surface and extracting the DNA with 150 μl SEVAG.

Unincorporated radiolabelled nucleotides were separated from DNA by Sephadex G-50 chromatography. The amount of synthesized cDNA was calculated from the counts determined in 1 μl aliquots of the samples. These samples of amplified insert obtained from the λgt11 library were then used to probe the λgt10 library as has been described. (Wong et al., 1989)

PRODUCTION OF POLYCLONAL ANTIBODY AGAINST 17β-HSDFOR SCREENING OF λgt11 LIBRARY

Initially three adult white leghorn roosters were used for the production of antibody against 17β-HSD. Two samples of control serum were obtained from each animal by withdrawing 1-2 ml of blood from the wing vein 4 days prior to immunization (ie. day -4) and again on day 0 just prior to immunization. In all cases of serum collection the blood was kept at 4°C in a glass test tube for at least 2 hours and then centrifuged for 15 minutes at 4000 rpm. The serum was drawn off with a pasteur pipette and stored in plastic test tubes at -20°C. The serum of each rooster was kept separate.

Immunization:

200 μl of 17β-HSD-III solution (0.5 mg/ml) was diluted in Tris pH 8.0 to 1.25 ml and emulsified to high viscosity with an equal volume of Freund's Complete Adjuvant (FCA). 0.5 ml of the final emulsion (20 μg enzyme) was injected intramuscularly into the breast muscle of each animal on day 0 of the study.

On day 14 each rooster was bled from the wing vein (2 ml) and the sera were collected and assayed for anti-enzyme activity (see below). Similarly, blood was drawn periodically until the primary antibody response was seen to diminish. At this time (day 48) re-immunization was performed. The immunization solution was prepared as above except that incomplete adjuvant (FIA) was employed. Animal #1 was also sacrificed at this time as it had become quite ill.

Two weeks following the re-immunization the remaining two animals were sacrificed by heart puncture under halothane. A total of 50-70 ml of blood was withdrawn from each animal and 12-15 ml of serum per animal was obtained. The final rooster anti-sera were stored at -20°C until used for Western blot identification and screening of the λgt11 library.

ASSAY FOR ANTI-ENZYME ACTIVITY

Serum dilutions up to 1/1000 in 10mM Tris buffer pH 8.0 were prepared in a total volume of 50 μl. 17β-HSD activity was assayed as previously described except that KCl was replaced with NaCl required for optimal antibody function. The medium contained per assay, 0.54 μM $NADP^+$, 70 mM glycine/NaOH pH 9.5, and 8.0% (w/v) NaCl in a total volume of 1.0 ml. To this solution was added one of the serum dilutions and 3 μl of the enzyme solution used for immunization (1.5 μg). Blank incubations contained no enzyme while control incubations contained 50 μl Tris buffer in place of the diluted serum. The samples were incubated for 30 minutes at 37°C to allow binding of the antibody to enzyme. [^{3}H]-Testosterone (3 nmol in 30 μl EtOH) was then added to all tubes mixed and the reaction continued for 30 minutes at 37°C. The reactions were stopped by the addition of 2.0 ml of ethylacetate and the quantitation of enzyme activity was carried out as described earlier. Inhibition of enzyme activity over that observed in the control tube was interpreted as anti-enzyme activity due to binding of the enzyme to the antibody.

WESTERN BLOT ANALYSIS

Western blot analyses were carried out to verify the hybridization of the rooster anti-sera to 17β-HSD. This involved electroelution of the protein run on SDS-PAGE onto a nitrocellulose membrane, incubation of the membrane filter with anti-sera and detection of hybridization of antibody to the filters.

SDS-PAGE:

A 1.5 mm slab gels (9.0% w/v) were prepared and run with stacking gels (0.3% w/v) as described by P. Blackshear (1984). SDS denatured protein samples (10-15 μg) were applied mixed with 2.0 μl bromophenol blue (0.2% w/v) to the polymerized gel. Electrophoresis was carried out at 30-40 mA for 3-4 hours.

Hybridization and peroxidase reaction:

After SDS-PAGE the resolving gel was assembled into the Transblot sandwich apparatus (Bio-Rad) and the resolved proteins were transferred onto the nitrocellulose membrane overnight in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3) at 100 mA. The assembly was dismantled and the nitrocellulose was rinsed in water. Proteins were stained by gently shaking the filter for 5 minutes in a 0.2% w/v solution of Ponceau Red in 3.0% TCA w/v and rinsing briefly in water. Their positions were noted before destaining the filter with PBS (137.0 mM NaCl, 3.0 mM KCl, ...) mM $Na_2HPO_4{\cdot}7H_2O$, 1.5 mM KH_2PO_4).

The filters were first blocked by incubation for 1 hour at room temperature with shaking in 25 ml BLOTTO (1.0% w/v skim milk powder, 0.001% w/v thiomersol in PBS). This was then poured off and replaced with 25 ml BLOTTO containing 250 μl antiserum (1/100 dilution) and incubation continued for a further 2 hours. After removing the primary antibody solution the filters were washed 3 x 10 minutes in PBS-Tween 20 (0.1% v/v Tween 20 in PBS) and rinsed twice in PBS.

The rinsed filters were incubated for 2 hours at room temperature with shaking in a 1/1000 dilution of anti-chicken IgG prepared in BLOTTO. The filters were washed as above except the three washes were for 20 minutes each. The peroxidase reaction to confirm that antibody reacted with the 17β-HSD was performed as described in the procedure for screening the λgt11 library.

RESULTS

PURIFICATION OF 17β-HSD

The procedure for the purification of 17β-HSD described by Antoun et al. (1985a) involved affinity chromatography on Procion Red HE3B matrix, size fractionation on Sephadex G-75, and chromatofocusing chromatography on polybuffer exchanger 94. The modification of this procedure was undertaken to reduce the number of manipulations and the time taken to purify the individual isoenzymes. The sensitivity of 17β-HSD to oxidation affects the yield of the closely related and low abundancy iso-forms. For this reason a more rapid purification procedure than the method previously established was required.

Sephadex G-50 column chromatography of the crude liver cytosol was found not to significantly improve the subsequent resolution of the 17β-HSD by affinity chromatography. 17β-HSD was effectively bound to the column even in the presence of the large number of proteins present in the cytosol. The elution of total 17β-HSD from a column of agarose-immobilized Procion Red HE-3B was also modified from the original elution pattern requiring linear salt gradients since this time consuming process increased the exposure time of the enzyme preparation to denaturing conditions. A stepwise increase in the salt concentration of the eluting buffer could result in the total elution of the enzyme in less time than would be required by the gradients. It was therefore necessary to first establish the conditions of the affinity column which would result in satisfactory elution of the enzyme in the shortest time period.

Following the application of the crude enzyme preparation to the equilibrated affinity column, the column was washed with 200 ml of buffer containing no NaCl. This longer washing, compared to that used previously (150 ml), removed the additional bound or weakly bound proteins

from the column. The column was then washed with 500 ml of buffer containing an intermediate concentration of NaCl; that is, a salt concentration which would result in the elution of most cytosolic proteins while the majority of the 17β-HSD activity is retained on the column.

As shown in Fig. 5 use of either 0.8 M or 1.5 M NaCl in the eluting buffer resulted in significant elution of enzyme activity from the column. However only a small amount of 17β-HSD activity was eluted at an NaCl concentration of 0.3 M. Attempts to isolate the enzyme eluted in the buffers containing 0.8 M and 1.5 M NaCl were unsuccessful due to the high levels of contaminating proteins also eluting in these fractions.

Following the establishment of an adequate washing method for the affinity chromatography elution of the total 17β-HSD from the column was carried out by passing 500 ml of Tris buffer containing 2.5 M NaCl through the column. As shown in Fig. 6 the enzyme is eluted from the column immediately following the increase in salt concentration. Those fractions showing high enzyme activity were pooled and were found to sufficiently cleaned of other proteins to allow for an adequate separation of the isoenzymes in the subsequent chromatofocusing. The observed increase in the 17β-HSD activity with the increased salt concentration of the eluting buffer accompanied an increase in the amount of total protein as reflected in protien concentration determination. The pooled fractions of total 17β-HSD were concentrated down to less than 10 ml before continuing the purification. This concentration step facilitated subsequent handling of the enzyme for dialysis and titrations. Following concentration of the solution in an amicon under N_2, insoluble material was removed by centrifugation. The solution was then transferred into dialysis tubing of cut off 10,000 daltons and dialyzed against 1.0 l of 25 mM histidine buffer of pH 6.15 containing 0.5 mM DTT. As the pH of the enzyme solution decreased a precipitate formed within the tubing. Dialysis was continued for 2-4 hours only as the enzyme is not stable at the lower pH. The white precipitate was removed by centrifugation.

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Figure 5

Elution of 17β-HSD from Procion Red HE 3B by buffers of increasing ionic strength. Affinity chromatography of liver cytosol with buffers (10 mM Tris, 0.5 mM DTT, 5 mM $MgCl_2$) containing different NaCl concentrations was carried out as described in Methods. All fractions were 10 ml.

— 1.5 M NaCl, ---- 0.8 M NaCl, ···· 0.3 M NaCl

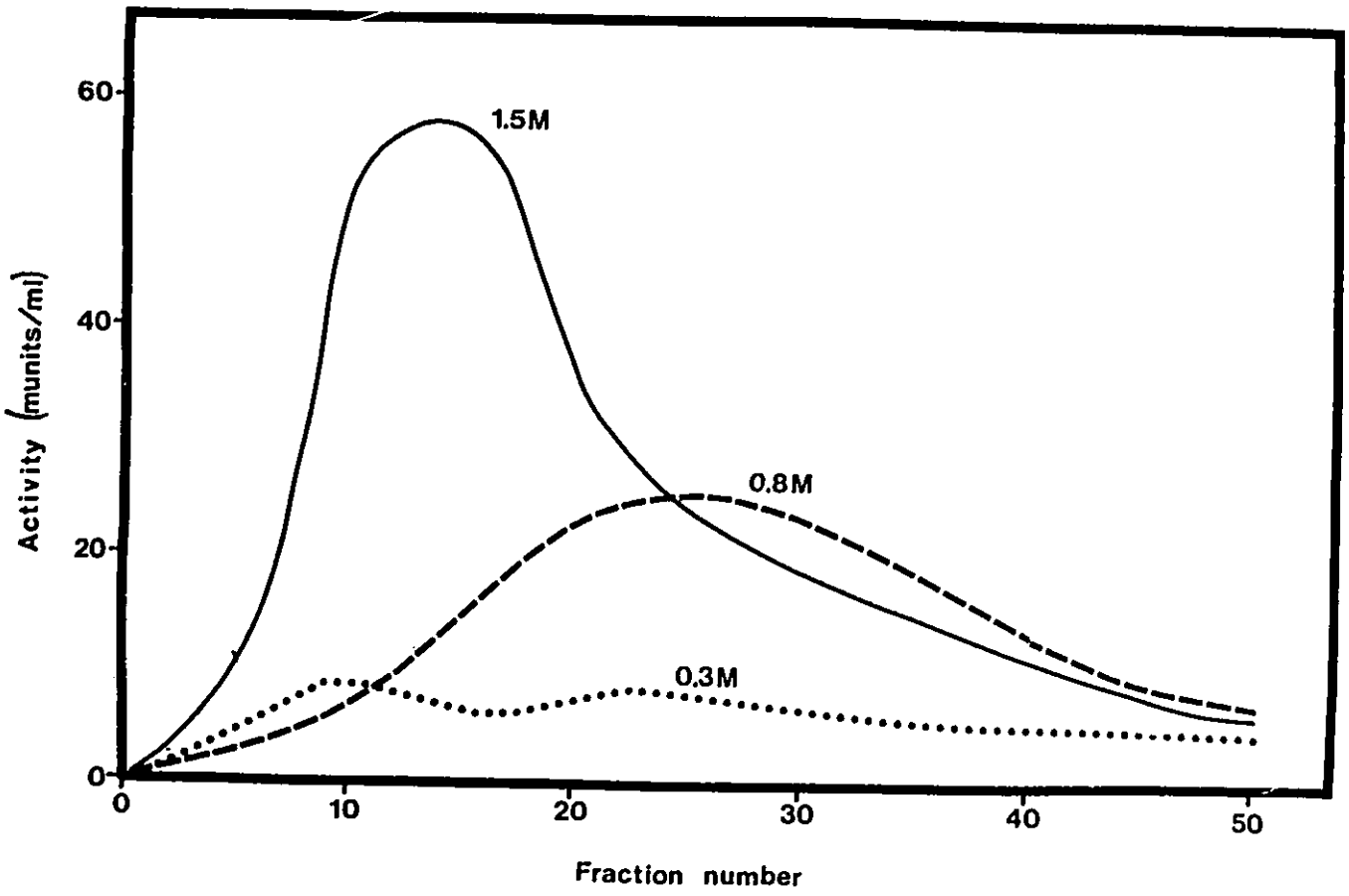
60
40
20
0
Activity (munits/ml)
1.5M
0.8M
0.3M
0
10
20
30
40
50
Fraction number

FIGURE 5

Figure 6

Partial purification of total 17β-HSD by affinity chromatography. Concentrated liver cytosol was applied to a column equilibrated with buffer of 10 mM Tris pH 8.0, 0.5 mM DTT. The column was washed with 200 ml of 10 mM Tris buffer pH 8.0, containing 0.5 mM DTT and 5 mM $MgCl_2$. The low salt buffer was as the wash buffer and contained 0.3 M NaCl. The eluting high salt buffer was as described for the wash buffer except $MgCl_2$ was 10 mM and NaCl was 2.5 M.

Fractions 1-50 were 10 ml each while fractions 51-100 were each 5 ml. Highest activity was detected in fractions 60 through 95 which were pooled as total 17β-HSD.

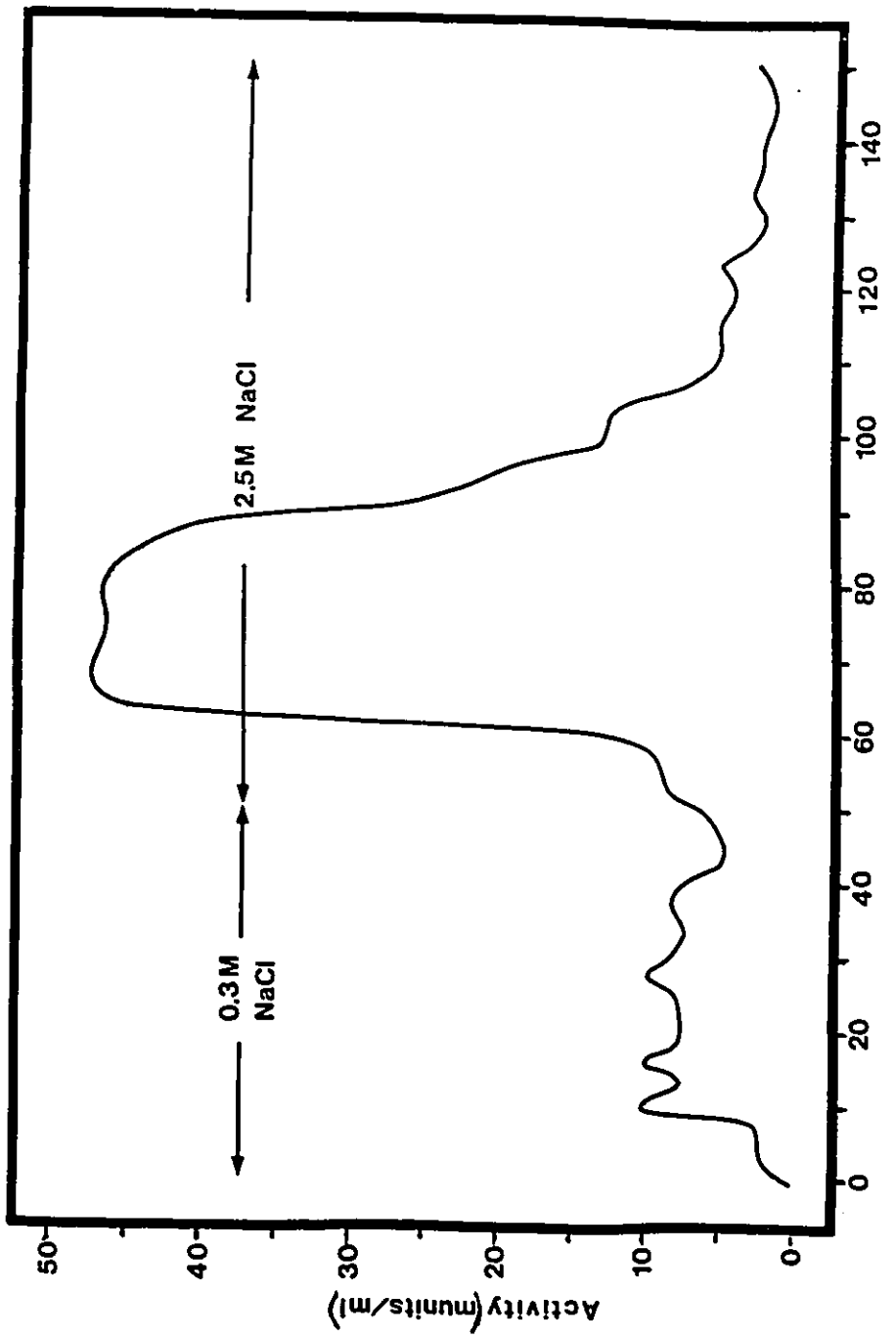
Activity (munits/ml)
0
10
20
30
40
50
0.3M NaCl
2.5M NaCl
0
20
40
60
80
100
120
140
Fraction number

FIGURE 6

Initial attempts to apply the enzyme mixture at this point in the procedure to the chromatofocusing column proved futile. During either the application to the column or at some point in the elution, some protein or proteins in the mixture would precipitate and result in the loss of the sample. This was corrected by removing the proteins which precipitated between pH 6.15 and 5.5 before undertaking the chromatofocusing procedure. The dialyzed enzyme solution was titrated carefully to pH 5.5 with dilute HCl. Further acidification of the solution was avoided so as not to denature the 17β-HSD. Once a pH of 5.5 was attained the mixture was left on ice for 15-20 minutes to allow for complete precipitation. The precipitate was removed by centrifugation and the solution was brought back up to a pH of 6.0 with dilute NaOH. Further precipitate formation occurred at this point and subsequently if the enzyme solution was kept at 4°C longer than one hour. These precititates were removed by centrifugation.

Enzyme activity was measured after dialysis at pH 8.0 and concentration of the enzyme solution to 8.0 ml as well as after titration to pH 6.0 and again to pH 5.5. These assays were performed following removal of the insoluble material, and volumes of the enzyme solution remained at 8.0 ml. Table 2 indicates the activity calculated from assaying 1 μl of the 8.0 ml solutions. Titration of the enzyme mixture to remove the precipitating proteins did not affect the 17β-HSD activity. Once the titration had been performed on the enzyme mixture it could be successfully applied to and eluted from the chromatofocusing column. After applying the solution to the column equilibrated with 25 mM histidine pH 6.15, 0.5 mM DTT it was eluted with polybuffer 74 pH 5.15, 0.5 mM DTT. The elution pattern of the column was followed by absorbance at 280 nm. Fig. 7 indicates the separation of two enzymes termed 17β-HSD-I and 17β-HSD-III on the basis of the enzyme forms identified by Antoun et al. (1985a). The off-scale absorbance observed in the first 20 fractions is attributed to (a) coloured protein(s).

Th. activity of the corresponding fractions is also indicated in Fig. 7. No enzyme activity

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The activity of the corresponding fractions is also indicated in Fig. 7. No enzyme activity

Table 2

Enzyme preparation	pH 8.0	pH 6.0	pH 5.5
Activity (munits/ml)	81.8	81.7	80.1

Titration of total 17β-HSD enzyme mixture with removal of the precipitating proteins. Enzyme activity was measured for 1 μl of each enzyme preparation whose total volume remained at 8.0 ml and was carried out as described in the text.

Figure 7

Separation of forms I and III of 17β-HSD by chromatofocusing chromatography. Equilibrating buffer was 25 mM histidine pH 6.15, 0.5 mM DTT. Eluting buffer was polybuffer 74 (dilution 1/10) containing 0.5 mM DTT. All fractions were 0.4 ml. 0.5 µl of every third fraction was assayed as described in the text to determine enzyme activity.

— Abs_{280} , ···· 17β-HSD activity

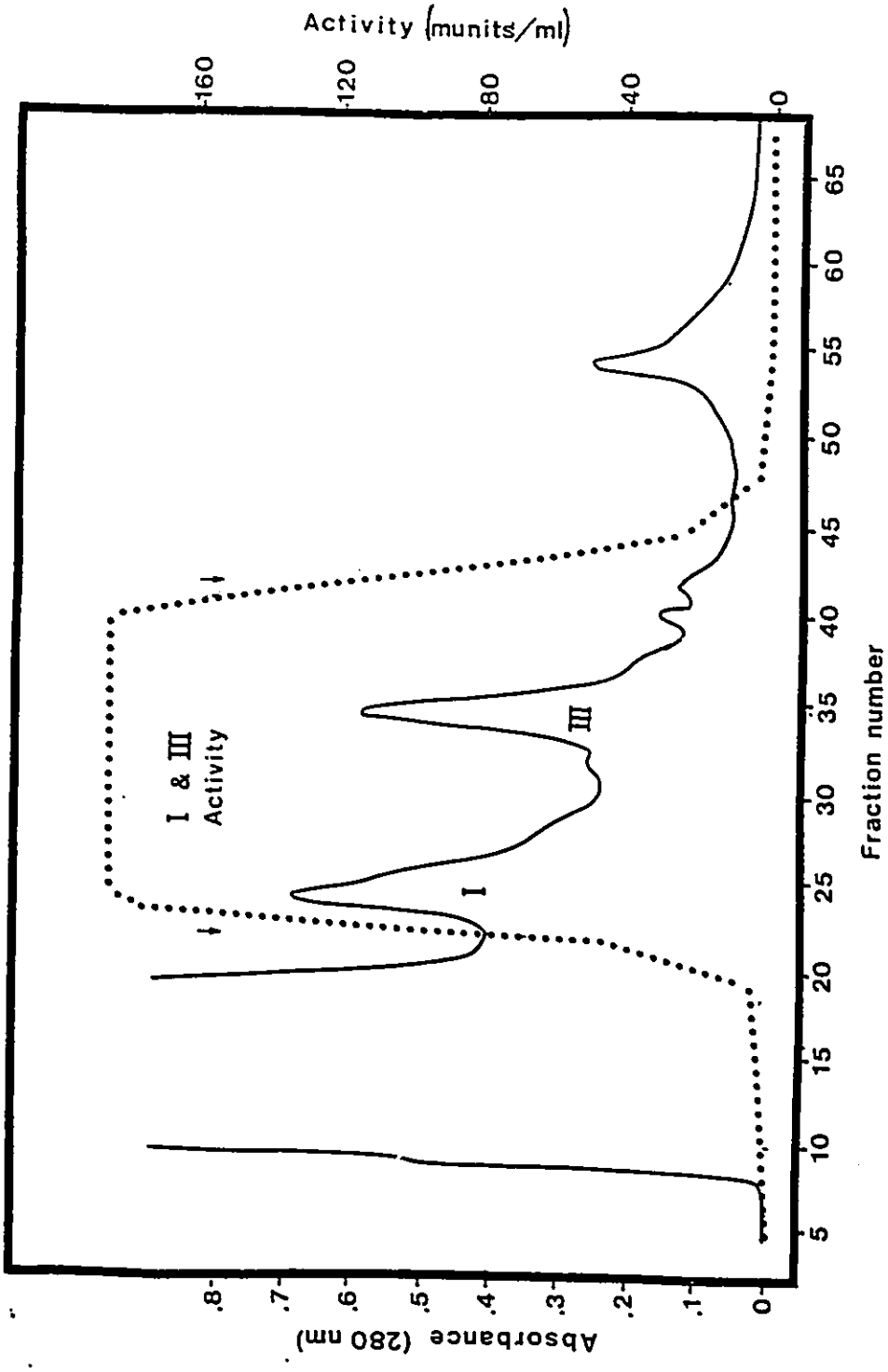
Activity (munits/ml)
160
120
80
40
0
Absorbance (280 nm)
.8
.7
.6
.5
.4
.3
.2
.1
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Fraction number
I & III
Activity
I
III

FIGURE 7

was detected in the initial high absorbancy peak. The activity monitored in these fractions is not a sufficient method for detection of the different forms of the enzyme. This is due to the sensitivity of the assay. Elution of the enzyme from the MONO P column is in a small volume, the fractions being only 0.4 ml each. Therefore the enzyme is no longer as dilute as it had been at previous steps. Despite assaying only 0.5 μl of the fractions the assay is sensitive enough that even a very low level of the enzyme in the fraction will convert essentially all the substrate used in the assay to product. It was also noted by following the enzyme both by absorbance and by activity that form I shows less activity than does form III when comparing their respective absorbances. In addition, the activity of form I was found to decrease, ie. the enzyme denatured, much faster than form III.

The precipitating proteins mentioned earlier in the titration of the enzyme solution can be demonstrated in the difference observed between Figs. 7 and 8 which represent consecutive chromatofocusings of aliquots of the same enzyme preparations. The absorbance peaks detected at fractions 40 and 54 of Fig. 7 are no longer observed in Fig. 8. The proteins corresponding to these peaks had intermittently precipitated before the sample in Fig. 8 had been subjected to chromatofocusing and were removed by centrifugation. The elution of the enzyme sample in Fig. 8 is shifted slightly from the elution of the sample in Fig. 7 due to application of the sample at a higher pH in Fig. 8 compared with Fig. 7.

Fig. 8 also shows the pH gradient established during chromatofocusing. From this the pI's of the isoenzymes I and III were determined to be 5.7 and 5.4 respectively. Appropriate fractions were pooled from the chromatofocusing chromatography based on the absorbance (280 nm) peaks within those column fractions having 17β-HSD activity. The pooled fractions were analyzed for purity by IEF. These fractions required additional focusing before homogeneity could be achieved. Figs. 9 and 10 illustrate the absorbance$_{280}$ profiles of the second chromatofocusing of 17β-HSD-I

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Figure 8

Chromatofocusing chromatography pII gradient with separation of forms I and III of 17β-HSD. Chromatofocusing chromatography profile, conditions as described in Fig. 7, indicating the pH gradient along the elution pattern of the column.

— Abs_{280} , ···· pH

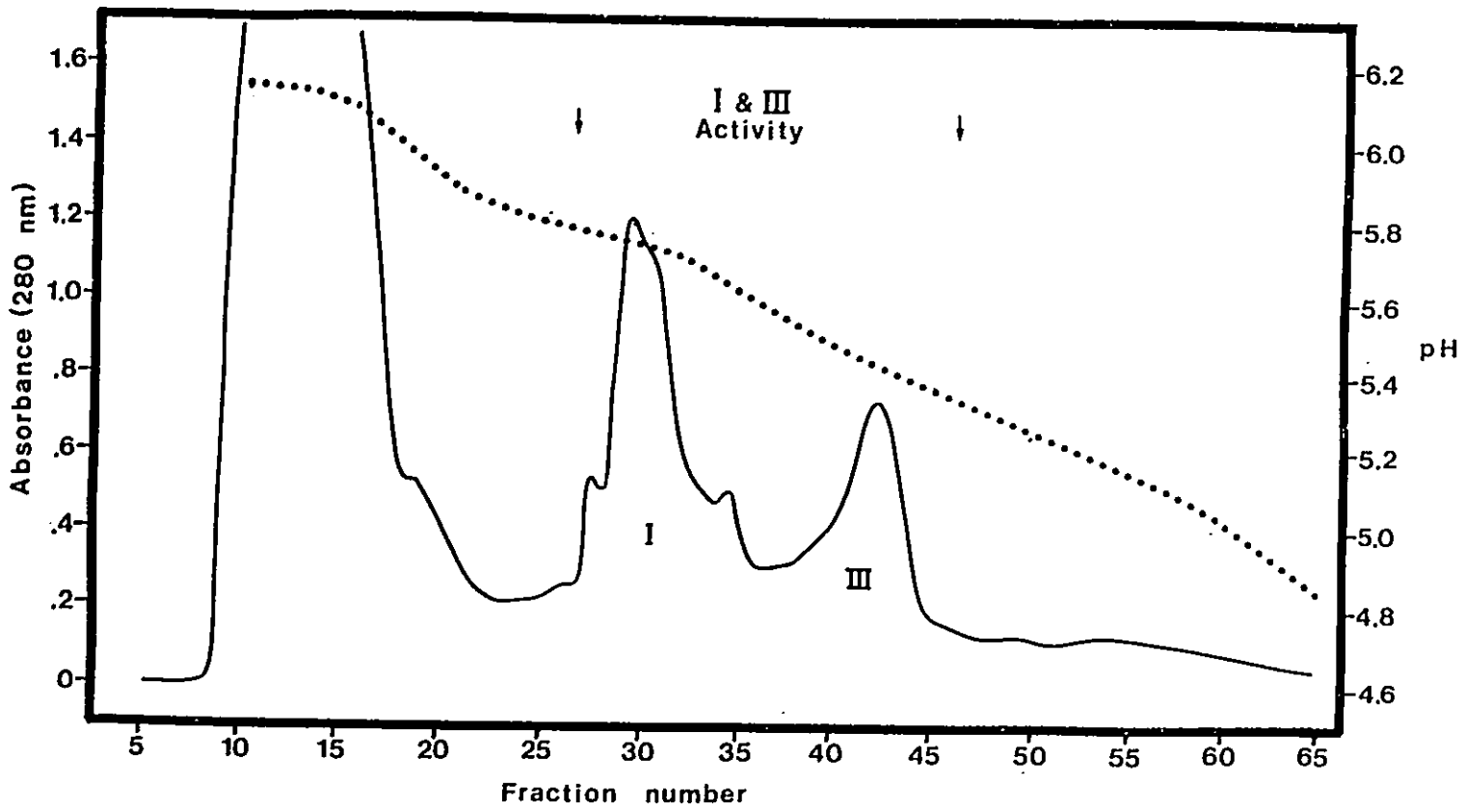
I & III
Activity
I
III
Absorbance (280 nm)
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
pH
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
5
10
15
20
25
30
35
40
45
50
55
60
65
Fraction number

FIGURE 8

Figure 9

Purification of enzyme I by chromatofocusing chromatography. Conditions were as described in Fig. 7. 0.5 μl of every fraction between 42 and 55 was assayed for activity as well as 0.5 μl from every third fraction from the remainder of the fractions.

Those fractions that contained high 17β-HSD activity and which corresponded only to isoenzyme I (46-49) are indicated by arrows and were pooled and analyzed by IEF gels.

— Abs_{280} , ···· 17β-HSD activity

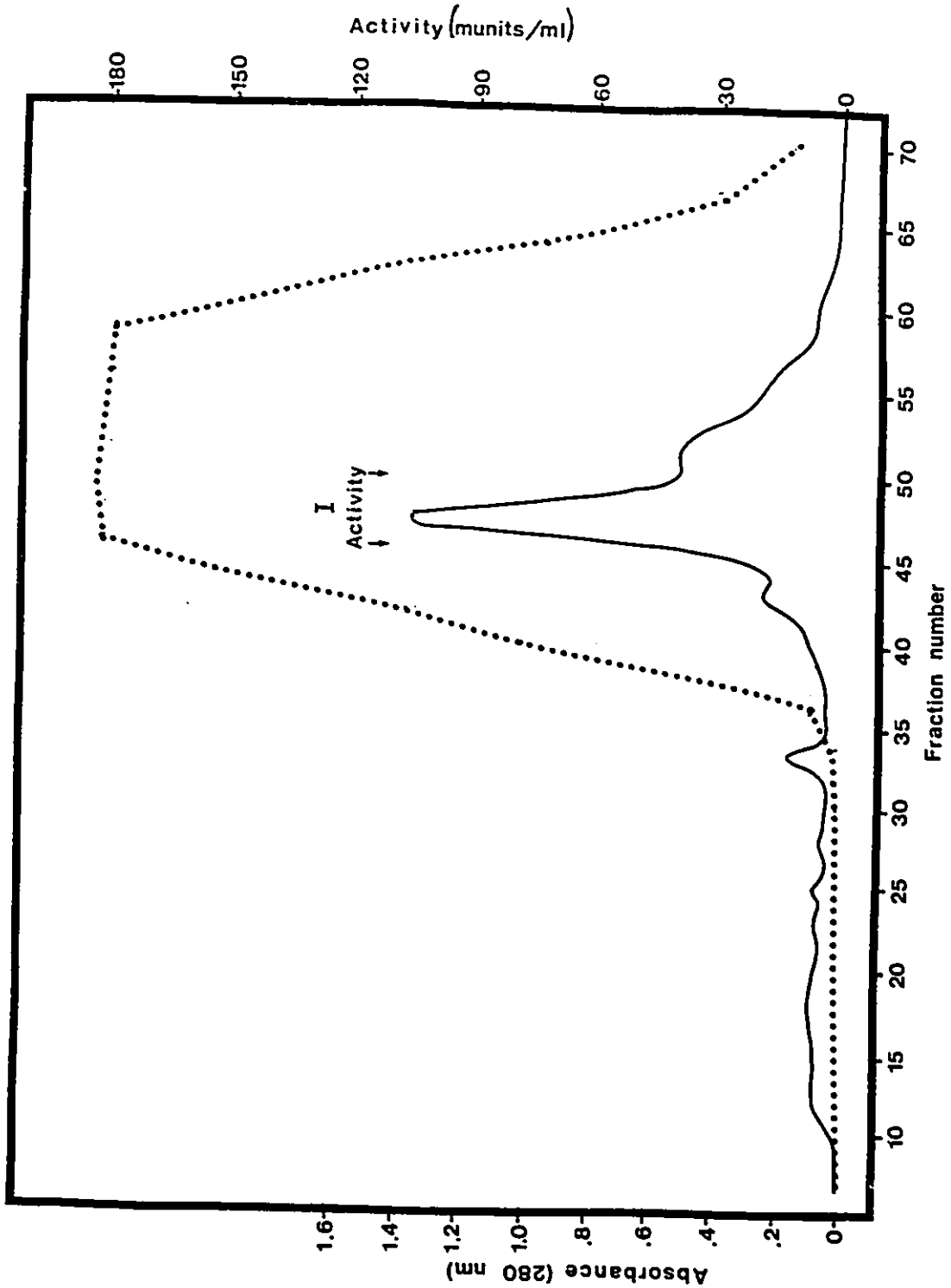
Activity (munits/ml)
180
150
120
90
60
30
0
I
Activity
Absorbance (280 nm)
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
10
15
20
25
30
35
40
45
50
55
60
65
70
Fraction number

FIGURE 9

Figure 10

Purification of enzyme III by chromatofocusing chromatography. Conditions were as described in Fig. 7. 0.5 μl of every fraction between 53 and 65 was assayed for activity as well as 0.5 μl from every third fraction from the remainder of the fractions.

Those fractions that contained high 17β-HSD activity and which corresponded only to isoenzyme III (57-65) are indicated by arrows and were pooled and analyzed by IEF gels.

— Abs_{280} , ···· 17β-HSD activity

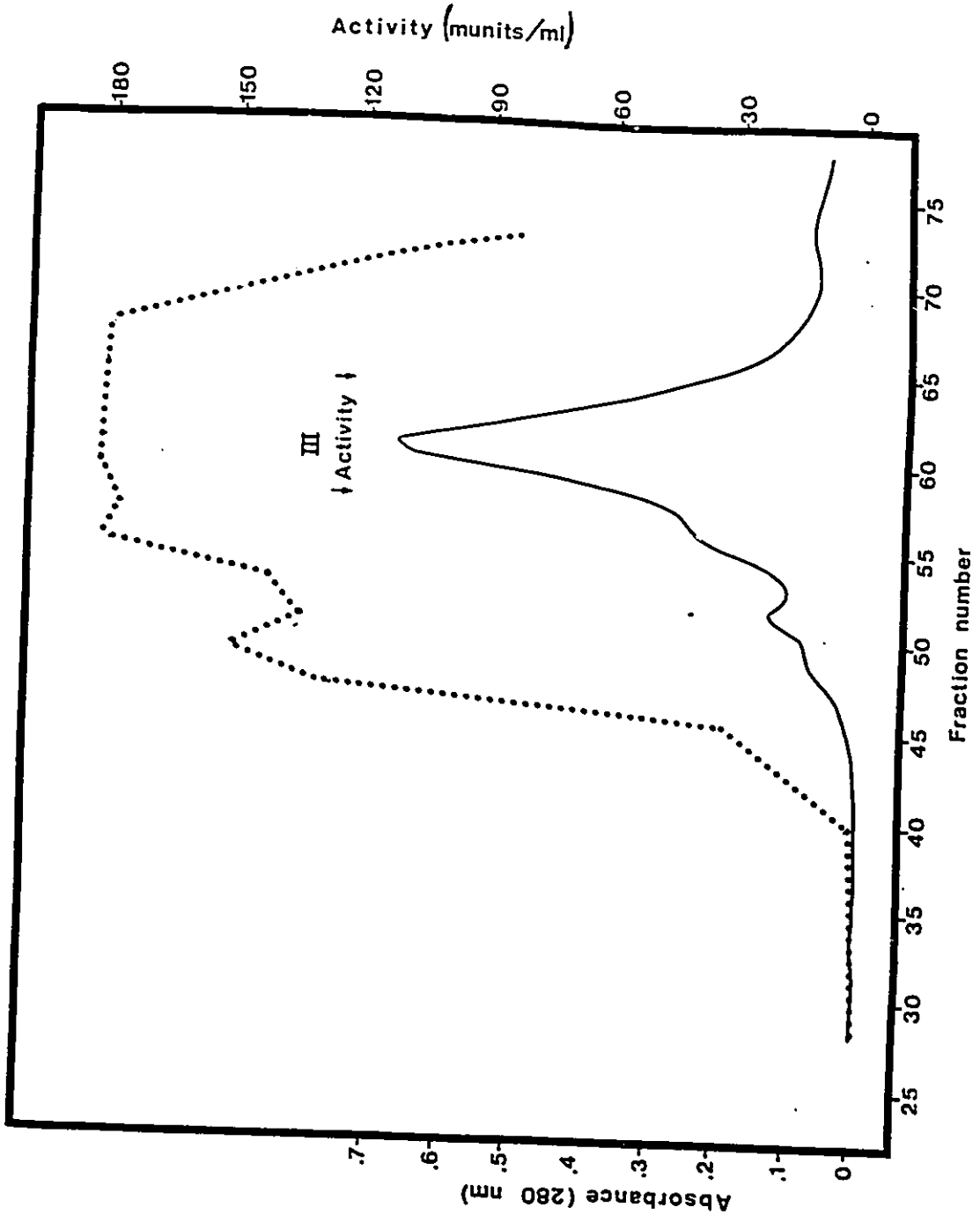
Activity (munits/ml)
180
150
120
90
60
30
0
III
Activity
Absorbance (280 nm)
.7
.6
.5
.4
.3
.2
.1
0
25
30
35
40
45
50
55
60
65
70
75
Fraction number

FIGURE 10

and 17β-HSD-III respectively. Those fractions having enzyme activity were pooled according to the absorbance patterns measured.

When comparing the initial resolution of the enzyme mixture (Figs. 7 and 8) with the profiles obtained upon further purification (Figs. 9 and 10) both enzyme forms are eluted in later fractions during the second chromatofocusing than in the first elution. This phenomenon can be explained by the pH of the solution being applied to the column. The enzyme mixture solution had been titrated to a pH of 6.0 just prior to being applied to the column. The partially purified solutions of forms I and III were applied to the column while still at pH 8.0. The temporary increase in pH on the column increased the volume of eluting buffer required to decrease the pH to 5.15. This was not found to significantly alter the resolution and this eliminated one step which would leave the enzyme susceptible to denaturation.

Fig. 11 shows the IEF gels of the final purified enzyme solutions. As reflected by the absorbance patterns of the focusing columns, the less acidic 17β-HSD-I focusses at a higher pH than does 17β-HSD-III. Once the purified forms of isoenzymes I and III were obtained in sufficient quantities they were prepared for peptide sequencing.

17β-HSD-II identified by Antoun et al. (1985a) in a previous study has a pI slightly more acidic than 17β-HSD-I. This enzyme form appears as a shoulder on the 17β-HSD-I peak (Figs. 7, 8, and 9). Further purification of this enzyme form was not attempted due to its low abundance.

DIGESTION OF 17β-HSD AND SEQUENCING OF THE PEPTIDES

The isoenzymes I and III purified to homogeneity were in a solution composed of 50% polybuffer 74 (v/v), 25 mM Tris pH 8.0, 0.5 mM DTT, and 10% glycerol (v/v). The enzymes were separated from these components by precipitation with ammonium sulphate and the precipitate was

Figure 11

Isoelectricfocusing polyacrylamide gels of 17β-HSD-I and 17β-HSD-III. 15 μg of each enzyme form was applied to a 10% IEF gel previously run to establish a gradient of pH 5.0-7.0 as described in Methods. The focusing was carried out initially for 30 minutes at 400 V followed by 3 hours at 500 V.

The less acidic isoenzyme I is shown on the left and isoenzyme III is shown on the right.

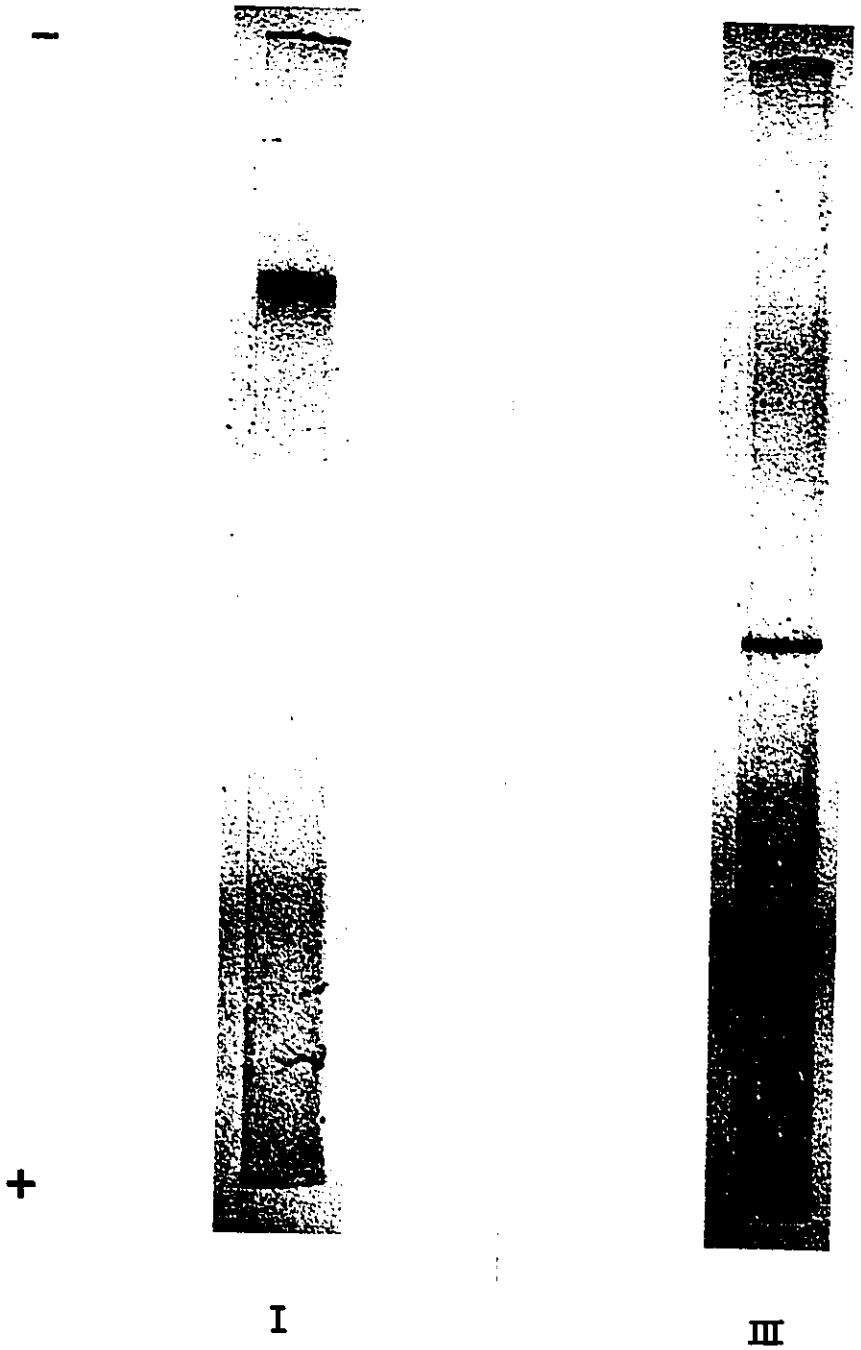

FIGURE 11

redissolved into 1-2 ml 10 mM Tris pH 8.0. The enzyme solutions were then extensively dialyzed against distilled H_2O to remove all buffer and lyophilized.

Isoenzyme I (24 nmol) and isoenzyme III (30 nmol) were prepared for sequencing as described in Methods. The enzyme used for digestion of the enzymes, endoproteinase Lys-C, cleaves the proteins at the amino side of any lysine residues such that all peptides obtained from the digest possess N-terminal lysine residues with the exception of the N-terminus of the proteins.

Fig. 12 shows the initial resolution of the endoproteinase Lys-C digests of 17β-HSD-I (panel A) and 17β-HSD-III (panel B) by reverse phase HPLC. These profiles reflect only one of the runs for each sample in which different amounts of digest were used. The height of peaks between samples can therefore not be compared, however it is clear that many more peptides are present in the enzyme III digest than in that for enzyme I. As previous amino acid analyses have indicated that the two forms have nearly the same number of lysine residues it seems likely that the digestion of enzyme III did not occur specifically at lysine residues. Each of these peaks was isolated and further purified by reverse phase HPLC with increasing acetonitrile percentage (v/v) with the exception of peak I (enzyme I) which was sufficiently pure after the first chromatography. The purification of these peaks is depicted in Fig. 13. Panels A through F illustrate those peaks which were subsequently sequenced following purification.

Three peaks from the digestion of isoenzyme I were sequenced. These include those fractions labelled as peaks A, I and G from the original resolution in Fig. 12 (panel A) and also represented in panels A and B in Fig. 13. None of these sequences were found to describe the N-terminus of the protein as all terminated in lysine. The N-terminal residue of enzyme I has previously been analyzed to be serine (Kaplan et al., 1983) Below are the sequences of peaks A,

Figure 12

Resolution of the endoproteinase Lys-C digests of 17β-HSD-I and of 17β-HSD-III. Digests were resolved by HPLC on a C18 reverse phase column. The column was equilibrated with dH_2O containing 0.1% TFA and the digests were then resolved with a linear gradient of increasing percentage acetonitrile containing 0.1% TFA.

Resolution of enzyme I peptides are shown in panel A, and those of enzyme III are shown in panel B. All major peaks were collected manually and prepared for further purification where necessary.

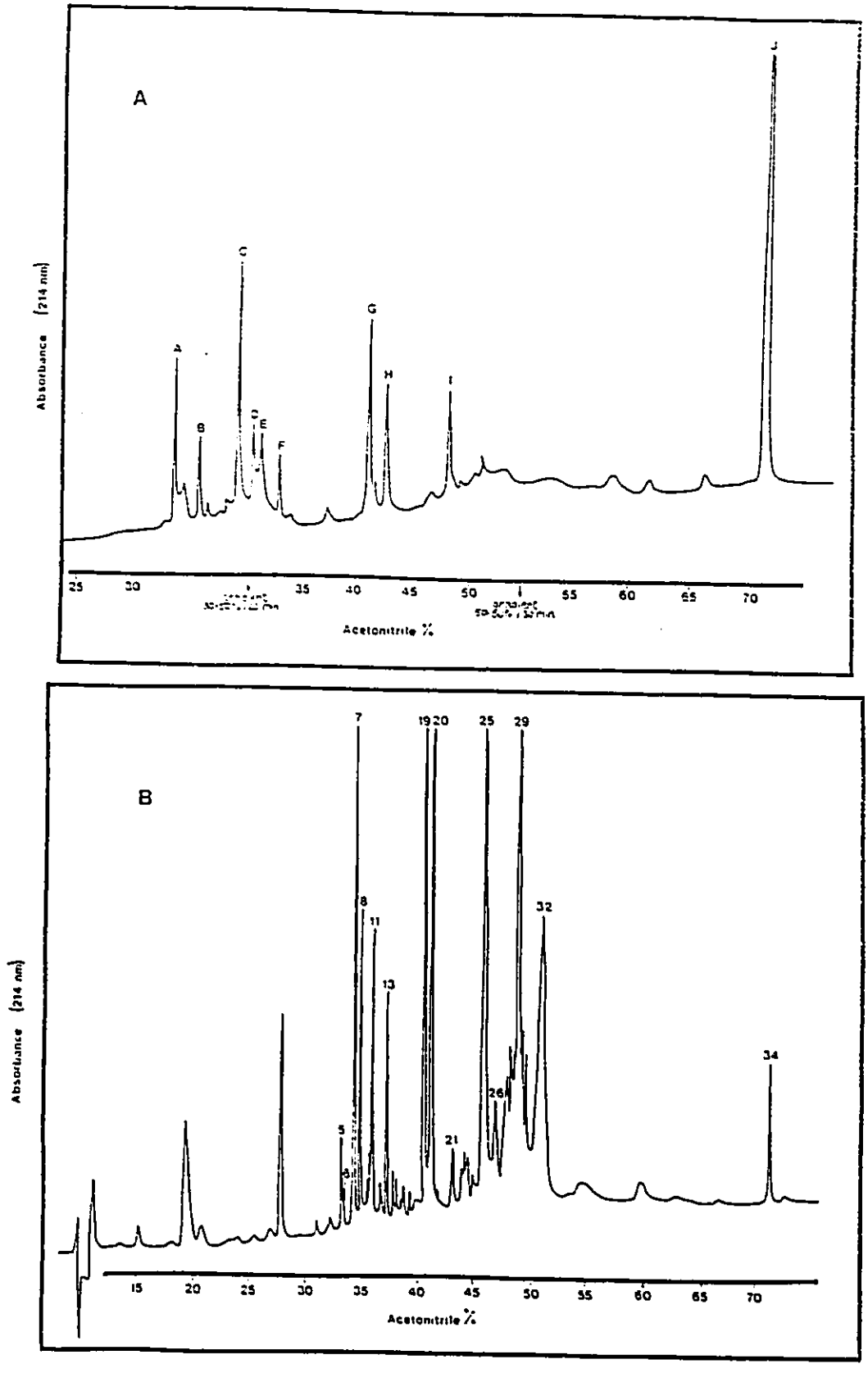
A
Absorbance (214 nm)
A
B
C
D
E
F
G
H
I
J
25
30
35
40
45
50
55
60
65
70
Acetonitrile %
B
Absorbance (214 nm)
7
19
20
25
29
8
11
32
13
34
26
5
21
15
20
25
30
35
40
45
50
55
60
65
70
Acetonitrile %

FIGURE 12

Figure 13

Repurification of endoproteinase digestion peptides of enzyme I and of enzyme III. The samples were repurified by HPLC on a C18 reverse phase column equiibrated with dH^2O containing 0.1% TFA and were resolved with a linear gradient of increasing percentage acetonitrile containing 0.1% TFA. Illustrated are those peaks which were subsequently sequenced. Peak I of enzyme I which was also sequenced did not require repurification.

Panels A and B illustrate two repurified peaks of enzyme I (peaks A and G) as labelled in figure 12. Panels C, D and E indicate the repurified peaks from enzyme III (peaks 5, 6 and 7).

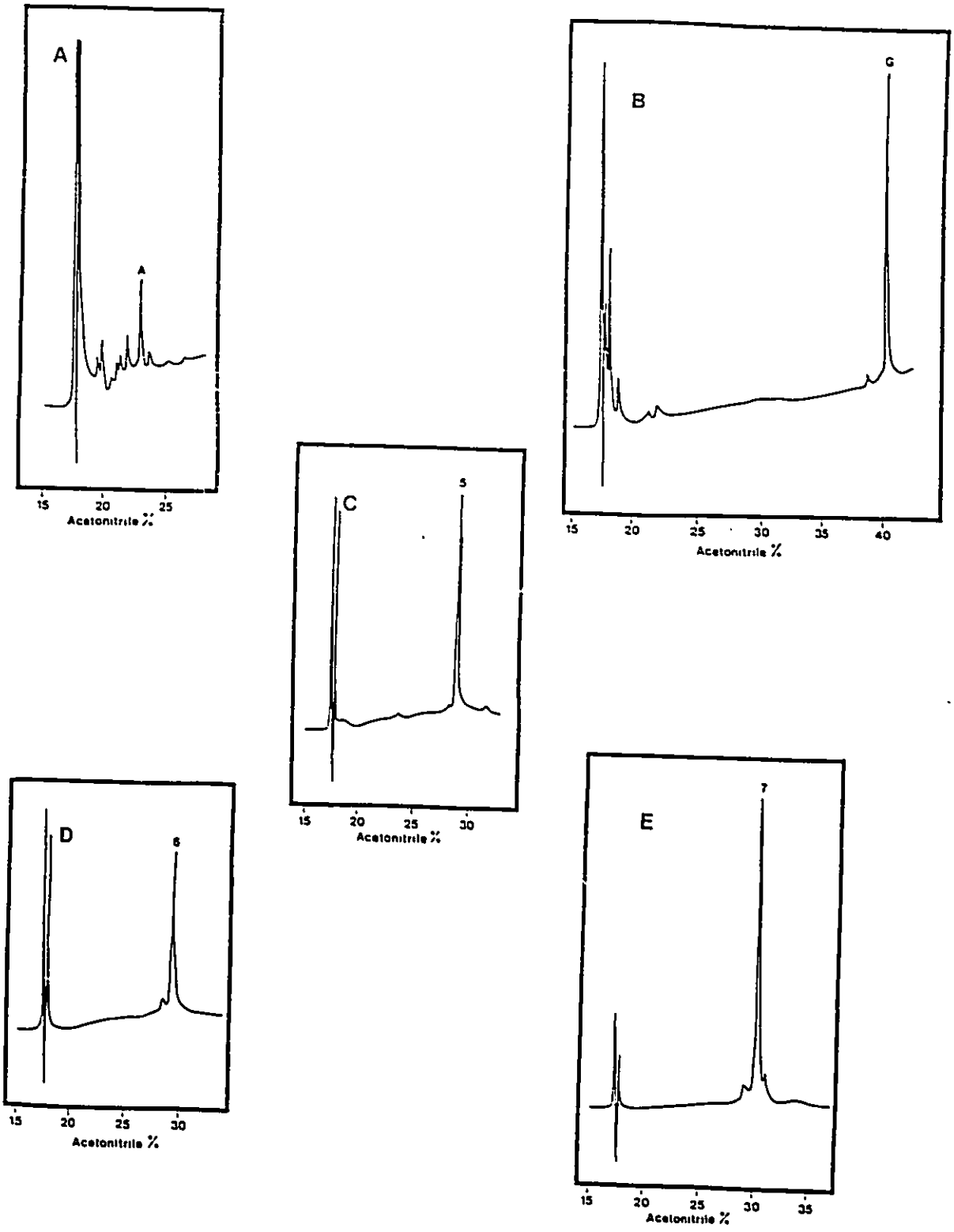
A
A
15
20
25
Acetonitrile %
B
G
15
20
25
30
35
40
Acetonitrile %
C
5
15
20
25
30
Acetonitrile %
D
6
15
20
25
30
Acetonitrile %
E
7
15
20
25
30
35
Acetonitrile %

FIGURE 13

G and I in both the one and three letter amino acid codes.

Peak A:

K - S - Q - N - L - Y - P - H - Q - E - V - Q - N - Q - V - P - G - G/A/L

Lys - Ser - Gln - Asn - Leu - Tyr - Pro - His - Gln - Glu - Val - Gln - Asn - Gln - Val - Pro - Gly - Gly/Ala/Leu

Peak G:

K - P - A - Y - T - F - F - G - L - I - P - I - F - H - G - D - N - L - E - L - Y - C ? - S/T/G

Lys - Pro - Ala - Tyr - Thr - Phe - Phe - Gly - Leu - Ile - Pro - Ile - Phe - His - Gly - Asp - Asn - Leu - Glu - Leu - Tyr - Cys ? - Ser/Thr/Gly

Peak I:

K - L - P - T - P - F - H - I - L - A - L - D - V - A - C - L - Q/M/F - V - S/G/A/L

Lys - Leu - Pro - Thr - Pro - Phe - His - Ile - Leu - Asp - Val - Ala - Cys - Leu - Gln/Met/Phe - Val -Ser/Gly/Ala/Leu

There is some uncertainty in the C-terminal residues of the peptides. These are the initial residues analyzed by the sequenator and the procedure frequently shows some discrepancy at this

position (personal communication, C. Lyons, Queen's University). The peptides analyzed have a high level of the relatively low frequency amino acids proline, histidine, phenylalanine and isoleucine.

Three sequences were also obtained from the digestion of enzyme III. The large number of peptides observed in the digestion of this enzyme is presumably due to incomplete digestion as 17β-HSD-III contains the same number of lysine residues as does 17β-HSD-I. In none of the sequenced peptides however were there any internal lysine residues. Peaks 5, 6 and 7 were analyzed as follows.

Peak 5:

- G - A - N - Y - R - L - N - R - N - L - G - D

- Gly - Ala - Asn - Tyr - Arg - Leu - Asn - Arg - Asn - Leu - Gly - Asp

Peak 6:

- D - S - D - S - D - H - P - H - P - D - F - Y - S - G - A - N/I -A/Y - ? - Y - N - L - N - L - G -D - I - I/T/G

- Asp - Ser - Asp - Ser - His - Asp - Pro - His - Pro - Asp - Phe - Tyr - Ser - Gly - Ala - Asn/Ile - Ala/Tyr - ??? - Tyr - Asn - Leu - Asn - Leu - Gly -Asp - Ile - Ile/Thr/Gly

Peak 7:

- H - N - P - H - G - D - F - Y - S - G - A - N - Y - M - L - N - P - N - L - G - D - I - G

- His - Asn - Pro - His - Gly - Asp - Phe - Tyr - Ser - Gly - Ala - Asn - Tyr - Met

- Leu - Asn - Pro - Asn - Leu - Gly - Asp - Ile - Gly

The peptides described for 17β-HSD-III, peaks 5 - 7, were all incomplete. That is, sequencing was not successful to the N-terminus of the peptide. As these were incomplete it is not possible to determine if any of them represented the N-terminus of the whole protein. Again a high incidence of proline is noted in the sequences. Also abundant are asparagine and histidine. It is unfortunate that these had been the samples chosen for sequencing. Comparison of these three peptides leads to the speculation that they are one in the same.

5; D-G-L-N-R-N-L-R-Y -N -A-G
6; I/T/G-I-D-G-L-N-L-N -Y-?-A/Y-N/I-A-G-S-Y-F-D-P-H-P-D-H
7; G-I-D-G-L-N-P-N-L-M-Y -N -A-G-S-Y-F-D-G-H-P-N-H

The above rearrangement of the sequences of peaks 5, 6 and 7 emphasizes the homogeneity between these sequences. Residues common to all three peptides are bolded while those residues which are identical between peaks 6 and 7 are also underlined. Sequence 5 had in fact not been completely analyzed due to equipment breakdown and may in fact show as much homogeneity as seen between 6 and 7. The homology is consistent with the consecutive elution of these peptides from the C18 column during HPLC (Fig. 12).

SYNTHESIS OF A λGT10 LIBRARY

Extraction of total RNA from 7.5 g of female adult rabbit liver was carried out as described in Methods. The quantity and purity of this RNA was analyzed by $OD_{(260/280)}$ as well as by glyoxal gel electrophoresis.

- His - Asn - Pro - His - Gly - Asp - Phe - Tyr - Ser - Gly - Ala - Asn - Tyr - Met

- Leu - Asn - Pro - Asn - Leu - Gly - Asp - Ile - Gly

The peptides described for 17β-HSD-III, peaks 5 - 7, were all incomplete. That is, sequencing was not successful to the N-terminus of the peptide. As these were incomplete it is not possible to determine if any of them represented the N-terminus of the whole protein. Again a high incidence of proline is noted in the sequences. Also abundant are asparagine and histidine. It is unfortunate that these had been the samples chosen for sequencing. Comparison of these three peptides leads to the speculation that they are one and the same.

5; D-G-L-N-R-N-L-R-Y -N -A-G
6; I/T/G-I-D-G-L-N-L-N -Y-?-A/Y-N/I-A-G-S-Y-F-D-P-H-P-D-H
7; G-I-D-G-L-N-P-N-L-M-Y -N -A-G-S-Y-F-D-G-H-P-N-H

The above rearrangement of the sequences of peaks 5, 6 and 7 emphasizes the homogeneity between these sequences. Residues common to all three peptides are bolded while those residues which are identical between peaks 6 and 7 are also underlined. Sequence 5 had in fact not been completely analyzed due to equipment breakdown and may in fact show as much homogeneity as seen between 6 and 7. The homology is consistent with the consecutive elution of these peptides from the C18 column during HPLC (Fig. 12).

SYNTHESIS OF A λGT10 LIBRARY

Extraction of total RNA from 7.5 g of female adult rabbit liver was carried out as described in Methods. The quantity and purity of this RNA was analyzed by $OD_{(260/280)}$ as well as by glyoxal gel electrophoresis.

A sample (1.0 μl) of the total RNA was mixed in 1.0 ml of sterile water and its absorbance was measured at 260 nm and 280 nm.

$$Abs_{280} = 0.026$$

$$Abs_{260} = 0.046$$

$$\text{ratio}(260/280) = 1.770$$

$$\text{concentration}(\mu g/\mu l) = 1.840$$

$$\text{total RNA}(\mu g) = 17.296 \text{ mg}$$

Lane 1 of Fig. 14 reveals that the RNA obtained had not degraded at all as the 18s and 28s bands of the most highly abundant ribosomal RNA are clearly visible.

Subsequent passage of 8.0 mg of the total RNA preparation through an oligo(dT) column resulted in poly A$^-$ and poly A$^+$ samples. Again the amount and purity of the solutions were measured as above. Fig. 14 is a glyoxal gel on which has been run total RNA (1.84 μg), poly A$^-$ RNA (1.56 μg), and poly A$^+$ RNA (1.36 μg). The faint identity of bands in the poly A$^+$ lane indicates the successful removal of almost all ribosomal RNA from the total RNA. Bands in the poly A$^-$ lane are the same as those in the total RNA lane since the poly A$^+$ fraction comprises only a small percentage of the total RNA(~10%). The concentrations of the different samples were determined by absorbance measurements.

	Abs_{260}	$\mu g/\mu l$	total(mg)
poly A$^-$	0.039	1.560	6.710
poly A$^+$	0.034	1.360	0.136

Figure 14

Separation of poly A$^+$ and poly A$^-$ from total RNA. Samples of total RNA (lane 1, 1.84 μg), poly A$^-$ RNA (lane 2, 1.56 μg), and poly A$^+$ RNA (lane 3, 1.36 μg), were electrophoresed on a 1.0% glyoxal agarose gel and stained with ethidium bromide. Arrows indicate the bands of 18s and 28s ribosomal RNA.

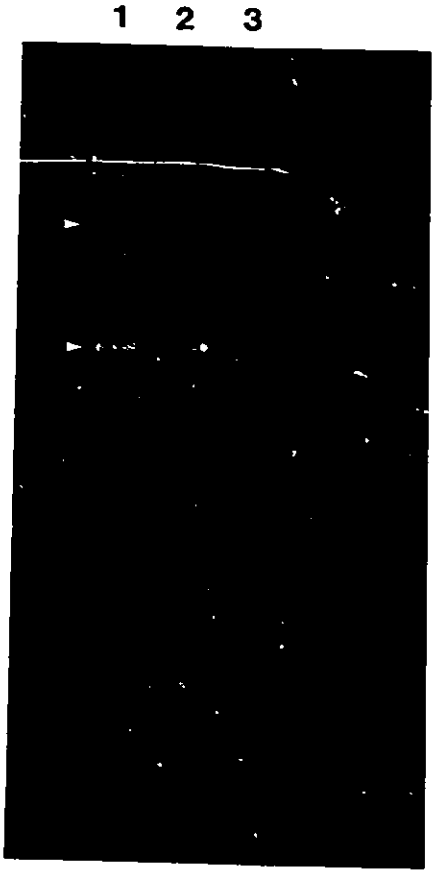

FIGURE 14

The poly A$^+$ RNA obtained was used for the synthesis of cDNA and ultimately for the synthesis of the λgt10 library. Synthesis of cDNA was carried using oligo(dT) primers in one case (sample dT) and random oligonucleotide primers as well as oligo(dT) primers in a second (sample RP). In both cases 10 μg of poly A$^+$ was used in the preparation of the cDNA which resulted in the production of 1.6 μg (dT) and 2.6 μg (RP) of cDNA.

The size fractionation on sepharose 4B columns was carried out for both cDNA preparations. As described in Methods the cDNA was eluted from the column and collected in 25 individual drops. Quantitation of the radioactivity in each of the fractions for both columns revealed, as shown in Fig. 15, that no cDNA eluted from the columns before the 13th (dT) or the 10th (RP) fractions. Fractions were pooled as indicated in table 3. Those column fractions following the radioactive peak were discarded as these contain the shorter segments of cDNA. A sample (1.0 μl) of each of the these fractions was removed and electrophoresed on a Tris-Acetate gel to determine the approximate sizes of the cDNA samples. The average lengths of the pooled cDNA fractions are indicated in Fig. 15. All the cDNA synthesized is between 0.4 and 2.0 kb.

Both cDNA preparations were precipitated and redissolved in 13 μl of sterile distilled H_2O. Synthesis of the cDNA library proceeded as described earlier with the methylation of the cDNA and addition of linkers to their ends. The samples were digested by EcoR I to produce cohesive ends and excess ends were then separated from the linkered cDNA on the columns supplied in the λgt10 library kit. Those fractions from 3 - 6 in both cases were pooled and precipitated. A total of 200 ng of dT cDNA and 160 n[illegible] of RP cDNA were redissolved in 2.0 μl and 1.6 μl STE buffer respectively for a concentration of 0.1 μg/μl each.

The remaining steps involved in the synthesis of the library were carried out as described in Methods. No further monitoring of the progress of the procedure could be performed until the

Figure 15

Size fractionation of cDNA on Sepharose 4B columns. The size separation of cDNA prepared with oligo(dT) primers (panel A) and of cDNA prepared with both the oligo(dT) and the random primers (panel B). Samples were eluted from the columns with TE buffer and collected in 25-1 drop fractions. Radioactivity eluting at the end of the collection comprised the smaller cDNA segments which were discarded. Fractions for pooling were as described in the text. The average lengths of the cDNA segments as determined by non-denaturing Elfo-tris agarose electrophoresis as described in Methods are indicated (arrows) with lengths in kilobases.

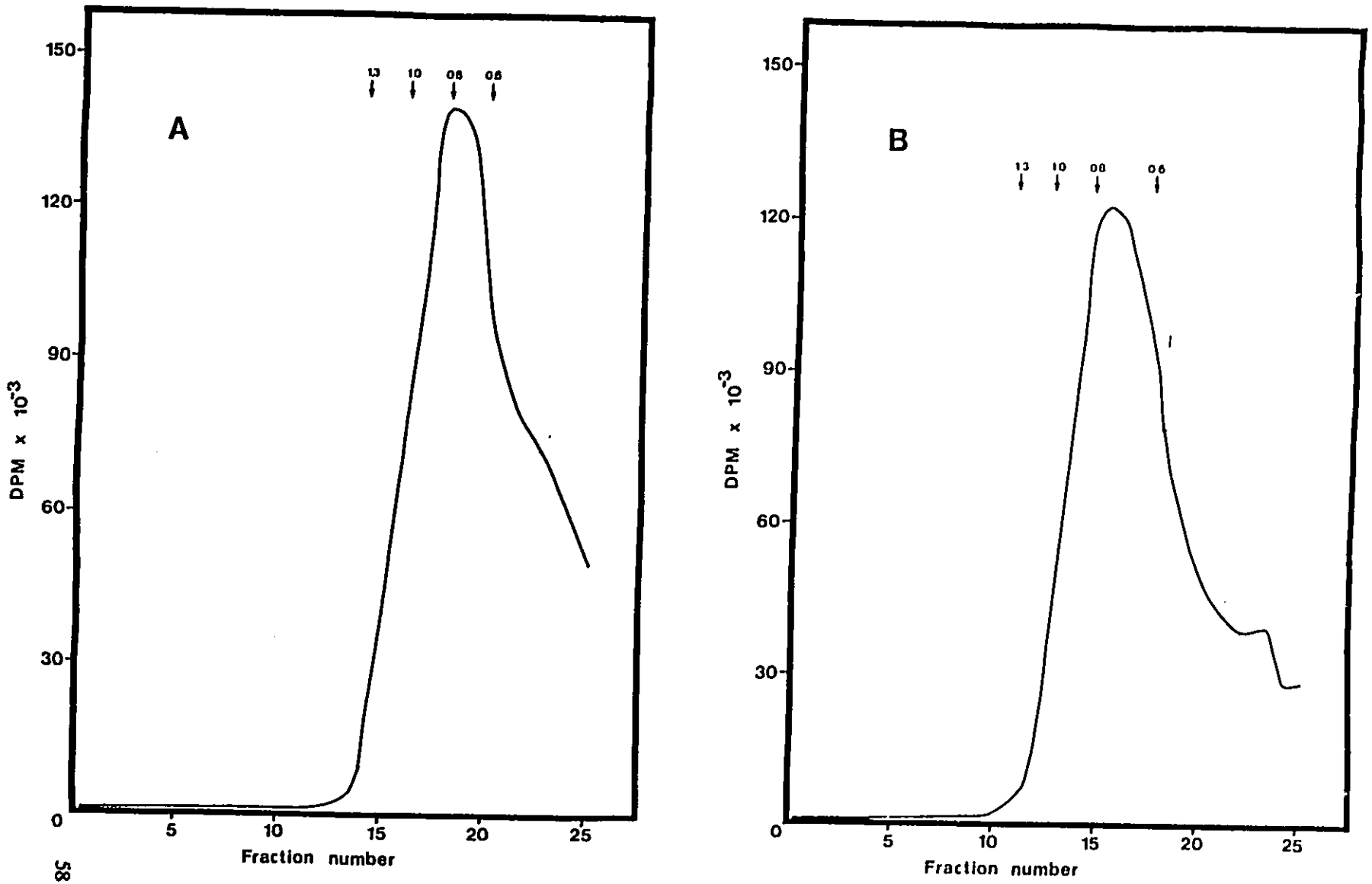
A
B
13
10
08
06
150
120
90
60
30
0
DPM x 10^{-3}
5
10
15
20
25
Fraction number

FIGURE 15

Table 3

	Sample		
	dT	RP	Fraction
Drops	13, 14	10, 11	I
	15, 16	12, 13	II
	17, 18	14, 15	III
	19, 20	16, 17,18	IV

Size fractionation of cDNA by sepharose 4B chromatography. That cDNA prepared using only oligo(dT) nucleotide primers is labelled dT and the sample prepared with both the oligo(dT) nucleotides and the random primers is labelled RP. Fraction numbers correspond to eluant drop number and appropriate fraction drops were pooled as determined by the DPM counts and these pooled fractions were identified as I, II, III or IV.

final infection of the E. coli cells with the recombinant λgt10 phage. Infection of competent L87 and NM514 cells with different dilutions of phage led to the calculation of the percentage of recombinants as 99% and 97% for dT and RP respectively. The cloning efficiency was in the order of 10^7 plaque forming units per ml (pfu/ml) for both which is optimal for probing. Replica filters were prepared of the plated libraries at 10^3 and 10^4 dilutions which were probed with total cDNA freshly prepared from poly A$^+$ RNA. Fig. 16 shows the hybridization of the radioactively labelled total cDNA to the λgt10 DNA verifying the insertion of cDNA into the phage DNA.

SCREENING OF THE λgt10 LIBRARY WITH THE NUCLEIC SEQUENCE OF PEAK A

Nucleic probes (both the sense and anti-sense) corresponding to a portion of the sequence of peak A obtained by the enzyme digest of 17β-HSD-I (Fig. 12, page 50) were synthesized. These probes allowed for redundancy of the triplet codes and included all possible combinations. The following sequences were synthesized:

```
   5'                                    3'
#1 :      TGQTCQTCNACQTGPTTQTG
#2 :      CAPAAQCAPGTNGAPGAPCA
#3 : TANGGPTGQTCQTCNACQTGPTTQTG
#4 :      CAPAAQCAPGTNGAPGAPCAQCCNTA
```

Where ; P = A or G
Q = C or T
N = A,C,G or T

Sequences 3 and 4 represent a portion of the peak A peptide two amino acid residues (six nucleotides) longer than sequences 1 and 2.

The two sense probes, numbers 2 and 4, were labelled by the T_4 polynucleotide kinase reaction as described in Methods. Replica filters were lifted from plates on which had been grown up both the dT and the RP libraries, both at either 10^3 or 10^4 dilutions. Three plates of each type were prepared and grown up overnight at 37°C. After preparation of two filters from each plate

Figure 16

Hybridization of total cDNA to the λgt10 library. Competent NM514 cells were infected with λgt10 library and grown up to lysis at 37°C. Filters were lifted as described in Methods and were probed with total labelled cDNA. Hybridization was carried out at 65°C. Autoradiography of hybridization verifies the insertion of cDNA into the λgt10 phage. Filters lifted from the RP and dT libraries are indicated. Control filters (phage arms only) labelled C show only background levels.

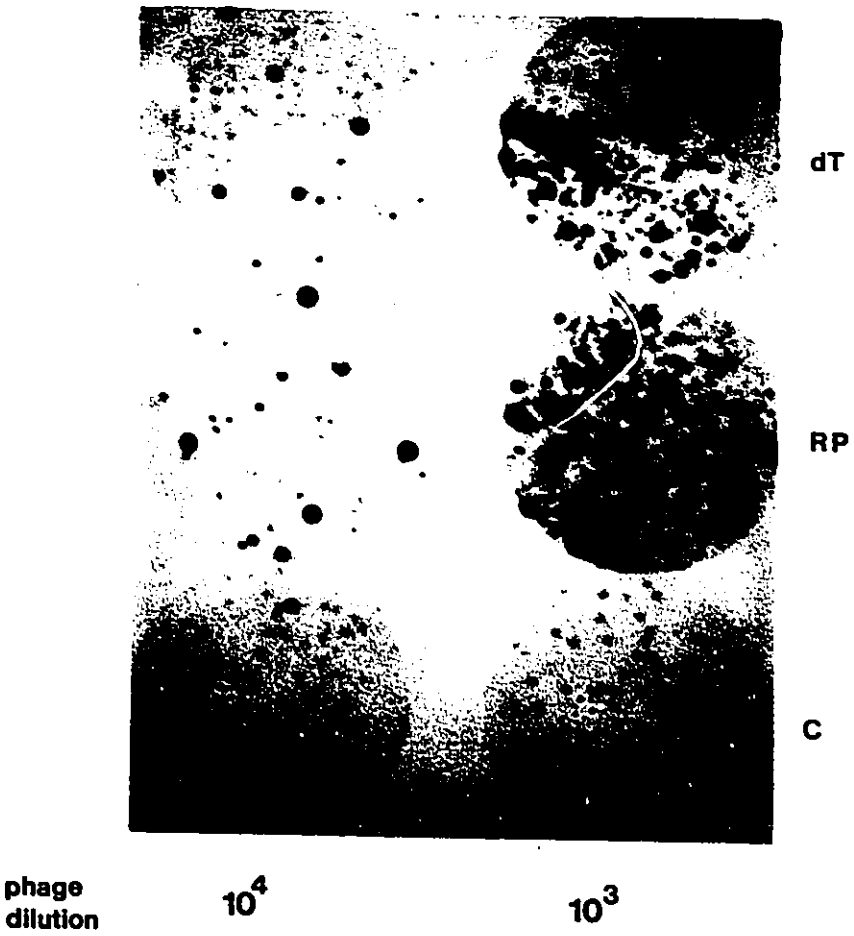

FIGURE 16

(one for the sense probe and a second for the anti-sense probe) one of each was incubated in pre-hybridization buffer at 65°C for four hours. The filters were first probed with the sense sequences. Thirteen colonies were found to be positive. Should any of these be true positives they would also hybridize to the anti-sense probes. The anti-sense probes were therefore prepared in the same way as the sense probes and the second of the filters which had been prepared were probed with these. In all, 24 colonies were found to hybridize to this probe but none of these corresponded to the positive colonies observed in the previous screening. All of the possible positive plaques were cored out and stored as described in Methods.

All of the colonies that had been picked were used to re-infect competent NM514 cells. These were grown on plates, filters were lifted and again probing was carried out with the sense probes. No colonies tested positive to this screening. All initial positives were therefore negative. The failure to obtain true positives from this screening could either be due to the very low abundance of the 17β-HSD proteins or to the high redundancy of the probe which prevented specific hybridization.

PRODUCTION OF POLYCLONAL ANTIBODY AGAINST 17β-HSD

Adult white leghorn roosters were immunized with 20 μg each of enzyme III and re-immunized with the same amount of this antigen after six weeks in order to take the sera following the secondary response. A procedure in which the effect of immune serum on the activity of the 17β-HSD was developed in an attempt to monitor antibody production during the immunization period. Establishment of a reliable anti-enzyme assay which would produce consistent results was difficult as the sera of these animals produced unpredictable effects on the 17β-HSD activity. In designing this assay it was necessary to establish an optimal NaCl concentration, a series of serum dilutions and a volume of 17β-HSD solution which would produce an intermediate level of activity and the proper controls and blanks. The latter refers to the magnitude of conversion measured

of testosterone to androstenedione. An amount of enzyme yielding a percent conversion near 50% for a control assay in which no serum were used would be adequate to successfully be able to distinguish either enhancement or inhibition of the enzyme.

First a series of assays were performed in which the concentration of NaCl was varied. The KCl normally present in the enzyme assays was replaced with NaCl, a component often required for proper antibody function. The KCl is not a requirement for function of the 17β-HSD and its level of activity was not in fact altered if the incubation mixture contained only glycine buffer. In most cases a NaCl concentration of 0.8% has been found to be adequate for antibody activity however up to 8.0% has been used (personal communication, Mr. E. Saley, Animal Disease Research Institute). A series of four incubation mixtures were prepared for each of serum 2 and 3 in which the salt concentration was varied between 0.8% and 8.0% (w/v). All incubations contained 0.7 ml of glycine buffer 0.1 M pH 9.0, 5 μl anti-serum in 45 μl 10 mM Tris pH 8.0 and 0.4 mg NADP+. To each was then added a 0.3 ml solution of NaCl to produce a final concentration of NaCl in the reaction mixture of 8.0, 4.0, 1.0 or 0.8%. To these was added 1.0 μl (0.5 μg) of the same enzyme solution as was used in the immunizations. The mixtures were incubated at 37°C with gentle shaking for 30 minutes before adding 30 μl of the ^{3}H-testosterone solution. The incubation continued for a further 30 minutes at this temperature before stopping the reaction and quantitating the activity as described in Methods. As indicated in Table 4 enhancement of enzyme activity was observed in all samples over the level of the control. The control mixture contains no serum and therefore the increase in enzyme activity in the other samples may be due to the increased protection of the enzyme by the additional proteins in the serum. NaCl affects the enzyme activity only at the highest concentration used (8.0%). NaCl at a concentration of 0.8% was used in all subsequent incubations.

In order to more fully determine the effects of anti-sera on the 17β-HSD a series of assays

Table 4

Salt Concentration (%)	Enzyme Activity (nmol product/μg enzyme) A2	A3
0.8	9.9	10.0
1.0	10.0	12.0
4.0	9.1	10.2
8.0	6.5	8.3

Determination of NaCl concentration in anti-enzyme assays. Activity of 17βHSD pre-incubated for 30 minutes in a 1/10 dilution of anti-serum at 37°C prior to a 30 minute incubation with 30 nmol ^{3}H-testosterone at 37°C was measured as nmol androstenedione produuced in the 30 minute incubation per μg of 17β-HSD-III used. A control assay prepared in 0.8% (w/v) NaCl but with no anti-serum added had an activity measured at 3.4 nmol of androstenedione produced per μg enzyme. A2 and A3 refer to the two week post-immunization anti-sera of roosters 2 and 3 respectively.

Table 4

Salt Concentration (%)	Enzyme Activity (nmol product/μg enzyme) A2	A3
0.8	9.9	10.0
1.0	10.0	12.0
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Determination of NaCl concentration in anti-enzyme assays. Activity of 17βHSD pre-incubated for 30 minutes in a 1/10 dilution of anti-serum at 37°C prior to a 30 minute incubation with 30 nmol ^{3}H-testosterone at 37°C was measured as nmol androstenedione produced in the 30 minute incubation per μg of 17β-HSD-III used. A control assay prepared in 0.8% (w/v) NaCl but with no anti-serum added had an activity measured at 3.4 nmol of androstenedione produced per μg enzyme. A2 and A3 refer to the two week post-immunization anti-sera of roosters 2 and 3 respectively.

was carried out in which the serum dilution ranged from 0 to 10^{-3}. In addition 3.0 μl of the enzyme solution (1.5 μg) was used as this seemed to give an intermediate level of activity. Assays of control (pre-immune) sera were always carried out at the same time as assays of the anti-sera due to the fluctation observed in the activity of the enzyme. It was found that the pre-immune sera of all the animals produced virtually identical results therefore only the pre-immune serum of animal #2 was used as the control in subsequent assays. Table 5 summarizes the results obtained from the week #4 serum of all three animals. The level of activity measured in all anti-sera is lower than that measured for the control serum. All enzyme activities measured for C2 (table 5) are close to the control reaction value (9.4 nmol product formed per μg enzyme) but are both above and below this level. All activities for the anti-sera are below the control value. This is particularly notable at the lowest dilution of serum. This dilution would offer the least protection of the enzyme as it adds the smallest amount of additional proteins to the incubation mixture. These data interpreted to indicate some inhibition of the enzyme activity presumably attributed to the production of antibody. Measurement of anti-enzyme activity was repeated at six weeks post-immunization. Table 6 summarizes these results. The lack of significant inhibition of 17β-HSD activity was interpreted to indicate the end to this primary response. At this time animals #2 and #3 were re-immunized with the same amount of 17β-HSD to which they were initially exposed. The third rooster was sacrificed at this time due to illness.

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Table 5

Serum dilution (%)	Enzyme Activity (nmol product formed/μg enzyme) C2	A1	A2	A3
neat	9.8	8.2	7.0	7.4
1/10	9.2	8.8	7.3	6.9
1/50	10.3	8.3	6.8	7.1
1/100	8.4	7.7	6.8	6.7
1/500	7.7	6.5	6.2	6.2
1/1000	8.4	6.0	5.1	6.4

Primary response of antibody production. The four week post-immunization anti-sera of animals #1, #2 and #3 designated as A1, A2 and A3. The control (pre-immune) serum of animal #2 is designated as C2. Enzyme activity was measured as described in the text and is expressed in nmol of androstenedione produced in the 30 minute incubation per μg of enzyme used. A control assay containing no serum yielded a value of 9.4 nmol androstenedione per μg enzyme.

Table 6

Serum dilution (%)	Enzyme Activity (nmol product formed/μg enzyme) C2	A1	A2	A3
neat	14.7	14.9	16.8	14.3
1/10	16.7	15.4	15.8	14.5
1/50	16.6	15.5	15.7	16.0
1/100	16.9	17.2	15.6	15.8
1/500	15.5	15.3	16.4	15.4
1/1000	12.3	15.0	17.0	12.1

Decline of antibody production in the primary response. 17β-HSD activity was measured as nmol of androstenedione produced in the incubation per μg enzyme and is compared to the control value of 12.9 nmol androstenedione per μg enzyme measured for an assay prepared with no serum. The designations A1, A2, A3 and C2 are as in Table 5.

The time course of the secondary response differs from the primary response in that no lag period preceeds the burst of antibody production. Therefore the time between re-immunization and collection of serum was predicted to require no longer than 2 weeks. After this length of time had passed and the assay results did not indicate any inhibition of the 17β-HSD activity the roosters were sacrificed as described in Methods and serum was collected and stored at -20°C. Western analysis of the sera was carried out to establish whether antibody had been raised against 17β-HSD. This was carried out as described earlier. Figs. 17 and 18 illustrate the presence of antibody to 17β-HSD in the sera of both animals. In Fig. 17 samples of the same 17β-HSD-III as used for immunization were run on SDS-PAGE and probed with the serum. Binding to the enzyme is clearly visible by the antisera from both roosters (A2 and A3) but no reaction occurred when probing was carried out with the control sera of the two animals. Two bands are visible in each of the antisera lanes. The lower molecular weight band reflects the degradation of the enzyme which occurred during storage at -20°C. Fig. 18 shows that the anti-sera reacts also with 17β-HSD-I. A pure solution of the isoenzyme had not been successfully isolated in this preparation but this Western shows how the antibody binds only to the enzyme I band. None of the other protein bands contaminating the enzyme I preparation which are indicated in Fig. 18 cross react with the anti-serum.

SYNTHESIS OF λgt11 EXPRESSION LIBRARY

The synthesis of ds-cDNA and insertion of this into λgt11 phage for construction of the expression library was carried out as described in Methods. A total of 2.78 μg cDNA was prepared and passed through a Sepharose 4B 1 ml column for size fractionation. Fractions 10-15 of the total 25 were pooled yielding 1.26 μg of the size fractionated cDNA. This cDNA fraction was precipitated and redissolved in 12.5 μl TE buffer to give a final concentration of 100 ng/μl.

The final plating of the Y1090 cells infected with the packaged λgt11 phage revealed

Figure 17

Western analysis of anti-sera raised against 17β-HSD-III. 15 μg of the enzyme solution used for immunization was electrophoresed on SDS-PAGE (9.0%), electroeluted onto a nylon filter at 100 V for 12 hours, and hybridized with rooster anti-serum as described in Methods.

Lane 1; total protein staining with ponceau red and all proteins observed are indicated by arrows, lanes 2-5: colour reaction performed following screening with, lane 2; pre-immune serum of animal #2, lane 3; antiserum of animal #2, lane 4; pre-immune serum of animal #3, and lane 5; antiserum of animal #3.

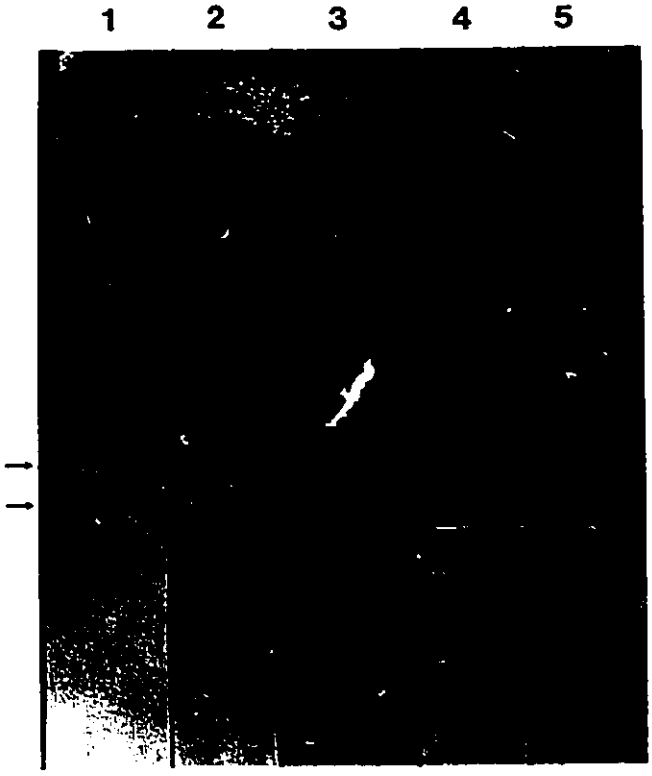

FIGURE 17

Figure 18

Western analysis of anti-sera raised against 17β-HSD-III showing binding to 17β-HSD-I. 15 μg from each of three pooled fractions obtained from a second chromatofocusing column for the purification of isoenzyme I were resolved by SDS-PAGE (9.0%). The proteins were electro-eluted onto a nylon filter at 100 V for 12 hours. Proteins were visualized by ponceau red staining and are indicated by arrows. Following destaining the filter was hybridized with rooster #2 anti-serum as described in Methods.

Lanes 1-3; three different fractions pooled from the 2nd focusing of an enzyme I preparation, Lane 4; enzyme III solution as in figure 17.

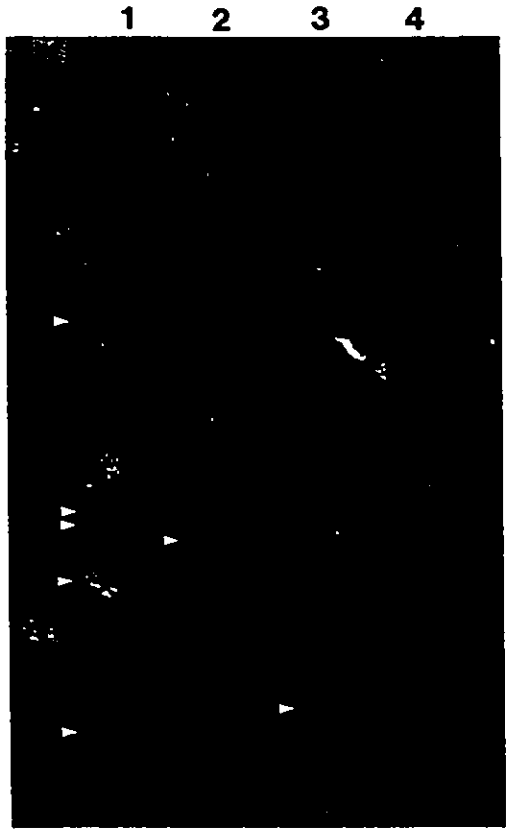

FIGURE 18

growth of a significant percentage of non-recombinants. The percentage of recombinants approached only 50-55% as compared to the high 99% obtained in the λgt10 library. This corresponds to a cloning efficiency in the order of only 10^6 pfu/ml. Despite the low titre sufficient recombinants exist for screening of the library provided that a large number of colonies are screened.

SCREENING OF THE λgt11 LIBRARY WITH ROOSTER ANTI-SERUM

Competent Y1090 cells were prepared and infected with either a 10^3 or 10^4 dilution of the library in SM buffer. Following the infection the cells were plated out as earlier described without the addition of IPTG or X-gal. In this way expression of the recombinant genes (or of the β-galactosidase gene in the case of the non-recombinants) would be repressed. The plates were incubated at 43°C for 3 hours to initiate growth of colonies and then continued overnight at 37°C after overlaying the lawns with nylon filters impregnated with IPTG. This allowed for expression of any recombinant genes and extension of the elongating protein sequence into the filter.

Probing of the expression library was as described in Methods and seven positive colonies were detected. These colonies were cored out, stored at 4°C in 0.5 ml SM buffer with 20 μl $CHCl_3$ as the phage stocks and re-screened. For re-screening it was determined that only 0.01 μl of the phage stocks was required for infecting 100 μl of competent Y1090 E. coli cells to produce well dispersed colonies when plated. All positive phage were therefore plated out at this density and filters were prepared and probed. Simultaneously a plate prepared from the entire library was grown up and screened to serve as a control.

Probing of the control filter showed no positive colour reaction. Probing of the filters from the seven positive phage revealed positive reactions to colonies grown from four of these seven. Not all colonies growing on these plates reacted positively to the hybridization. This suggested

some contamination of the phage stocks with negative phage types. Those colonies (1, 2, 3 and 4) still being positive were cored out and stored as before. To further ensure that these phage were indeed recombinant they were each used to infect competent Y1090 cells and were grown up on IPTG/X-gal plates to establish the recombinant white plaques. Characterization of phage DNA was then carried out by EcoR 1 digestion, Kpn 1/Sac 1 combined digestion and by PCR.

Initial attempts at DNA purification of the four positive phage samples resulted in DNA which proved to be undigestible by the sensitive EcoR 1 preventing the identification of any inserts. Samples of this DNA were therefore digested with BamH 1 to ensure that the DNA was digestible by other restriction enzymes. As shown in lanes 2-5 of Fig. 19 the BamH 1 digestion was successful and produced bands of the predicted sizes. As the size of the band containing the insert is relatively large in the non-recombinant DNA the increased length added by the insert can not be distinguished by agarose gel. The combined digestion of the DNA with both Kpn 1 and Sac 1 was chosen as this would produce a fragment of 2.08 Kb in non-recombinant DNA compared with a fragment up to 3.0 Kb in the recombinant DNA. The size difference can be identified by agarose gel analysis as the fragments are small and can be resolved. Fig. 20 is a restriction map of the gt11 phage DNA which reveals the sites of cleavage of the restriction enzymes used in this characterization.

This combined enzyme digestion would produce five fragments as predicted from Fig. 20. The fragments of lengths 17.07 Kb and 18.61 Kb would not be resolved on a 1.0% agarose gel and thus only four bands should be visible for the Kpn 1/Sac 1 digestion of a pure sample of the phage DNA. The digestion of the samples 2 and 3 in lanes 7 and 8 of Fig. 19 was not successful but that of samples 1 and 4 shown in lanes 6 and 9 reveal the digestion bands. It is apparent from this gel that an additional band is included in these two lanes. The top thick band comprises the 17.07

Figure 19

BamH 1 and Kpn 1/Sac 1 digestions of λgt11 DNA isolated after positive screening with anti-sera to 17β-HSD. Elfo-tris agarose gel (1.0%) electrophoresis of the restriction digests (BamH 1) of λgt11 DNA found to hybridize with rooster anti-serum stained by ethidium bromide.

Lanes 1 and 10 contain lambda HindIII markers (Lengths in kilobases, 23, 9.4, 6.5, 4.3, 2.3 2.0, 0.56 and 0.125 which can not be seen on this gel). Lanes 2-5 show the BamH 1 digestion of DNA samples 1 through 4 respectively. Lanes 6-9 show the Kpn 1/Sac 1 digestion products of the same DNA samples.

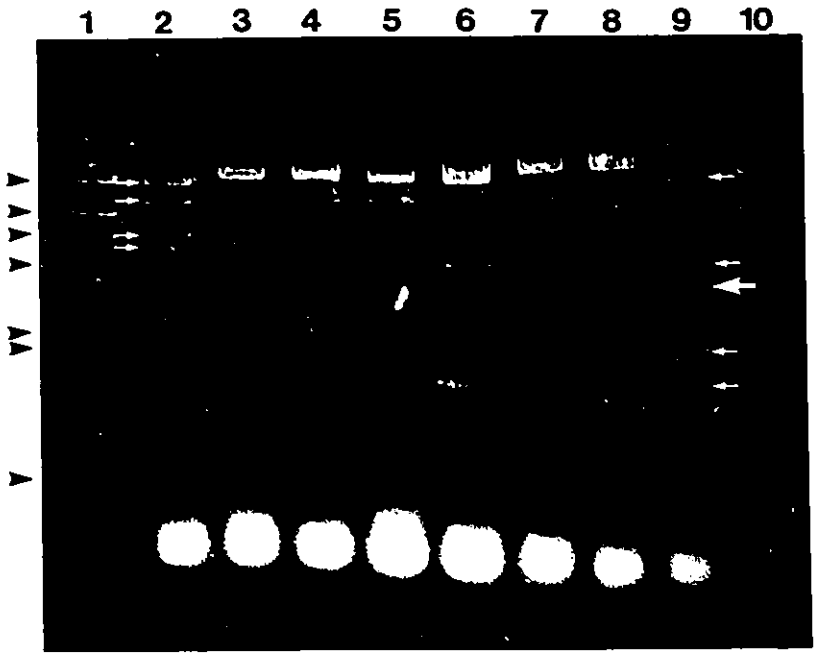

FIGURE 19

Figure 20

Restriction enzyme maps of both λgt10 and λgt11. Indicated are the sites of cleavage of EcoR 1 and BamH 1 for both the phage DNA sequences. In addition the cleavage positions for Sac 1 and Kpn 1 for λgt11 are shown. Distances indicated to the right of the figures are in kilobase pairs. The "phage DNA arms" supplied by Amersham refer to the two fragements obtained of each phage type DNA upon EcoR 1 digestion.

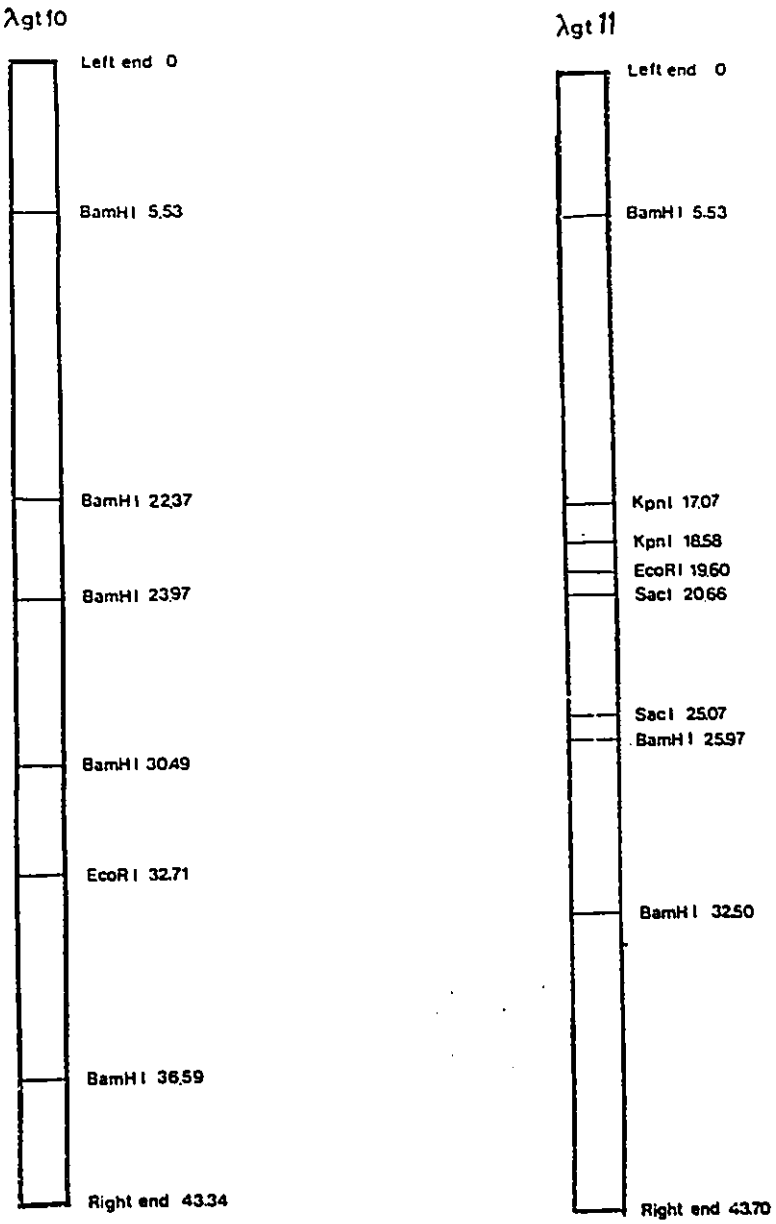
λgt10
Left end 0
BamHI 5.53
BamHI 22.37
BamHI 23.97
BamHI 30.49
EcoRI 32.71
BamHI 36.59
Right end 43.34
λgt11
Left end 0
BamHI 5.53
KpnI 17.07
KpnI 18.58
EcoRI 19.60
SacI 20.66
SacI 25.07
BamHI 25.97
BamHI 32.50
Right end 43.70

FIGURE 20

Kb and the 18.61 Kb lenghts. Bands corresponding to sizes of 4.43 Kb, 2.08 Kb and 1.51 Kb are labelled. These correspond to the cleavage products of non-recombinant λgt11 phage DNA. An additional band is also apparent. In recombinant phage the insert would be contained in the 2.08 Kb fragment causing it to migrate to a distance equal to approximately 3.0 Kb if the size fractionation of the cDNA at the beginning of the library synthesis was successful. A band below that of the 4.43-Kb fragment in lane 6 (sample 1) is clearly visible. This represents the fragment containing the insert. The existence of a 2.08 Kb band in this sample suggests that the phage stock was contaminated with non-recombinant phage DNA.

In order to determine the size of the inserts the modified DNA purification method described in Methods was carried out in order to obtain DNA which could be successfully digested with EcoR 1. Following the purification of the phage DNA by the plate lysate method it was digested with EcoR 1 as described in the λgt11 library synthesis kit. The success of the digestion was monitored by electrophoresing a sample of these digests on a 0.5% agarose gel. The low percentage gel would allow for resolution of the two arms of sizes 19.6 Kb and 24.1 Kb. Fig. 21 shows the resolution of the two arms on this gel stained with ethidium bromide. The small insert would not be visible here for one of two reasons. The low percentage gel used here would cause rapid migration of the small insert and therefore it may have run off the gel by this time. Secondly, should the insert fragment not yet have migrated off the gel it was only stained with ethidium bromide. The very low amount of the insert would not be visible by this method of staining.

The digests were labelled using the Klenow fragment of DNA polymerase I and ^{32}P-dATP. The samples were then resolved on a 1.5% agarose gel as indicated by the autoradiograph of Fig. 22. This does not reveal any distinct band of an insert as would be expected. Sample 1 (lane 5) may contain an insert but it is of a smaller size than would be predicted by the Kpn 1/Sac 1 digest or by the size fractionation of the cDNA. In order to establish the presence of inserts within these

Kb and the 18.61 Kb lengths. Bands corresponding to sizes of 4.43 Kb, 2.08 Kb and 1.51 Kb are labelled. These correspond to the cleavage products of non-recombinant λgt11 phage DNA. An additional band is also apparent. In recombinant phage the insert would be contained in the 2.08 Kb fragment causing it to migrate to a distance equal to approximately 3.0 Kb if the size fractionation of the cDNA at the beginning of the library synthesis was successful. A band below that of the 4.43 Kb fragment in lane 6 (sample 1) is clearly visible. This represents the fragment containing the insert. The existence of a 2.08 Kb band in this sample suggests that the phage stock was contaminated with non-recombinant phage DNA.

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The digests were labelled using the Klenow fragment of DNA polymerase I and ^{32}P-dATP. The samples were then resolved on a 1.5% agarose gel as indicated by the autoradiograph of Fig. 22. This does not reveal any distinct band of an insert as would be expected. Sample 1 (lane 5) may contain an insert but it is of a smaller size than would be predicted by the Kpn 1/Sac1 digest

Figure 21

EcoR 1 digest of λgt11 DNA. The EcoR 1 digests of λgt11 DNA samples found through antibody screening to be positive were electrophoresed on a 0.5% Elfo-tris agarose gel. Lanes 1 and 7 each contain 1 μg of lambda Hind III markers, lengths as described for figure 19. Lanes 3-6 are the digestion products of phage DNA samples 1-4 respectively, lane 2 contains undigested sample 1 for comparison. 2 μg of DNA sample was applied in each lane. The arrows indicate the two arms of the λgt11 DNA of 19.60 and 24.10 Kbp. The undigested phage DNA has a length of approximately 44 Kbp.

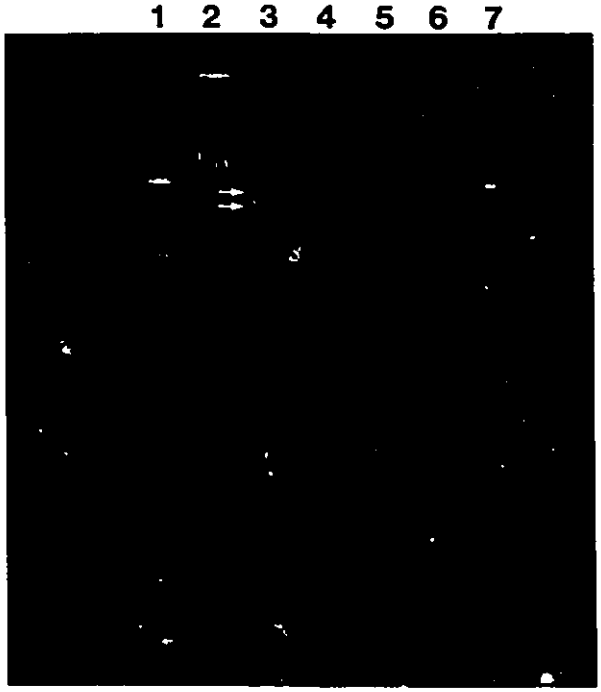

FIGURE 21

Figure 22

EcoR 1 digestion of the positive λgt11 DNA samples. The digests were electrophoresed on a 1.5% Elfo-tris agarose gel and autoradiographed. Lambda Hind III markers of lengths as described for figure 19 are shown in lanes 1 and 6. Lanes 2-5 contain phage DNA samples 4-1 respectively. Indicated in lane 5 (sample 1) is a possible band of insert of approximately 0.2 Kb.

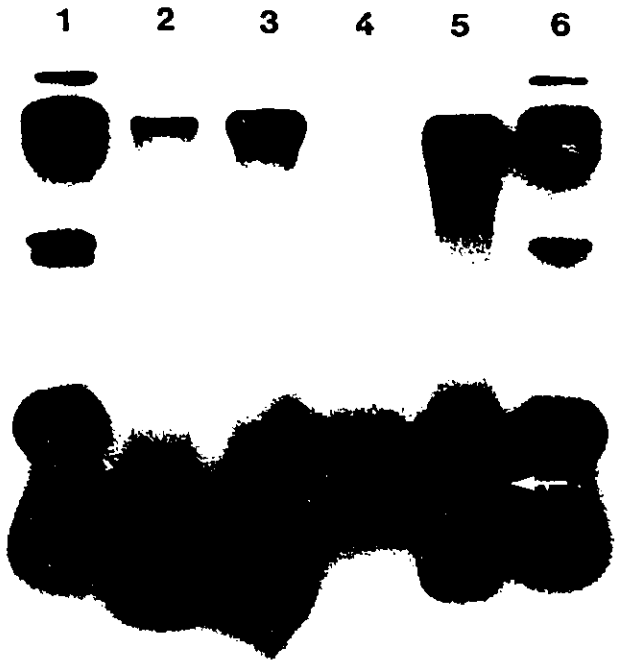

FIGURE 22

phage samples the PCR method was employed. This would result in a large amount of insert being synthesized without the need for a restriction digest. This would also negate the possibility that the insert contains internal EcoR 1 sites.

As described in Methods all "positive" phage DNA samples were subjected to the polymerase chain reaction and the amplified insert samples were analyzed by 1.0% agarose gel electrophoresis. As indicated in lane 2 of Fig. 23 sample 1 phage DNA is the only one which appears to contain insert. This was also suggested by the Kpn 1/Sac 1 digestion. The size of the insert shown in lane 2 is approximately 190 bases. The successful isolation of the 17β-HSD clone allows for its future use as a probe for the detection of longer clones in the λgt10 library.

Figure 23

Polymerase chain reaction products of the positive λgt11 DNA samples. The products of the polymerase chain reaction for the four λgt11 DNA samples identified through antibody screening as positive were electrophoresed on a 1.0% Elfo-tris agarose gel. Lanes 1 and 6 contain 1.0 μg of the lambda Hind III markers of lengths as described for figure 19. Lanes 2-5 contain the PCR products of DNA samples 1-4 respectively. The band shown in all of these four lanes corrresponds to the portion of the DNA obtained when the DNA contains no insert. The band above this in lane 2 corresponds to the cDNA insert of 150-190 bp in length.

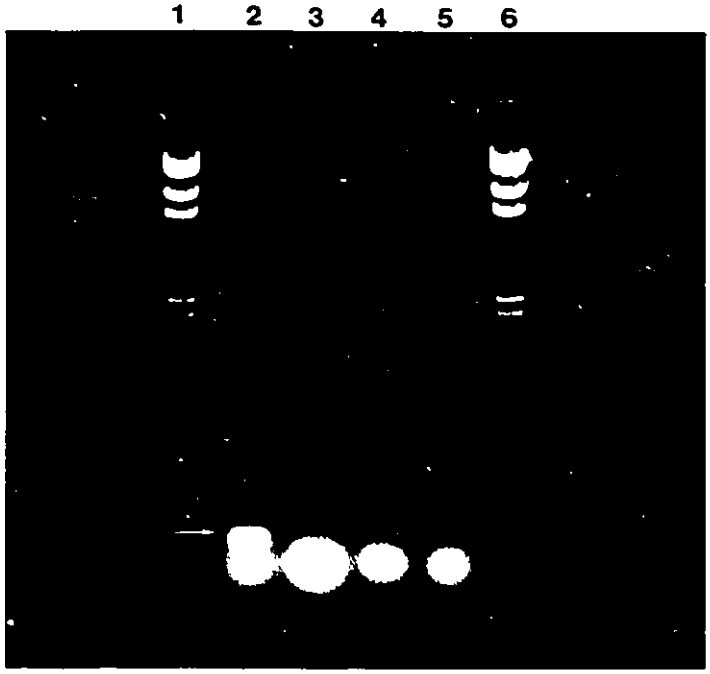

FIGURE 23

DISCUSSION

Antoun et al. (1985) demonstrated that the 17β-HSD of rabbit liver cytosol has at least three isozymic forms (labelled I, II, III). The acidic enzyme forms are charge isomers and vary in pI from 5.4 to 5.7. An additional 17β-HSD is separated from these three forms during the affinity chromatography procedure. This form elutes from the column before the initiation of the NaCl gradient reflecting its higher affinity for NAD^+ as a cofactor as compared to $NADP^+$. The Procion Red HE3B matrix has been shown to have a higher affinity for $NADP^+$ dependent dehydrogenases than for the NAD^+ dependent dehydrogenases (Watson et al., 1978).

In the studies described in this thesis two of the enzyme forms identified by Antoun et al. (1985a), namely forms I and III were purified to homogeneity. Form II of the enzyme was consistently found only in very low amounts and sufficient quantities of this enzyme could not be isolated for further investigation. All homogenous samples purified were from a single liver and proteins from different animals were not pooled. The purification procedure of Antoun et al. was modified to reduce the time required for isolation of the 17β-HSD. Affinity chromatography of the crude liver cytosolic fraction was effectively carried out using a stepwise increase in NaCl concentration of the eluting buffers. This adequately separated all 17β-HSD from contaminating proteins, including the NAD^+-dependent 17β-HSD. while eliminating the time consuming elution with salt gradients and the previously used step of gel filtration on Sephadex G-75.

The 17β-HSD activity eluted by high salt from the affinity column was subsequently subjected to chromatofocusing between pH 6.15 - 5.15 to separate the charge isomers of the enzyme. The enzyme preparation ws first briefly titrated to pH 5.5 to remove contaminating protein which precipitated under the experimental conditions used for chromatofocusing and interfered with application of the enzyme sample to the chromatofocusing column.

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Following the initial chromatofocusing of the enzyme mixture two major enzyme peaks were identified. These corresponded to isoenzymes I and III by IEF. Neither of the enzymes were homogenous at this stage. Cross-contamination of the isoenzymes as well as other contaminating proteins was evident. Small amounts of isoenzyme II were also present. The consistent low amounts of this enzyme form and its close proximity to form I following elution from the MONO P column prevented its isolation. A second chromatofocusing of each of the enzyme peaks was usually sufficient to achieve purity. Typical yields of isoenzymes I and III would be 1 and 3 mg respectively. The modified purification procedure shortened the time required to obtain homogenous enzyme samples by approximately 50 percent.

Sequencing of the two isoenzymes required further modifications to the sample solutions. The pure enzyme solutions were subjected to ammonium sulphate precipitation to separate the enzyme from the polybuffer 74 which would interfere with the sequencing technique. This precipitation step resulted in further loss of protein. The precipitated protein was then redissolved in buffer (10 mM Tris pH 8.0, 0.5 mM DTT) and extensively dialyzed against distilled water to eliminate all buffers and salts present. The enzyme samples, of low solubility in water, were subsequently lyophilized and weighed. A total of 0.5-0.7 mg of enzyme I and 0.8-1.0 mg of enzyme III were obtained. Attempts to verify protein homogeneity by gel electrophoresis were unsuccessful due to the insolubility of the lyophilized products.

Digestion of the 17β-HSD isoenzymes with endoproteinase Lys-C and purification of the peptides generated was undertaken in order to sequence and compare the peptides of isoenzyme I with those of isoenzyme III. This would reveal any differences between the two enzymes, which are structurally similar on the basis of their molecular weights, amino acid compositions and peptide maps (G.R Antoun, PhD thesis, 1983). Unique peptide sequences for 17β-HSD-I and 17B-HSD-

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Purity of the enzyme fractions was verified by IEF gels and supported by the single band observed on the SDS gels carried out for Western analysis. Those chromatofocusing profiles determined by Antoun match those found in this study. In previous analyses the purity of the isoenzymes was established through Davis gels, peptide maps and N-terminal analysis in addition to IEF and SDS-PAGE. As chromatofocusing yielded the same profiles here (figures 7-10) as by Antoun, IEF analysis was considered adequate to ascertain homogeneity. The possiblity that antibody had been produced against some contaminating protein in the enzyme sample is unlikely as only a single band is observed in the Western analyses to both forms I and III of the enzyme. This would mean that the same contaminant would have to have been present in the samples of both isoenzymes.

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III could then be back-translated into their nucleic acid equivalents and used to probe the λgt10 library. In this way it could be determined if the different enzyme forms derive from separate messages. Identification of the gene(s) responsible could also be pursued. However the limited sequencing data obtained was not sufficient to synthesize suitable probes for thorough screening of the λgt10 library and no positive cDNA clone was isolated. All plaques which showed hybridization to the nucleic probes synthesized from a peptide sequence derived from enzyme I were shown to be negative upon further screening.

Peptide sequences of enzyme III were obtained for those peaks corresponding to 5, 6 and 7. Comparison of these sequences revealed that they were derived from the same enzyme sequence. A possibility for the elution of the peptides at different acetonitrile percentages is arbitrary cleavage during the digestion process. The endoproteinase lys-C may have become contaminated resulting in peptide hydrolysis at sites other than at lysine residues. These digestions had been performed at different times and contamination of the proteinase may have occurred prior to the second digestion, that of enzyme III. Additional sites of cleavage resulted in peptide 7 being hydrolyzed internally, producing peptides 5 and 6 also. The shorter peptides had slightly more hydrophilic character than peak 7 and eluted from the HPLC column earlier. In addition the questionable cycles obtained in the sequencing of peak 6 (page 54) may in fact not exist within the peptide and could be attributed to failure of the sequenator to switch efficiently from the automatic sequencer to the PTH analyzer. Had the digestion produced only cleavages at the lysine residues only 24 or 25 peaks should have been observed as is the case with enzyme I. As almost 40 peaks were obtained from digestion of enzyme III this indicates additional sites of cleavage. It is unlikely that the digestion was incomplete due to the extensive incubation time.

Purified 17β-HSD was used to induce an immunogenic response in animals. Adult white leghorn roosters were used for the production of polyclonal antibodies against 17β-HSD. The

in buffer (10 mM Tris pH 8.0, 0.5 mM DTT) and extensively dialyzed against distilled water to eliminate all buffers and salts present. The enzyme samples, of low solubility in water, were subsequently lyophilized and weighed. A total of 0.5-0.7 mg of enzyme I and 0.8-1.0 mg of enzyme III were obtained. Attempts to verify protein homogeneity by gel electrophoresis were unsuccessful due to the insolubility of the lyophilized products.

Digestion of the 17β-HSD isoenzymes with endoproteinase Lys-C and purification of the peptides generated was undertaken in order to sequence and compare the peptides of isoenzyme I with those of isoenzyme III. This would reveal any differences between the two enzymes, which are structurally similar on the basis of their molecular weights, amino acid compositions and peptide maps (G.R. Antoun, PhD thesis, 1983). Unique peptide sequences for 17β-HSD-I and 17B-HSD-III could then be back-translated into their nucleic acid equivalents and used to probe the λgt10 library. In this way it could be determined if the different enzyme forms derive from separate messages. Identification of the gene(s) responsible could also be pursued. However the limited sequencing data obtained was not sufficient to synthesize suitable probes for thorough screening of the λgt10 library and no positive cDNA clone was isolated. All plaques which showed hybridization to the nucleic probes synthesized from a peptide sequence derived from enzyme I were shown to be negative upon further screening.

Sequences for enzyme III were obtained for those peptides corresponding to peaks 5, 6 and 7. Comparison of these sequences revealed that they were derived from the same enzyme sequence. A possibility for the elution of the peptides at different acetonitrile percentages is arbitrary cleavage during the digestion process. The endoproteinase lys-C may have become contaminated resulting in peptide hydrolysis at sites other than at lysine residues. These digestions had been performed at different times and contamination of the proteinase may have occurred prior to the second digestion, that of enzyme III. Additional sites of cleavage resulted in peptide 7 being hydrolyzed

animals were immunized with 20 μg 17β-HSD-III and in six weeks were given a booster injection of the same amount of antigen. Western analysis of the antisera of two roosters indicated the presence of serum antibodies which bound to both isozymic forms of the enzyme. The serum was then used for screening of the gt11 library following induction of expression of the recombinant fusion proteins.

Anti-enzyme assays which were performed throughout the antibody production proved to be difficult to interpret, particularly following the booster shot. 17β-HSD activity measured in the absence of serum varied at the times of the different assays (ie. tables 5 and 6) although these should be considered duplicates as they were prepared in exactly the same way. The 17β-HSD employed in the studies is very sensitive to any variation in incubation factors which would be manifested by fluctuations in activity values. Variations in the level of the enzyme's activity could also be attributed to the storage of the enzyme solution. This was stored in 1 ml aliquots at -20°C such that not all of the solution would have to be brought to room temperature each time assays were carried out. It has been found that the enzyme can exhibit an abrupt change in activity following a repetitive freeze/thaw cycle. Use of a fresher aliquot in the assays of table 5 may account for the discrepancy. Similarly the absence of increased activity of the control (pre-immune) serum activities over that of the control assay (no serum added) in table 4 can be interpreted to also be due to denaturation of the enzyme. The 17β-HSD at this point was beyond enhancement by the serum proteins.

The anti-enzyme assays performed following the re-immunization proved to be less interpretable. The enzyme activity measured was higher than in the previous assays and no inhibition of this activity by the immune serum could be detected. This could be attributed either to high enzyme activity in the incubated samples or to the molecular nature of the antibodies. The amount of 17β-HSD assayed produced maximal conversion of substrate and partial inhibition of this

activity by antibody may not be apparent. Another possibility is the nature of the antibodies produced in response to the secondary immunization. In the secondary response not all of the types of antibodies produced initially are amplified after re-immunization. The antibodies comprising the majority of the secondary response may not inhibit enzyme activity. The assays for measuring anti-enzyme activity allowed for a 30 minute pre-incubation of sera with 17β-HSD. The design of the assay made the assumption that binding of antibody to the enzyme would prevent its catalytic activity. That is that the antibody would either bind to the steriod binding site of 17β-HSD, or would bind to the enzyme in such a way as to prevent oxidation of the testosterone. Antibody which has been raised to the testicular form of the enzyme has been shown to bind to or in close proximity to the co-factor binding site of the enzyme (Inano et al., 1980). If the immunogenic site of 17β-HSD-III in this study was also at the co-factor binding site then the anti-enzyme assay would have to be altered to inhibit the enzyme's activity. The pre-incubation of enzyme and serum not only should exclude testosterone but $NADP^+$ as well. The possibility also exists that the antibody produced in this study does not prevent the enzyme's activity when bound to the protein, regardless of the antibody binding site.

Despite the difficulty in following the antibody production, western analysis showed successful binding of a, or multiple, rooster serum immunoglobulins to both isoenzymes I and III of 17β-HSD. The samples of 17β-HSD-III used for immunization of the animals was the same as that used six and eight months later in the two western analyses shown in Figs. 17 and 18 respectively. At the time of the first analysis the beginning of breakdown of the protein can be seen by the faint presence of a faster migrating band. Verification of the hybridization of anti-serum to 17β-HSD-I as shown in Fig. 18 also includes a lane with 17β-HSD-III for comparison. This analysis was carried out two months following the previous one and the almost complete alteration of isoenzyme III can be seen. The protein had been stored at -20°C and taken out for use three or four times.

Synthesis and screening of a λgt11 expression library was then pursued once polyclonal antisera against 17β-HSD was obtained. The library sythesized had quite a high level of non-recombinant phage. This observation meant that although the library could still be used for probing, more plaques would have to be screened for identification of positive clones. Hybridization of rooster antiserum to nylon filters onto which were transferred the fusion proteins of recombinant phage resulted in the initial identification of seven positive plaques. Subsequent screening of these phage left four positive samples. Of these four, insert could be positively identified in only one of the phage DNA samples. The reason for this is not known considering that extensive probing of the expressed proteins with antiserum remained positive and that the samples produced white colonies (recombinant) when infected into cells and grown with IPTG and X-gal to induce expression of the proteins and allow colour determination of the non-recombinant plaques.

The insert which was identified by the PCR assay was found to be considerably smaller than anticipated. The size fractionation step of the cDNA synthesis procedure shoud have ensured inserts of at least 1Kb in length. The positive clone isolated here has a length of only 0.150 - 0.190 Kb. DNA of this length would produce a polypeptide of only 50-70 amino acids in length if this is all translated message. This corresponds to only approximately 20% of the protein's native size. It would be unlikely that all antibodies produced against 17β-HSD were against the same epitope. However the variety of the antibodies obtained in the polyclonal antiserum could feasibly contain those which would react with this segment of the enzyme.

Non-specific association of antisera with phage plaques did not appear to be a problem. Initial re-screening of positive phage picked from a high density plate showed some to be negative while other plates from positive phage did not hybridize to all plaques. This reflected the heterogeneity of plaques cored out initially. Screening of those samples still showing hybridization

with antiserum remained positive throughout three additional rounds of screening. To verify that any small variations in the screening procedures had not produced artifactual hybridization, a control filter was also prepared. This corresponded to a plate prepared from total λgt11 library stock at medium density (10^3 plaques). The filter lifted from the plate was treated identically to the filters lifted from positive plates. Screening of the control filter indicated no hybridization of antiserum antibodies to λgt11 fusion proteins.

The results obtained for inserts 2,3 and 4 are difficult to interpret. The fragments observed from the combined Kpn I/Sac I digest of the positive phage DNA indicate the presence of insert clearly in both samples 1 and 4 (Fig. 19). An insert was therefore anticipated in the PCR assay for at least sample 4 in addition to sample 1.

It would be beneficial to synthesize a large quantity of the insert from positive phage DNA 1 so as to rescreen the λgt10 library. Rescreening of the cDNA library with the clone from the expression library would allow isolation of longer inserts. Once the isolation of a full length segment has been accomplished this can be probed with the nucleic sequence of one of the peptides identified earlier to verify its identity to 17β-HSD. A full length message for 17β-HSD would be at least 1 Kb for the isoenzymes of about 325 and 350 amino acids for forms I and III respectively.

The clone obtained in this study may either be specific to one of the enzymes if separate messages exist, or could also be common to both. This is because the antibodies used for screening of the expression library reacted with both forms of the 17β-HSD. Extensive screening of the library with a longer insert identified in the λgt10 library with the positive clone probe could reveal other full length inserts of similar but not identical sequence. This would reveal the presence of more than one mRNA species for the 17β-HSD enzymes. Post-translational modifications of one

form of the enzyme to produce the multiple forms, such as cleavage of small terminal sequences may also be responsible for the heterogeneity.

Failure to isolate more than one mRNA for the enzyme from the library synthesized in this study would not exclude the possiblity of their existence. Both libraries synthesized here were obtained using the liver RNA of a 16 week old female adult rabbit. At this age the rabbit has fully matured and the age dependent fluctuation in the levels of the isozymic forms of the enzyme (Antoun et al., 1985b) may be stabilized. Fluctuation of the isoenzyme levels was monitored up to an age of eight weeks. Purification of 17β-HSD from a four week old rabbit indicates the presence of a form IIIY and no form III. The two forms have very similar pI values as reflected by their similar eluting patterns on chromatofocusing columns and differ in molecular weight by only 1,500. In addition the enzyme composition of the immature rabbit includes very little form I. Screening of a cDNA library prepared from the RNA of immature rabbit liver may include an additional mRNA species coding for the form IIIY of the enzyme which is not present in the older rabbit. Again, post-translational mechanisms may produce the isoform III from form IIIY. This process does not seem to be a likely explanation for the existence of the different enzyme forms. The complete disappearance of form IIIY suggests a message no longer being expressed.

Identification of multiple mRNA species coding for the different 17β-HSD forms could be followed by quantitative studies. The synthesis of cDNA libraries from RNA of rabbits of different ages would allow for quantitative determination of the levels of the different 17β-HSD mRNAs. These levels may resemble the protein level profiles determined previously (Antoun et al., 1985b) and could reflect changes in the degradation of the mRNA or changes in the rate of expression of the different mRNAs. Isolation of mRNA transcripts encoding the 17β-HSD enzymes would also allow for their sequencing and therefore determination of the amino acid sequences of the different enzymes.

Speculation as to the purpose of the different enzyme forms has not yet been made. The enzyme profile variations observed from female rabbit liver at different ages may bear little similarity to the profile from a male source. Assays of these 17β-HSD enzymes indicated greater specific activity towards testosterone as compared with 17β-estradiol (Antoun et al., 1985a). This suggests that the enzyme forms may show a more drastic fluctuation in their levels within the tissues of a male animal.

Fetal production of testosterone will be increased during gonadal differentiation, development of the Wolffian duct into seminal vesicles, vas deferens, epididymus and differentiation of external genitalia. In the immature animal testosterone production will be suppressed while its level will increase during the animals maturation. Measurements of the specific activities of the different isoenzymes with various steroid substrates indicated that 17β-HSD-I has a higher specific activity than form III. In the immature rabbit it is form IIIY which is most abundant with very little form I and no form III. Measurement of the specific activity of this isoenzyme proved it to possess even lower activity than those of forms I and III. Assuming the same fluctuations in 17β-HSD activity with age in both sexes of the rabbit it is reasonable to suggest that during the immature stage of development of the animal at a time when steroid production is suppressed those enzymes of higher specific activity are also suppressed.

Isozymic forms of 17β-HSD have been determined in human endometrium (Pollow et al., 1976). The concept of organ specific isoenzymes has been speculated through studies of patients with male pseudohermaphroditism due to 17β-HSD defiency. In these patients testicular 17β-HSD activity is decreased, however its peripheral activity is normal (Peterson and Imperato-M^{c}Ginley, 1984, Gross et al., 1986, Imperato-M^{c}Ginley et al., 1987). During the fetal development of these patients the absence of testicular 17β-HSD activity prevents the formation of gonadal testosterone,

the source of androgens in the developing fetus. This loss prevents the proper development of the urogenital tract and external genitalia. The appearance of ambiguous genitalia at birth usually results in patients being raised as females. At puberty however with increasing levels of androstenedione in the testes virilization occurs and increased levels of testosterone in peripheral tissues causes significant masculinization.

The genetic basis for this deficiency has not been studied beyond its pattern of heredity. This is inconsistent but has been described as autosomal recessive. Only one genetic female has been described with the deficiency following an active search for a female sufferer of the deficiency (Pang et al., 1987). Examination of the afflicted's siblings revealed two sisters who were also affected and an unaffected brother. The nature of the deficiency appears to be a defective testicular 17β-HSD rather than to the absence of the enzyme altogether as described by Wilson et al., 1987 where testicular conversion of androstenedione to testosterone was almost absent but the oxidative direction of the catalysis approached normal values. The existence of distinct 17β-ketosteroid reductase (catalyzing androstenedione to testosterone conversion) and 17β-hydroxysteroid dehydrogenase (for testosterone to androstenedione conversion) have been suggested (Bogovich and Payne, 1980). The enzyme preparation in this study from rat testicular tissue was extremely unstable and no further work supports this suggestion. Work presented by Oshima et al., 1980 indicates the presence of separate binding sites for the two steroids within human testicular 17β-HSD. The identification of the source of this deficiency depends on isolation of the corresponding mRNA species and genes. Sufficient enzyme forms cannot be purified from samples of human tissues (except placental) for direct structural analysis.

The presence of isozymic forms of 17β-HSD and determination of their range in specific activities together with the observation that only the gonadal tissue is deficient in 17β-HSD in patients with the above mentioned affliction support the theory of organ specificity of the enzyme.

The work presented here describes the initial steps in the elucidation of the genetic basis of 17β-HSD. Further experimentation can be carried out from the results achieved here to isolate the full length cDNAs corresponding to the messages encoding the sequences for all 17β-HSD isoenzymes. Purification of these messages and their ensuing use as probes to quantitate the levels of the messages within different tissues of animals of both sexes and at various stages of development can then be pursued to reflect the organ specificity of the isoenzymes of 17β-HSD.

REFERENCES

Akesode, F. A., Meyer, W. J., Migeon, C. J. (1977) Male pseudohermaphroditism with gynaecomastia due to testicular 17-ketosteroid reductase deficiency. Clinical Endocrinology, 7: 443-452.

Antoun, G. R., Brglez, I., Williamson, D.G. (1985a) A 17β-hydroxysteroid dehydrogenase of female rabbit liver cytosol. Purification and characterization of multiple forms of the enzyme. Biochemical Journal, 225: 383-390.

Antoun, G. R., Brglez, I., Williamson, D.G. (1985b) Age-dependent changes in multiple forms of the soluble 17β-hydroxysteroid dehydrogenase of female rabbit liver. Biochemical Journal, 225: 391-398.

Auffrey, C. & Rougeon, F. (1979) Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Europian Journal of Biochemistry, **107**: 303-314.

Aviv, H. & Leder, P. (1972) Purification of Biologically Active Globin Messenger RNA by Chromatography on Oligothymidylic acid-Cellulose. Proceedings of the National Academy of Sciences, USA, **69**: 1408-1412.

Balducci, R., Toscano, V., Wright, F., Bozzolan, F., Di Piero, G., Maroder, M., Panei, P., Sciarra, F., Boscherini, B., (1985) Familial male pseudohermaphroditism with gynaecomastia due to 17β-hydroxysteroid dehydrogenase deficiency. A report of 3 cases. Clinical Endocrinology, **23**: 439-444.

Blackshear, P.J. (1984) Systems for Polyacrylamide Gel Electrophoresis. Methods in Enyzmology, **104**: 237-255.

Bogovich, K. & Payne, A. H. (1980) Purification of Rat Testicular Microsomal 17-Ketosteroid Reductase. Evidence that 17-ketosteroid reductase and 17β-hydroxysteroid dehydrogenase are distinct enzymes. Journal of Biological Chemistry, **256**: 5552-5559.

Burns, J. W., Engel, L. L., Bethune, J. L. (1972) Amino Acid Composition and Subunit Structure. Human Placental 17β-Estradiol Dehydrogenase. Biochemistry, **11**: 2699-2703.

diJeso, F. (1968) Ammonium Sulfate Concentration Conversion Nomograph for 0°C. Journal of Biological Chemistry, **243**: 2022-2023.

Dumic, M., Plavsic, V., Fattorini, I., Ille, J. (1985) Absent Spermatogenesis despite Early Bilateral Orchidopexy in 17-ketoreduction Deficiency. Hormone Research, **22**: 100-106.

Goebelsmann, U., Hall, T. D., Paul, W. L., Stanczyk, F. Z. (1975) In Vitro Steroid Metabolic Studies in Testicular 17β-Reduction Deficiency. Journal of Clinical Endocrinology and Metabolism, **41**: 1136:1143.

Grunstein, M. & Hogness, D. S. (1975) Colony hybridization: A method for the isolation of cloned DNA that contain a specific gene. Proceedings of the National Academy of Sciences, USA, **72**: 3961-3965.

Gubler, U. & Hoffman, B. (1983) A simple and very efficient method for generating cDNA libraries. Gene, 37: 263-269.

Hadley, M. E., Endocrinology, Prentice-Hall, Inc., New Jersey, 1984.

Hasnain, S., Williamson, D. G. (1974) The Separation and Partial Purification of the Soluble 17α- and 17β-Hydroxysteroid Dehydrogenases of Rabbit Liver. Canadian Journal of Biochemistry, 52: 120-125.

Hayes, M. B. & Wellner, D. (1969) Microheterogeneity of L-Amino Acid Oxidase. Separation of multiple components by polyacrylamide gel electrofocusing. Journal of Biological Chemistry, 244: 6636-6644.

Imperato-M'Ginley, J. & Peterson, R. E. (1976) Male pseudohermaphroditism: The complexities of male phenotypic development. American Journal of Medicine, 61: 251-272.

Imperato-M'Ginley, J., Akgun, S., Ertel, N. H., Sayli, B., Shackleton, C. (1987) The coexistence of male pseudohermaphrodites with 17-ketosteroid reductase deficiency and 5α-reductase deficiency within a Turkish kindred. Clinical Endocrinology, 27: 135-143.

Inano, H., Hayashiyama, J., Tamaoki, B. (1982) Solubilization Δ^5-3β-hydroxysteroid dehydrogenase with Δ^5-Δ^4 isomerase and 17β-hydroxysteroid dehydrogenase from rat testicular microsomal fraction by several detergents. Journal of Steroid Biochemistry, 16: 587-593.

Inano, H., Kawakura, K., Tamaoki, B. (1977) Active form of the 17β-hydroxysteroid dehydrogenase of porcine testes. Journal of Steroid Biochemistry, 8: 787-789.

Inano, H., Ohba, H., Tamaoki, B. (1981) Porcine testicular 17β-hydroxysteroid dehydrogenase: multiple forms of the enzyme. Journal of Steroid Biochemistry, 14: 1347-1355.

Inano, H., & Tamaoki, B. (1974) Purification and Properties of NADP-Dependent 17β-Hydroxysteroid Dehydrogenase Solubilized from Porcine-Testicular Microsomal Fraction. Europian Journal of Biochemistry, 44: 13-23.

Inano, H., & Tamaoki, B. (1975) Relationship between Steroids and Nucleotides in the Oxido-Reduction Catalyzed by the 17β-Hydroxysteroid Dehydrogenase Purified from the Porcine Testicular Microsomal Fraction. Europian Journal of Biochemistry, 53: 319-326.

Inano, H., & Tamaoki, B. (1986) Testicular 17β-hydroxysteroid dehydrogenase: molecular properties and reaction mechanism. Steroids, 48: 1-26.

Inano, H., Tamaoki, B., Hamana, K., Nakagawa, H. (1980) Amino acid composition and immunochemical properties of porcine testicular 17β-hydroxysteroid dehydrogenase. Journal of Steroid Biochemistry, 13: 287-295.

Kaplan, H., Oda, G., Antoun, G.R, Williamson, D.G., Tattrie, B., Rabin, E.Z. (1983) A highly sensitive method for identification of amino termini of proteins: application to multiple forms of poly (C)-avid ribonuclease and 17β-hydroxysteroid dehydrogenase. Canadian Journal of Biochemistry and Cell Biology, 61: 323-327.

Karavolas, H. J., Baedecker, M. L., Engel, L. L. (1970) Human Placental 17β-estradiol Dehydrogenase. Journal of Biological Chemistry, 245: 4948-4952.

Kurosumi, M., Ishimura, K., Yoshinaga, T., Fujita, H., Tamaoki, B. (1986) Immunocytochemical localization of 17β-hydroxysteroid dehydrogenase in porcine testes. Histochemistry, 85: 287-289.

Lapeyre, B. & Amalyric, F. (1985) A powerful method for the preparation of cDNA libraries: isolation of cDNA encoding a 100-KDal nucleolar protein. Gene, 37: 215-220.

Leinonen, P., Dunkel, L., Perheentupa, J., Vihko, R. (1983) Male pseudohermaphroditism due to deficiency of testicular 17-ketosteroid reductase. Acta Paediatrica Scandinaviea, 72: 211-214.

Liu, D. K., Kochakian, C. D. (1971a) Partial purification and some properties of guinea pig kidney 17β-hydroxy-C_{19}-steroid dehydrogenase. Steroids, 19: 701-720.

Liu, D. K., Kochakian, C. D. (1971b) Heterogeneity of guinea pig kidney 17β-hydroxy-C_{19}-steroid dehydrogenase activity observed by disc gel electrophoresis. Steroids, 19: 721-729.

M^{c}Indoe, J. H., Woods, G. R. (1981) Steroid-Metabolizing Enzymes in Human Breast Cancer Cells. II. 5α-Reductase, 3α-Hydroxysteroid oxidoreductase, and 17β-Hydroxysteroid oxidoreductase. Endocrinology, 108: 1407-1413.

Maniatis, T., Fritsch, E.F., Sambrook, J., Molecular Cloning. A laboratory manual, Cold Spring Harbor Laboratory, New York, 1982.

Martin, C. R., Endocrine Physiology, Oxford University Press, New York, 1985.

Mendoza-Hernandez, G., Lopez-Solache, I., Rendon, J. L., Diaz-Zagoya, J. C. (1988) 17β-hydroxysteroid dehydrogenase activity in canine pancreas. Biochemical and Biophysical Research Communications, 152: 376-382.

Michel, F., Nicolas, J.-C., Crastes de Paulet, A. (1975) 17β-hydroxysteroid dehydrogenase of the sheep ovary: purification, properties and substrate binding site. Biochimie, 57: 1131-1140.

Milewich, L., Stephens-Parker, P., M^{c}Donald, P. C. (1978) Testosterone metabolism by human lung tissue. Journal of Steroid Biochemistry, 9: 29-32.

Milewich, L., Whisenant, M. G., Sawyer, M. K. (1982) Androstenedione metabolism by human lymphocytes. Journal of Steroid Biochemistry, 16: 81-85.

Mulder, E., Lamers-Stahlhofen, G. J. M., Van der Molen, H. J. (1972) Isolation and Characterization of 17β-Hydroxy Steroid Dehydrogenase from Human Erythrocytes. Biochemical Journal, 127: 649-659

Murono, E. P. & Payne, A. H. (1976) Distinct Testicular 17-ketosteroid reductases, one in interstitial tissue and one in seminiferous tubules. Biochimica et Biophysica Acta, 450: 89-100.

Musto, N., Hafiez, A. A., Bartke, A. (1972) Prolactin Increases 17β-Hydroxysteroid Dehydrogenase Activity in the Testes. Endocrinology, 91: 1106-1108.

Nicolas, J.C., Pons, M., Descomps, B., Crastes de Paulet, A. (1972) Large-scale purification of the soluble oestradiol-17β dehydrogenase of human placenta. FEBS Letters, 23: 175-179.

Ohba, H., Inano, H., Tamaoki, B. (1982) Kinetic mechanism of porcine testicular 17β-hydroxysteroid dehydrogenase. Journal of Steroid Biochemistry, 17: 381-386.

Okayama, H. & Berg, P. (1982) High Efficiency Cloning of Full-Length cDNA. Molecular and Cellular Biology, 2: 161-170.

Oshima, H. & Ochiai, K. (1972) On testicular 17β-hydroxysteroid oxidoreductase product activation of testosterone formation from androstenedione in vitro. Biochimica et Biophysica Acta, 306: 227-236.

Oshima, H., Yoshida, K., Troen, P. (1980) A Further Study of 17β-Hydroxysteroid Oxidoreductase in the Human Testis: Mechanism of in vitro Activation. Endocrinologica Japonica, 27: 107-115.

Pang, S., Softness, B., Sweeney, W. J., New, M. I. (1987) Hirsutism, polycystic ovarian disease, and ovarian 17-ketosteroid reductase deficiency. New England Journal of Medicine, 316: 1295-1301.

Peltoketo, H., Isomaa, V., Maentausta, O., Vihko, R. (1988) Complete amino acid sequence of human placental 17β-hydroxysteroid dehydrogenase deduced from cDNA. Federation of Europian Biochemical Societies, 239: 73-77.

Plotti, G., Menini, E., Bompiani, A. (1972) Serum levels of oestradiol-17β dehydrogenase in normal and abnormal pregnancies. Journal of Obstetrics and Gynaecology of the British Commonwealth, 79: 603-611.

Pollow, K., Lübbert, H., Boquoi, E., Kreutzer, G., Jeske, R., Pollow, B. (1975) Studies on 17β-hydroxysteroid dehydrogenase in human endometrium and endometrial carcinoma. Acta Endocrinologica, 79: 134-145.

Pollow, K., Lübbert, H., Pollow, B. (1976) Partial purification and evidence for heterogeneity of the cytoplasmic 17β-hydroxysteroid dehydrogenase (17β-HSD) from normal human endometrium and endometrial carcinoma. Journal of Steroid Biochemistry, 7: 315-320.

Ranelletti, F. O., Piantelli, M., Carbone, A., Rinelli, A., Scambia, G., Benedetti, P., Mancuso, S. (1988) Type II Estrogen-Binding Sites and 17β-Hydroxysteroid Dehydrogenase Activity in Human Peripheral Blood Mononuclear Cells. Journal of Clinical Endocrinology and Metabolism, 67: 888-892.

Rosler, A. & Kohn, G. (1983) Male pseudohermaphroditism due to 17β-hydroxysteroid dehydrogenase deficiency: Studies on the natural history of the defect and effect of androgens on gender role. Journal of Steroid Biochemistry, 19: 663-674.

Rutledge, R. G., Seligy, V. L., Cote, M. J., Dimock, K., Lewin, L. L., Tenniswood, M. P. (1988) Rapid synthesis cloning of complimentary DNA from any RNA molecule into plasmid and M13 vectors. Gene, 68: 151-158.

Saez, J. M., de Peretti, E., Morera, A. M., David, M., Bertrand, J. (1971) Familial Male Pseudohermaphroditism with Gynecomastia Due to a Testicular 17-Ketosteroid Reductase Defect. I. Studies in vivo. Journal of Clinical Endocrinology, 32: 604-610.

Shen, C. C. & Kochakian, C. D. (1978) Purification and Properties of 17β-Hydroxy-C_{19}-Steroid Dehydrogenases of Adult Male Guinea Pig Kidney. Endocrinology, 103: 2040-2052.

Siiteri, P. K. & Wilson, J. (1974) Testosterone Formation and Metabolism During Male Sexual Differentiation in the Embryo. Journal of Clinical Endocrinology and Metabolism, 38: 113-125.

Sluyterman, L. A. Æ. (1982) Chromatofocusing: a preparative protein separation method. Trends in Biological Sciences, 67: 168-170.

Storey, D., Dimock, K., Kang, Y. (1984) Structural characterization of virion proteins and genomic RNA of human parainfluenza virus. Journal of Virology, 52: 761-766.

Strickler, R. C., Tobias, B. (1980) Estradiol-17β-dehydrogenase and 20α-hydroxysteroid dehydrogenase from human placental cytosol: one enzyme with two activities? Steroids, 36: 243-253.

Strickler, R. C., Tobias, B., Covey, D. F. (1981) Human Placental 17β-Estradiol Dehydrogenase and 20α-Hydroxysteroid Dehydrogenase. Two activities at a single enzyme site. Journal of Biological Chemistry, 256: 316-321.

Thaler-Dao, H. & Breuer, H. (1974) Gonadal factors regulating the activity of the 17β-hydroxysteroid oxidoreductase in rat liver. Acta Endocrinologica, 77: 727-736.

Thaler-Dao, H. & Descomps, B., Saintot, M., Crastes de Paulet, A. (1972) The 17β-hydroxysteroid NAD(P) oxidoreductase from female rabbit liver cytosol: separation and characterization. Biochimie, 54: 83-91.

Watson, D. H., Harvey, M. J., Dean, P. D. G. (1978) The Selective Retardation of $NADP^+$-Dependent Dehydrogenases by Immobilized Procion Red HE-3B. Biochemical Journal, 173: 591-596.

Wilson, S. C., Hodgins, M. B., Scott, J. S. (1987) Incomplete masculinization due to a deficiency of 17β-hydroxysteroid dehydrogenase: comparison of prepubertal and peripubertal siblings. Clinical Endocrinology, 26: 459-469.

Wit, J. M., van Hooff, C. O. M., Thijssen, J. H. H., Van den Brande, J. L. (1988) In vivo and in vitro Studies in a 46,XY Phenotypically Female Infant with 17-Ketosteroid Reductase Deficiency. Hormone Metabolism Research, 20: 367-374.

Wong, P., M^{ac}Donald, I., Tenniswood, M. (1989) Gene, in press.

Young, R. A. & Davis, R. W. (1982) Efficient isolation of genes by using antibody probes. Proceedings of the National Academy of Sciences, USA, 80: 1194-1198.