# INVESTIGATION OF 11β-HYDROXYSTEROID DEHYDROGENASE IN SALIVARY GLAND

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by

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## SUMMARY

The enzyme responsible for catalysing the oxidation-reduction of the 11-oxygen functions of steroids is 11β-hydroxysteroid dehydrogenase (11β-HSD). Previous physiological and histochemical studies have shown that the enzyme occurs in salivary gland, as demonstrated by the conversion of cortisol to cortisone in significant quantities. The aims of this thesis were to report more fully on the investigation of 11β-HSD in rat submandibular gland.

A reverse isotope dilution technique using cortisol-4-$^{14}$C as substrate was used to assay the enzyme in homogenate, spectrophotometric and fluorometric assays being unsuitable. Cortisol was converted to cortisone, as identified by formation of cortisone derivatives and recrystallisation to constant specific activity.

A variety of assay parameters were investigated to find the optimal conditions of enzyme activity. Nicotinamide adenine dinucleotide was the preferred cofactor to nicotinamide adenine dinucleotide phosphate with the optimal pH conditions for the cofactor-linked enzyme being pH8.1-8.9 and pH7.6 respectively. Both cofactors were in saturating amounts at 1.25mM in the assay volume of 11ml. A phosphate buffer showed no side effects whereas borate and tris buffer showed chemical reactions and therefore phosphate buffer was used to control pH. The optimal temperature for the NAD- and NADP-linked 11β-hydroxysteroid dehydrogenase was 37°C for incubations of 2 mins. and 7 mins. respectively. Km and Vmax values for the NAD-linked 11β-hydroxysteroid dehydrogenase were 0.48μM and 1.27 n moles/min., the NADP-linked 11β-hydroxysteroid dehydrogenase values being 0.95μM and 0.37n moles/min. The substrate concentration chosen for enzyme assay was approximately 2xKm of the NAD-linked enzyme. Verification of enzyme assay for 100mg gland homogenates was shown by increasingly proportional enzyme activity with enzyme (gland) concentration around this value.

Reversibility of the reaction using cortisone-4-$^{14}$C as substrate could not be shown although reduced cofactors and a variety of pH values and incubation periods were used. One of the effects of 11β-HSD in salivary gland is, therefore, to convert physiologically active steroids into inactive steroids by 11β-hydroxyl group transfer to 11-oxo group steroids.

Corticosterone, the naturally secreted adrenocorticoid in the rat, was metabolised by the submandibular gland 11β-HSD.

Rat kidney was also used to investigate 11β-hydroxysteroid dehydrogenase activity. The conversion of cortisol to cortisone was confirmed but using cortisone as substrate it was converted to a product other than cortisol thus indicating the difference between kidney and salivary gland metabolism of cortisone.

The intracellular localisation of 11β-hydroxysteroid dehydrogenase was studied by incubating subcellular particles of the rat submandibular salivary gland for the NAD-linked 11β-hydroxysteroid dehydrogenase. The subcellular particles were isolated from homogenate by differential centrifugation in sucrose. The main enzyme activity was found in the crude nuclear fraction and this led to the investigation of what part of the nuclear fraction was responsible for the enzyme activity. Nuclei were isolated using 1.8M Sucrose and these were found to retain the major enzyme activity. Attempts to solubilise the nuclear enzyme using detergent and hypotonic media were unsuccessful. It is of interest to see that the enzyme is nuclear bound as the liver enzyme has been located in the microsomal (105,000g/hr.) fraction. The enzyme in the salivary gland may influence nucleic acid metabolism. However, further work will have to be undertaken to investigate this possibility.

It was recognised that the rat submandibular gland was a tissue of differentiated cell types. In the study of 11β-hydroxysteroid dehydrogenase it was considered important to investigate the activity of the steroid

enzyme in separated tubules using the enzyme, collagenase, to break up the gland. However, little activity was recovered in the tubules probably because the collagenase used included a number of protease and peptidase impurities.

The histochemical localisation of the enzyme was in the duct cells. It was not located in any single component of the salivary duct system but was evident in striated, lobular and interlobular excretory ducts. Acinar tissue did not exhibit any activity. Enzymes involved in central metabolic pathways were present principally in the ducts whereas alkaline phosphatase was present in the acinar tissue and not in the duct system. Acid phosphatase appeared in equal amounts in both the acinar and ductal tissue. The presence of these enzymes gives further proof of the concept of a highly active metabolic duct epithelium.

The culture of salivary gland ducts was investigated but no 11β-hydroxysteroid dehydrogenase activity was present in cultures which were 7 days and 3 weeks old. The enzyme may require the addition of various cofactors and hormones which are lacking in the tissue culture.

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# GENERAL INTRODUCTION

# GENERAL INTRODUCTION

## 1. 11β-Hydroxysteroid Dehydrogenase.

In the early 1950's, several groups of workers studied the metabolism of cortisone and cortisol in man and other mammalian species which gave evidence for the probable existence of an enzyme catalysing the oxidation of the 11β-hydroxy functions of naturally occurring steroids (Mason, 1950; Burton et al., 1953). Cortisol and corticosterone were identified as the major glucocorticoid secretory products of the mammalian adrenal cortex, whereas cortisone and other 11-oxo group steroids were predominant in human urine. Cortisone was also found in large quantities in the urine of patients suffering from Cushing's syndrome in which large doses of cortisol were being secreted. Morris & Williams (1953) were, however, the first to suggest that cortisone might arise by the peripheral oxidation of cortisol, having found that small quantities of cortisone were detectable in the peripheral blood.

In subsequent years a large number of studies have been done to establish the properties and behaviour of the enzyme responsible, 11β-hydroxysteroid dehydrogenase (11β-HSD). Fish et al. (1953) and Caspi et al. (1953) reported the presence of 11β-HSD in porcine, bovine and rat liver preparations. Mahesh & Ulrich (1960) centrifuged the particulate fraction from a rat kidney homogenate at 104,000g for 1 hour and showed that the enzyme activity was found mainly in the nuclear particles and microsomes. By addition of $5 \times 10^{-4}$M NADP (nicotinamide adenine dinucleotide phosphate) they increased the yield of cortisone from cortisol thirteenfold. They found that the system was not freely reversible. However, Hurlock & Talalay (1959) found that the microsomal 11β-HSD of rat, guinea pig and bovine liver carries out the reaction reversibly with NAD (nicotinamide adenine dinucleotide) or NADP approximately equally effective as cofactors. They were the first to obtain a fairly highly purified 11β-HSD by using acetone-dried powders of microsomes

from rat livers. Their preparation was not soluble, although it needed centrifuging at 105,000g to sediment the enzyme. Sweat & Bryson (1960) found in mouse muscle an 11β-HSD whose activity was inhibited by NAD, whereas NADP increased the activity. NADP($H_2$) was found to be the preferred cofactor for the 11β-HSD's found in rat liver and kidney (Mahesh & Ulrich, 1960), rat brain (Grosser & Bliss, 1966), rat lung and testis (Koerner, 1966), human placenta (Osinski, 1960), human liver (Meigs & Engel, 1961), and human skin (Hsia & Hao, 1966). Other sites of 11β-HSD activity reported are in the murine thymus (Dougherty et al., 1960), the wall of the small and large intestine of the rat (Stahl & Tapley, 1963), and human testicular tumour tissue (Smith et al., 1964).

Bush et al. (1968) demonstrated that the relative substrate efficiencies and Km values of the enzyme were similar in whole (debris-free) homogenates, washed microsomes and acetone-dried powders of washed microsomes of rat liver. They reported that the optimal pH for the oxidative reaction of the liver microsomal 11β-HSD was pH9 - 9.15. The optimal pH reported by Koerner (1969) for the cortisol 11β-HSD in liver microsomes was pH10. Most other workers have chosen their assays to be carried out at the 'physiological' pH7.4.

Peterson and his coworkers (1955, 1957) demonstrated that cortisone and cortisol were rapidly interconverted in normal humans, whereas one steroid analogue, 2α-methylcortisone, and its related 11β-hydroxylic derivative were not interconvertible in the same way. Glenn et al. (1957) made similar findings using rat liver preparations in vitro. Bush and coworkers (1959, 1968) have shown with man in vivo, and with rat liver microsomes in vitro, that 11β-hydroxysteroids and 11-oxosteroids with a trans A/B ring fusion are a structural requirement for 11β-HSD enzymic activity.

11β-HSD activity can be demonstrated histochemically (Baillie et al., 1965c) and is present in two categories of tissue; firstly, where this

activity coexists with several other hydroxysteroid dehydrogenases and, secondly, where it is present to the exclusion of all other hydroxysteroid dehydrogenases. The first group is exemplified by the adrenal cortex (Calman et al., 1966; Hart et al., 1966) and in this instance the 11β-HSD is thought to be involved in general steroid biosynthesis and metabolism. With regard to the second group, the specific tissue sites where 11β-hydroxysteroids are utilised only are rat salivary gland ducts (Ferguson, 1967), renal collecting tubules (Baillie et al., 1965a; Baillie et al., 1966a), epididymal epithelium (McGadey et al., 1966), and certain placental membranes (Ferguson & Christie, 1967); each of these epithelial tissues is also considered to be involved in water and electrolyte transfer.

Baillie et al. (1965b) demonstrated weak 11β-HSD activity in the sebaceous glands from areas prone to acne vulgaris, along with strong $\Delta^5$-3β-, 16β- and 17β-HSD activity in the same region. Human and murine Leydig cells (Baillie et al., 1965c) utilised 11β-hydroxyandrostenedione and 11β-hydroxyprogesterone as substrates of choice for 11β-HSD activity while moderate 11β-HSD activity was apparent consistently in human interstitial cells (Baillie & Mack, 1966). The enzyme has also been histochemically demonstrated in mouse ovary and human placental trophoblast (Baillie et al., 1965c), kidney and liver (Baillie et al., 1965a), and mouse kidney and thymus (Hoyer & Andersen, 1970). Ferguson (1967) and Ferguson et al. (1970) studied cortisol utilisation by salivary glands, kidneys, and adrenals of various mammals by using a standard histochemical technique for the demonstration of hydroxysteroid dehydrogenase and found no obvious relationship between the levels of 11β-HSD activity in either of the tissues. Neither was the presence of 11β-HSD in salivary glands particularly associated with mucous or serous secretion, nor were sex differences in levels of activity evident. They reported that "rat submandibular gland homogenates converted cortisol to cortisone in significant quantities". (This gland can also be called the submaxillary gland.) Shannon & Prigmore (1960) described the presence of free

(non conjugated) 17-hydroxycorticosteroids (17-OHCS) in human parotid fluid before and after oral administration of synthetic adrenocortical analogues. They suggested that parotid fluid could be used as a medium to determine human adrenocortical status. Katz & Shannon (1964) reported that cortisol to cortisone transfer in dog parotid tissue was indicative of 11β-HSD in salivary gland tissue.

2. The Hydroxysteroid Dehydrogenases.

The hydroxysteroid dehydrogenases act by carrying out stereospecific conversions of hydroxyl and oxo groups with the addition, or removal, of hydrogen atoms using cofactors, NAD or NADP. The enzyme is named according to the number of the C-atom in the steroid nucleus to which the attacked hydroxyl group is attached and it is necessary to stipulate whether this hydroxyl group is on the α- or β- face of the steroid molecule. The position at which hydroxysteroid dehydrogenases are active are 3α-, 3β-, 6α-, 6β-, 7α-, 7β-, 11β-, 16α-, 16β-, 17α-, 17β-, 20α-, 20β- and 21-. Reviews have been written on several of these enzymes by Talalay (1962) and Dorfman & Ungar (1965). The histochemical documentation of these enzymes has been reported by Baillie et al. (1966b).

A. 3α-Hydroxysteroid Dehydrogenase.

Whether this enzyme is obtained from bacterial or mammalian sources it is specific for the 3α-hydroxyl function of 5α- and 5β- steroids. Talalay & Marcus (1965) were able to isolate a soluble NAD-linked 3α-HSD from Pseudomonas testosteroni that utilised various steroid substrates.

From mammalian sources Ungar & Dorfman (1954) and Tomkins & Isselbacher (1954) isolated the enzyme. Tomkins (1956) reported that the enzyme was in the soluble fraction of rat liver and the hydrogen acceptors could be either NAD or NADP. Dorfman & Ungar (1965) state that the enzyme occurs in rat liver, kidney and testis but not in lung, spleen, muscle or brain. Histochemically the enzyme will utilise androsterone and aetiocholanolone. In rat ovarian corpora lutea a strong 3α-HSD exists which appears to utilise 5α-steroids preferentially (Baillie et al., 1966b).

It has not been possible to separate completely mammalian 3α-HSD from 3β-HSD. Hurlock & Talalay (1958) could not separate the activities even after extensive purification. Hurlock & Talalay (1959) ascribed to the enzyme a transhydrogenase function.

B. 3β-Hydroxysteroid Dehydrogenase

This is a collective title which includes a $\Delta^5$-3β-HSD, a $\Delta^4$-3β-HSD, a 3β-HSD and a 3 (or 17) β -HSD.

The first enzyme, $\Delta^5$-3β-HSD, is concerned with steroid biosynthesis. Samuels et al. (1951) used both $C_{19}$ and $C_{21}$ steroids and found the enzyme present in ovarian, testicular, adrenal and placental tissue which irreversibly converts $\Delta^5$-3β-hydroxysteroids to $\Delta^4$-3-oxo group steroids. This enzyme, which is involved in a critical step in the sequence of biosynthetic reactions required to produce circulating corticosteroids from cholesterol in the adrenal cortex, involves the conversion of the pregnenolone, which is formed in the mitochondria (Koritz, 1968), into progesterone via 3β-oxidation and $\Delta^{5,4}$-isomerisation (Talalay, 1957). The first, and rate limiting, reaction is catalysed by 3β-HSD (3β-hydroxysteroid-NAD oxidoreductase; EC 1.1.1.51) (Neville & Engel, 1968). According to Beyer & Samuels (1956), the reaction is NAD mediated and occurs in the microsomal fraction of ox adrenal homogenate. McCune et al. (1970) and Basch & Finegold (1971) have found the enzyme present in the mitochondria of rat adrenal homogenate, and postulate the relationship of this enzyme system to the movement of steroid molecules. Histochemically (Baillie et al., 1966b) the reaction will develop in some tissues with NADP. Rosner et al. (1965) and Katkov et al. (1972) have found $\Delta^5$-3β-HSD activity in rat and boar salivary glands respectively.

The second enzyme is $\Delta^4$-3β-HSD. Dorfman & Ungar (1965) noted that this enzyme along with $\Delta^4$-3α-HSD which have been found in liver and testis (Ungar et al., 1957; Grosso & Ungar, 1964) convert $\Delta^4$-3-hydroxysteroids to the $\Delta^4$-3-oxo group. The activity may, however, be due to the action of other contaminating HSD's.

The third enzyme, 3β-hydroxysteroid dehydrogenase, is supposed to occur in mammalian liver. It is involved in the reversible oxidation of the 3β-hydroxyl group to the 3-oxo group of 5α- and 5β- steroids. It was obtained by Rubin & Strecker (1961) in the microsomal and soluble fractions of mammalian liver. The histochemical utilisation of various saturated 3β-hydroxysteroids has, however, been studied in detail by Baillie et al. (1966b). Baillie & Mack (1966) and Koide & Mitsudo (1965) have noted a formazan deposit in testicular Leydig cells and placental trophoblast respectively, when incubated with saturated 3β-hydroxysteroids and nitro blue tetrazolium.

The fourth enzyme, 3 (or 17) β-HSD, is bacterial in origin. Pseudomonas testosteroni can be grown with testosterone as the only source of carbon, and Dorfman & Ungar (1965) have indicated that the enzyme is somewhat non-specific. It has been shown to catalyse the oxidation of 3β-, 16β- and 17β-hydroxysteroids including ring A- aromatic compounds containing a 17β-hydroxyl group (Talalay & Marcus, 1956).

C. 6α- and 6β-Hydroxysteroid Dehydrogenases.

These enzymes have been reported in the urine of humans (Breuer & Breuer, 1965). However, little work has been done on these enzymes and their existence has largely been inferred from naturally occurring steroids and reaction products.

D. 7α- and 7β-Hydroxysteroid Dehydrogenases.

There has been little work done with these enzymes also and their presence can only be inferred from naturally occurring steroids (Dr. J. Campbell, Glasgow Royal Infirmary, personal communication).

E. 11β-Hydroxysteroid Dehydrogenase.

A fuller report on this enzyme, which is the main subject of this thesis, is given on p. 1-4.

F. 16α- and 16β-Hydroxysteroid Dehydrogenases.

These enzymes have been poorly documented biochemically. Histochemically, NAD is the usual cofactor in human testis; adrenal, ovarian and placental 16β-HSD will also use NADP, and an aromatic A-ring configuration is less well utilised than a $\Delta^5$A-ring configuration (Baillie et al., 1966b).

G. 17α- and 17β-Hydroxysteroid Dehydrogenases.

Corral - Gallerdo et al. (1966) have found a 17α-HSD in ovarian hilus cell tumour. Dorfman & Ungar (1965) have noted the presence of the enzyme in cow blood by the conversion of oestrone to 17α-oestradiol.

However, the literature on 17α-HSD's is very sparse compared to 17β-HSD's. Endahl & Kochakian (1962) using guinea pig liver and kidney found two 17β-HSD's, one requiring NADP present in the soluble fraction and the other requiring NAD in the mitochondrial fraction. Their enzymes were specific for $C_{19}$ neutral steroids, i.e. steroids without an aromatic ring A. Recently, Mulder et al. (1971) isolated and characterised the 17β-HSD from human erythrocytes.

A placental 17β-hydroxysteroid dehydrogenase has been studied biochemically (Langer et al., 1959; Adams et al., 1962; Jarabak et al., 1962, 1963) which is dependent on oestrogens. NAD or NADP can both react as cofactors. Talalay (1957), Crist & Warren (1966) and Williams - Ashman (1965) have discussed the implications of transhydrogenase action with this enzyme although this property has been vigorously disputed by Villee et al. (1960). Recently, Burns et al. (1971) have purified the human placental enzyme to a high degree of purity giving a single band by sodium dodecyl sulphate - polyacrylamide gel electrophoresis, corresponding to a molecular weight of approximately 33,500 daltons. Alanine was the only amino acid detected by N-terminal analysis and ultracentrifugation gave a molecular weight of 67,700 daltons which suggests that the enzyme exists in solution as a dimer.

Histochemically, 17β-HSD has been found in a wide variety of tissues (Baillie et al., 1966b).

H. 20α- and 20β-Hydroxysteroid Dehydrogenases.

Hubener et al. (1956) found a rat liver supernatant from a 17,500g centrifugate contained both 20α- and 20β-HSD's. Dorfman & Ungar (1965) have noted that these enzymes also occur in muscle, liver and kidney. Schoen & Samuels (1965) found a 20α-HSD in the testes of rat treated with ACTH. NADP is considered to be the biochemical cofactor for the 20α-HSD, although the histochemical system appears to be NAD dependent for the 20β-HSD (Baillie et al., 1966b).

20β-HSD has been crystallised from Streptomyces hydrogenans (Hubener et al., 1959). It required $NADH_2$ specifically and did not require $NADPH_2$. Ganguly & Warren (1971) studied 20β-HSD affinity labelling by cortisone 21-iodoacetate with a view to characterising the binding site of 20β-HSD, and a histidine residue was seen to react at that site.

I. 21-Hydroxysteroid Dehydrogenase.

Monder & White (1961) demonstrated that the 21-aldehydes of cortisone, cortisol and corticosterone were reduced to the corresponding alcohol function in rat and beef liver tissue.

3. Hormones in Salivary Glands.

Since the discovery by Lacassagne (1940) that there is sexual dimorphism in the murine salivary gland, it appears well established that steroid hormones influence salivary gland morphology and metabolism.

The work, however, has been carried out mainly with the androgens. Not only submandibular gland size (Lacassagne, 1941; Zebrowski, 1972) but submandibular gland sialoglycoprotein concentration (Zebrowski, 1972) and protease (Sreebny & Meyer, 1964) activity have been shown to be androgen sensitive.

The implication of female sex hormones in the regulation of rat submandibular gland metabolism is, however, less clearly understood.

Liu (1968) on the effect of steroid contraceptives, Liu et al. (1969) on the effect of oestrogen, and Liu & Lin (1969) on the effect of progesterone have outlined the implication of female sex hormones on the structural growth and development of the submandibular gland of female rats.

Shannon and coworkers (1959, 1964, 1967, 1969a, 1969b) have investigated the presence of adrenocorticosteroids in human saliva, and they concluded that measurements of cortisol and cortisone found therein possess the potential of monitoring adrenocortical responses.

4. Aims of the Work.

It has only been in recent years that the presence of the enzyme, 11β-HSD, was discovered in salivary gland. Katz & Shannon (1964) incubated radioactive cortisol with dog parotid gland slices and found it to be converted to cortisone. Ferguson (1967) has demonstrated the histochemical localisation of 11β-HSD in salivary gland duct cells. Ferguson et al. (1970) reported that rat submandibular gland homogenates converted radioactive cortisol to cortisone in large quantities.

This present work has set out to report more fully on the investigation of 11β-HSD in salivary gland with the purpose of verifying the previous physiological and histochemical reports.

The aims of the work are tabulated as follows:

A. (1) Provision of reliable and sensitive 11β-hydroxysteroid dehydrogenase assay system for the male rat submandibular salivary gland (SMSG) involving the isolation and positive identification of the product (cortisone). Examination of the kinetic aspects of 11β-hydroxysteroid dehydrogenase activity in submandibular salivary gland homogenate with a view to providing optimal assay conditions. The parameters are:

a. duration of incubation
b. buffer
c. pH
d. cofactor concentration and preference
e. substrate concentration
f. temperature of incubation
g. tissue (homogenate) concentration

(2) Investigation of other potential steroid substrates in submandibular gland.

(3) Identification of 11β-hydroxysteroid dehydrogenase in rat kidney and the comparison with the SMSG enzyme.

B. Separation of submandibular gland subcellular components and examination of 11β-hydroxysteroid dehydrogenase enzyme activity in each portion.

C. Histochemical examination of various enzymes including 11β-HSD in the submandibular salivary gland in order to differentiate between acinar cell and duct cell function.

D. Tissue culture of salivary gland duct epithelium with the intention of studying 11β-HSD present therein.

# PART I

## 11β-HYDROXYSTEROID DEHYDROGENASE ASSAY

# PART I

## 11β-HYDROXYSTEROID DEHYDROGENASE ASSAY

## INTRODUCTION

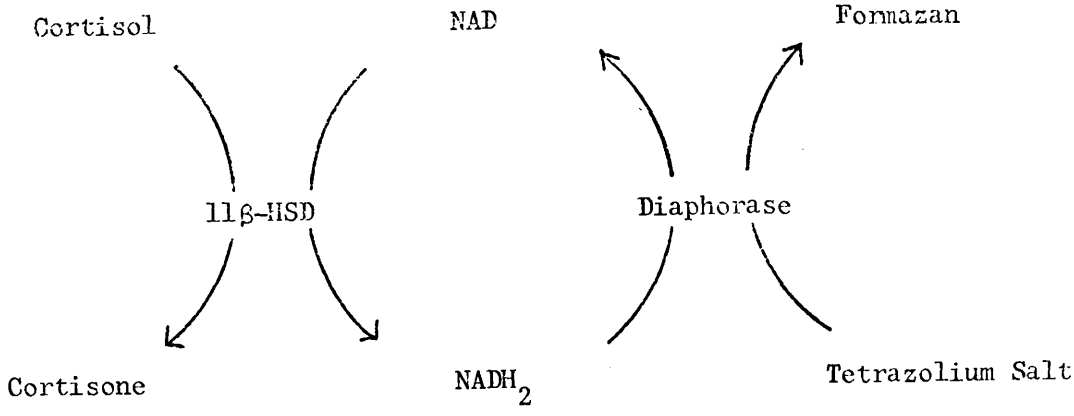
### 1. Methods of Enzymic Analysis

There are two categories of method used to follow enzyme reactions, i.e. continuous and sampling methods. Dixon & Webb (1964) and Bergmeyer (1963) have discussed the principles of enzymic analysis in detail, and Talalay (1962) reviewed enzymic analyses of hydroxysteroid dehydrogenases.

Continuous methods in the assay of dehydrogenases rely on coupling the enzyme reaction to another which involves the NAD- or NADP-oxidoreductase system. These are based on the extinction at 340nm of $NADH_2$ and $NADPH_2$ (Warburg & Christian, 1936) and the final amount estimated by using the molar extinction coefficient, $6.22 \times 10^3$ l/mole - cm. Hurlock & Talalay (1959), Bush et al. (1968) and Koerner (1969) have assayed liver microsomes for 11β-HSD using this principle. This method is often preferred in the case of purified enzymes but in the case of using homogenates or tissue slices this is erroneous, although Schoer & Glick (1967) have determined 3β-HSD in microgram samples of rat adrenal tissue and given a quantitative histological distribution of the fluorometric measurement of $NADH_2$ formed using an Aminco-Bowman Blank Subtract Spectrofluorometer. Bergmeyer (1963) has, however, discussed the problems in the use of coupled enzyme assays. The histochemical staining technique for 11β-HSD devised by Baillie et al. (1965c) uses a coupled enzyme system in which the dehydrogenase reaction is coupled with the reduction of the soluble and colourless tetrazoluim salt to the insoluble and coloured formazan which precipitates at the site of 11β-HSD activity (Fig. 1). However, it is not possible to have a reliable quantitation of 11β-HSD activity by histochemical studies.

In the sampling method of enzyme assays the reaction is stopped after a suitable time interval and then a measurement is made, either of the amount of product formed or of the substrate remaining. Koerner & Hellman

Figure 1. Principal of histochemical identification of 11β-HSD.


Cortisol
NAD
Formazan
11β-HSD
Diaphorase
Cortisone
NADH2
Tetrazolium Salt

(1964) used a method for the radiochemical analysis of 11β-HSD. However, they used a substrate concentration of $1.4 \times 10^{-4}$M which is an unphysiological level of cortisol: Symington (1969) has suggested that in steroid studies one should stay as near as possible to physiological conditions. For this reason the need for a reliable and sensitive method for the assay of 11β-HSD in submandibular homogenates is required.

The assay used in this report makes use of a reverse radioisotope dilution assay, where a tracer amount of steroid substrate is used but carrier steroid is added at the end of the incubation and before extraction of the media. This allows the amount of radioactivity incorporated into each isolated product to be assessed accurately.

2. Radioisotopes.

A. Definitions.

(1) Radioisotopes: Isotopes which are radioactive, i.e. undergo spontaneous disintegration or transformation with emission of radiation.

(2) Isotopes: Nuclides of an element, characterised by having the same atomic number but different mass numbers.

There are different types of radiation:

a. (α) Alpha radiation is emitted only by certain radioactive isotopes of the heavier elements. It consists of particles comprising two protons and two neutrons, i.e. a helium nucleus. It has very limited penetrating power, example - radium 226.

b. (β) Beta radiation consists of high speed electrons emitted from the nuclei of disintegrating atoms. Most β particles are negatively charged ($\beta^-$), these being electrons, but some may be positively charged ($\beta^+$), these being positrons. The penetrating power of beta radiation is low, examples - carbon-14, tritium, phosphorous-32.

c. (γ) Gamma radiation occurs when the product nucleus is formed in an excited state. It has high penetrating power and is electromagnetic, similar to X-rays.

d. X-radiation results from electron capture and internal conversion. It is identical with γ radiation of the same energy.

B. Types of Counter.

(1) Geiger - Muller Counter (or G-M tube).

This consists of a large round outer electrode with a fine wire stretched along the centre axis of the cylinder and this wire is maintained at a high potential with respect to the outer electrode. The inner space of the tube is usually filled with argon or an organic quenching compound such as ethanol. The open end of the tube is covered with an extremely thin window of mica beneath which is placed the radioactive material. When a charged particle enters the tube it causes the neutral counting gas to ionise, releasing electrons.

These free electrons are then accelerated to the positively charged central wire. As they progress through the gas additional molecules are ionised, with the result that a brief current surge is measured by a suitable electronic device and registered as one count. If the multiplication of the electrons is limited, the size of the pulse is proportional to the energy of the absorbed radiation. A chamber operated in this voltage range is known as a gas proportional counter.

The multiplication process reaches saturation when the voltage is further raised and the size of the resulting electrical pulse is independent of the energy of incident radiation. This region is known as the Geiger region of operation and it is in this voltage region that the G-M tube works. This has the advantage that the count rate is relatively independent of applied voltage and therefore the voltage supply does not have to be highly stable.

There are several disadvantages of using G-M tubes or proportional counters:

a. Self-absorption:- Weak radiation ($^{3}H$, $^{14}C$) is absorbed by the mass of its own source and inefficient counting will occur.

b. Geometry:- Poor spreading of samples on the sample holdings can lead to errors.

c. Coincidence Correction:- When the disintegrations per minute are so high the counter cannot register all the electrical surges, counts lower than the actual events will be recorded. Correction factors may be introduced.

d. Background:- Background count represents ionisation caused by either cosmic rays or radioactive contamination of the counting chamber striking the G-M tube.

For monitoring radioactive chromatograms a chart recorder is attached to the rate-meter, the peaks on the chart relating to the zones of activity.

(2) Scintillation Counters.

These are extremely effective counting systems since problems of geometry, coincidence, and self absorption can be avoided. It consists of the principle that when ionising radiation is absorbed by a suitable phosphor there is an emission of light, and the scintillations are counted by very sensitive photomultiplier tubes. The light falling on a photocathode inside the glass envelope causes the emission of one or more photoelectrons which undergo several stages of internal amplication so that a pulse emerges at the anode which can be recorded with a scaler or rate-meter. The size of the pulse emerging from the photomultiplier tube is proportional to the energy of the absorbed radiation.

a. Solid Scintillation Counters:- These are used for $\gamma$ radiation detection. The light emitting substance is a thallium activated crystal of sodium iodide which is external to the sample. The advantages of the solid scintillation counter are:-

(i) Economy.

(ii) Large volumes of solution can be counted. Urine activity can be counted in a well crystal scintillation counter (cylindrical crystal with hole cut in one end for the sample to be inserted in) (Hallberg & Veall, 1962) or an annular cup crystal scintillation counter (the sample in this case surrounds the crystal) (Bell, 1965).

(iii) Where the sample has to be left unchanged, this can be done by solid scintillation counters. Scintillation coffins have been constructed for in vivo counting of human beings and animals (Veall & Vetter, 1958).

b. Liquid Scintillation Counters:- A more detailed study of liquid scintillation counting theory is found in Bell & Hayes (1958) and Birks (1964). Liquid scintillation counting has considerable advantages over other forms of counting for isotopes such as carbon-14 and tritium which produce very weak beta rays.

(i) The sample is contained in the detection medium removing problems of proximity and geometry.

(ii) The exact efficiency of the samples can be determined.

(iii) One can differentiate between the radiation produced by two isotopes in the same sample.

In this process the energy passes first to the solvent (1, 4-Dioxane used in the present study) and then to the molecules of the primary fluor (PPO, i.e. 2, 5-Diphenyloxazole) which emits light at a wavelength of 363nm. This is not a suitable frequency for measurement and therefore a spectrum shifter or secondary

fluor is added to the vial (POPOP, i.e. 1, 4-Bis- (5-phenyloxazol-2-yl) benzene) which re-emits the light at a longer wavelength (430nm). These solvents and solutes have been reviewed by Turner (1967).

The liquid scintillation counters have the ability to discriminate between pulses of different energy. Sophisticated counters possess pulse height analysers with discriminators which may be set between energy limits so that undesired pulses can be eliminated, e.g. low energy background pulses.

There are certain sources of error which occur in the method, e.g.:-

(i) Inhomogeneity.

(ii) Chemical and colour quenching.

(iii) Insolubilisation of materials in organic solvents such as toluene and dioxane.

Steroids which are non polar may be readily dissolved in almost any of the available liquid scintillation mixtures. However, many biological molecules are polar and will not dissolve in normal scintillation solvents. There are several methods which allow one to count these molecules. They include:-

(i) Decolourisation of biological samples by bleaching agents for counting in solution.

(ii) Solubilisation methods by acid and alkali.

(iii) Combustion and oxidation procedures for biological materials.

(iv) Carbon-14 dioxide and Sulphur-35 dioxide absorption into a variety of bases.

(v) Emulsion counting in Toluene/Triton x-100 liquid scintillant.

(vi) Suspension counting - using Cab-O-Sil Gel.

(vii) Immersion counting - using filter paper.

Aqueous solutions of β-emitters of energy greater than 0.26 MeV will emit light in the spectral region 300nm to 700nm which can be detected in the liquid scintillation medium. This type of radiation is called Cerenkov Radiation, subject to colour quenching but not to polar or dilution quenching (Belchner, 1953).

C. Determination of Counting Efficiency.

The efficiency of counting of a sample is the ratio of the number of disintegrations recorded to those actually occurring in it, expressed as a percentage. The efficiency can be decreased by quenching, which is produced by processes that absorb some of the energy of radiation of the isotopes being counted. Examples of quenchers are polar substances such as water and ethanol or coloured samples which absorb light intended for the photocathode, i.e. around 430nm. Three main methods are used for determining counting efficiencies: internal standard, channels ratio and external standard. Peng (1966) has reviewed methods of determining counting efficiencies.

(1) Internal Standardisation involves the addition of a known amount of radioactivity to the sample. The efficiency of the sample can be determined from the difference between the count rate before and after adding the internal standard.

(2) The Channels - Ratio Method depends on a shift in the overall spectrum of pulse heights produced by the quenching agent. The count rate in each of two channels is recorded. One selects a channel with the whole of the energy spectrum generated by the isotope in question and the other channel has a portion of this spectrum. There are changes in these channel ratios with quenching. A calibration curve relating the concentration of quencher to the ratio of counts in the two channels is drawn up.

(3) External Standardisation involves the use of a gamma radiation source which generates a spectrum of Compton electrons in the sample vial. In the Nuclear Chicago liquid scintillation counter the gamma source is radioactive Barium and this spectrum is affected by quenching in the same way as the β particles in the sample. The count rate is automatically recorded in two of the channels at a given time and expressed as a ratio. A calibration graph of the ratio to the amount of quenching present is prepared.

D. The Principle of Isotope Dilution (Gorusch, 1966).

When a pure labelled compound is added to e.g. a biological fluid, it mixes with and becomes indistinguishable from the endogenous compound already present. If a sample of this compound is isolated in pure form the specific activity may be measured, and knowing the specific activity of and weight of the added material, it is now possible to calculate, regardless of the final yield, the amount of unlabelled compound already present in the mixture. In reverse dilution analysis, e.g. during in vitro study of the metabolism of labelled compounds by biological tissue, known amounts of unlabelled 'carrier' metabolites being studied are added at the end of the incubation period and then isolated in a radiochemically pure form.

Again, from their specific activity the original amounts of labelled metabolites can be calculated, regardless of losses during extraction and purification, e.g.:-

Let Y dpm/mole = final specific activity of purified metabolite

G mole = amount of unlabelled 'carrier' metabolite added

X dpm = original amount of radioactivity of labelled precursor added

then percentage conversion to metabolite

$$= \frac{YxG}{X} \times 100$$

assuming the labelled precursor to have negligible mass compared with the metabolite.

3. Tissue Homogenates.

A higher degree of fragmentation than tissue slicing or mincing is homogenisation. A homogenate is a whole tissue preparation in which the cell disruption is as complete as possible, while at the same time the cell particulates remain intact. For reviews of tissue homogenisation see Potter (1955, 1964).

One of the earliest homogenisers was that designed by Potter & Elvehjem (1936) which is a test tube with a close fitting glass pestle, the end of which has teeth. The tissue with some liquid medium is placed in the tube and the pestle, which is driven by an electric motor, is slowly moved up and down inside the tube. The tissue is macerated by the teeth and finely ground as it passes the pestle. There are pestles made of plastic such as Teflon, often in conjunction with a stainless steel shaft, which can withstand more rugged conditions.

Homogenates are also produced by machines that use high speed cutting knives:

(1) Waring Blendor: In this type of machine the blade assembly is fixed into the bottom of the vessel and driven from underneath. This device can deal with volumes of 25ml to 500ml. The vessels are of glass or stainless steel.

(2) Virtis Homogeniser: In this type of machine the blades are introduced through the top of the vessel. It can handle small volumes of homogenate.

(3) Colloid Mill: This device is more efficient than the Waring Blendor with regard to cell breakage, it can be efficiently cooled and it can rapidly process large quantities of material.

(4) Ultra-Turrax Homogeniser: In this device the blades cut up small quantities of tissue but there is a danger of too high a degree of fragmentation.

## 4. Principles of Isolation and Identification of Steroids.

### A. History.

Since the first steroid hormone, oestrone, was isolated in 1929 there have been many advances in techniques for isolation and identification of steroids. There was much work done on steroid metabolism in the 1930's with the classical technique of administering large doses of known crystalline steroids to experimental animals and isolating crystalline metabolites from body fluids. Girard & Sandulesco (1936) discovered two reagents known as Girard's reagents T and P, (trimethylaminoacetohydrazide hydrochloride and pyridylacetohydrazide hydrochloride) which could efficiently separate oxo form steroids from non-oxo form steroids. Reichstein (1936) introduced the use of absorption column chromatography for the separation of adrenocortical steroids, and chromatographic techniques advanced from there. Paper chromatography (Zaffaroni et al., 1950) introduced steroid analysis at the microgram level. Partition column chromatography (Martin & Synge, 1941), thin layer chromatography (Kirchner et al., 1951), gas chromatography (Eglington et al., 1959), and gel chromatography (Sjovall et al., 1968) are other chromatographic techniques developed for the isolation and identification of steroids.

Countercurrent distribution, pioneered by Engel et al. (1950) with oestrogens, has now been brought to a high level of efficiency for most steroids.

For the spectral identification of steroids there are many techniques available:

Infrared Spectroscopy (Furchgott et al., 1946);

U.V. Determinations (Dorfman, 1953);

Optical Rotary Dispersion (Djerassi, 1957);

Nuclear Magnetic Resonance (Shoolery & Rogers, 1958);

Mass Spectrometry (Reed, 1958);

X-ray Crystallography (Bernal, 1932).

There are also certain colour reactions which aid analytical determination of steroids: Kober reaction (Kober, 1938), Zimmermann reaction (Zimmermann, 1935) and fluorescense analysis (Cohen & Bates, 1947).

Isotope methods also have helped the isolation and identification of certain steroids.

B. Chromatography (used for isolation and identification).

Chromatography represents one of the greatest technical advances in biochemistry of the past several years. In its many methods it has revolutionised the separation and detection of steroid mixtures as well as the determination and identification of isolated steroids.

There are several kinds of chromatography:

(1) Adsorption Chromatography.

This involves separation of the steroid mixture between solid and liquid phases. There are two forces:-

a. Adsorption which tends to hold the solute to the surface of the adsorbent.

b. Elution which tends to remove the solute from the adsorbent.

A highly polar substance is that which exhibits more affinity for the adsorbent and a low-polar substance is that which exhibits more affinity for the fluid phase.

(2) Partition Chromatography.

This involves separation of the steroid mixture between two liquid phases or a gas - liquid phase according to the relative affinity between the solute and each of the two phases of the system. Under partition chromatography are the techniques of:-

a. Paper chromatography

b. G.L.C. (gas liquid chromatography)

(3) Thin Layer Chromatography (T.L.C.).

Thin layer chromatography has developed from column adsorption chromatography. It is performed on open layers of

adsorbent materials supported on glass plates. A thin uniform film of silica gel or aluminium oxide containing a binding medium, such as calcium sulphate, is spread on to a glass plate, the thin layer allowed to dry and then activated by heat at 110°-250°C. The steroid dissolved in solvent is spotted on to the plate and once the solvent has evaporated the plates are placed vertically in a glass tank which contains a suitable solvent, and in a short time, e.g. 30-60 minutes, a separation is produced by the solvent rising through the thin layer, differentially carrying the components of the spots from the origin depending on the adsorption of the components on the thin layer. The spots may be detected by a variety of reagents or dyes. Camag DSF - 5 silica gel with an added ultraviolet indicator shows corticosteroids absorbing under U.V. light. Thin layer chromatography has advantages in that it is speedy, efficient, sensitive and drastic detection agents can be used.

Neher (1964) reviewed thin layer chromatography. In the review he deals in detail with preparation of adsorbent, of the layer application of the specimen, the choice of suitable solvents and of detection agents.

(4) Paper Chromatography.

Developed in 1944 (Consden et al., 1944), it was not adopted for steroid separations until the introduction of special solvent systems by Zaffaroni et al. (1950) and Bush (1952). Several reviews have been written on paper chromatography (Bush, 1961; Neher 1963, 1964; Dominguez, 1967). Separation of steroids on paper is based on a liquid partition of the compounds. The steroid mixture is applied to a strip of filter paper (the support) on which is the stationary water phase. The steroid mixture is transported in a given direction by a suitable solvent system; it will travel along the paper at a certain fraction of the rate of travel of the solvent front, the

fraction depending on the distribution of the compounds between the two phases (stationary and mobile). The ratio of the distance travelled by the compounds to the distance travelled by the solvent front from the original spot is called the Rf value of the compound. Under strictly controlled conditions the Rf is an important constant for identification purposes.

Paper chromatography has several disadvantages in that it is slow and drastic detection agents cannot be used.

(5) Gas Chromatography.

Gas chromatography was postulated in 1941 and introduced into practice in 1952 by James & Martin. However, the first report of its successful application to the field of steroids did not appear until 1959 (Eglington et al., 1959).

There have been several reviews on the gas liquid chromatography (G.L.C.) of steroids (Neher, 1964; Wotiz & Clark, 1966; Brooks & Sabkiewicz, 1967; Horning et al., 1969). To enable identification of the structure of unknown compounds, G.L.C. has been standardised resulting in the Retention Index system of Kovats (1965). Menini & Norymberski (1965) introduced a method not only to separate steroids but also to quantitate them providing an internal standard is used. An ancillary technique used in direct conjunction with G.L.C. is the monitoring of the column effluent by a mass spectrometer (Eneroth et al., 1964). This gives excellent separation of compounds in a mixture and a reliable means of identification of each compound.

Gas chromatography has become the preferred method for rapidly and accurately analysing any volatile substance. It is a form of partition chromatography differing from the others in that the mobile phase is a gas and the stationary phase is a high boiling liquid with an inert solid as a support. A limitation of G.L.C. is that the components must be volatile. However, the method is now increasingly

being extended to include non-volatile and polar compounds by the conversion of these into volatile derivatives. This separation method is very fast, sensitive and usually an effective means of separation. The effluent gas passes through a monitoring device which measures changes in the gas and converts these to electrical responses on a recorder. There are several advantages of G.L.C.:-

a. Only small amounts of complex mixture, e.g. 1ul, are needed for efficient separation.

b. Greater ease of detecting traces of solute in a gas phase compared with a liquid phase.

c. Separations are reproducible provided the conditions are the same.

Disadvantages are:-

a. Components must be volatile.

b. Components may be decomposed by high temperature, e.g. the corticosteroids, cortisol and cortisone are degraded by G.L.C.

c. Cost of equipment.

C. Spectral Examination for Identification of Steroids.

(1) Colour and Fluorescence Reactions.

These reactions frequently indicate the presence of certain functional groups in the molecule on the steroid, e.g. $\Delta^4$-3-oxo group, 17-oxo group, phenolic nucleus etc. However, there are relatively few reactions suitable for the identification of individual steroids. There is a specific reaction for oestrogens, the Kober reaction which with sulphuric acid in the presence of phenol gives a yellow colour turning pink after dilution with water.

(2) U.V. Spectroscopy.

Engel (1963) has written a review on ultraviolet absorption, fluorescence spectra and absorption spectra in concentrated sulphuric

acid. These spectra can be easily measured but only provide useful information on functional groups, e.g. the $\Delta^4$-3-oxo group has a U.V. absorption at 240nm.

(3) Infra-Red Spectroscopy.

I.R. spectroscopy has the advantage that an I.R. spectrum is unique and characteristic for a compound by comparison of its spectrum with that of a reference sample. There is an atlas of steroid I.R. spectra (Neudart et al., 1965).

(4) Mass Spectroscopy.

This is perhaps the most sensitive method for the identification of micro-amounts of material. Spectra may be obtained with 1-10μg of a compound.

(5) X-Ray Crystallography.

This provides the most certain identity of a compound but it requires highly specialised equipment which is not readily available. There is also much time and labour involved in this technique. A survey of X-ray crystallography can be found in Parsons et al. (1956).

(6) Nuclear Magnetic Resonance.

This method is of great importance for the characterisation and localisation of substituents as well as for the configuration of individual rings. However, it does not normally reveal complete structure and has not been developed for microanalysis. Tables of spectra in a solution of deuterochloroform (which least disturbs the absorption of the measured substance) for steroids have been shown by Shoolery & Rogers (1958).

(7) Optical Rotary Dispersion.

The optical rotation of an organic compound depends on the wavelength of light used. The estimation of difference in the optical rotation dependent on the wavelength of ultraviolet radiation in the region of 280-700nm is carried out. From the measured values a

dispersion curve is constructed. This method is important especially for the localisation of oxo groups in the steroid molecule as well as for the configuration of rings (cis, trans) and was developed for steroids by Djerassi (1957, 1960). However, the method is not useful in the identification of an unknown compound.

D. Identification of Isotopically Labelled Steroids.

There has been a review article written on the identification of steroids by Brooks et al. (1970) and in this there are described methods of identification of isotopically labelled steroids. Berliner & Salhanick (1956) described a method where the isolated steroid is converted to derivatives and the specific activity of the derivatives compared with the isolated steroid. Recrystallisations, where the specific activity of the residues from the mother liquors agree with that of the last crop of crystals (Axelrod et al., 1965), also give positive identification and verification of purity of a steroid. The specific activities should agree within $\pm$ 5% of the mean value.

## MATERIAL AND METHODS

### 1. 11β-Hydroxysteroid Dehydrogenase Assay in Rat Submandibular Salivary Gland.

#### A. Materials.

Cortisol-4-$^{14}$C (Specific Activity, 52-58mC/mM) was purchased from the Radiochemical Centre, Amersham, Bucks and was purified by thin layer chromatography in the system of chloroform:ethanol (85:15v/v).

Reference steroids (cortisol, cortisone, cortisone acetate and adrenosterone), nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from the Sigma Chemical Company, London. The purity of the reference steroids was established by recrystallisation and thin layer chromatographic identification. Cortisol was recrystallised in methanol - water, cortisone in methanol - water, cortisone acetate in acetone - ether and adrenosterone in ether - ethanol. Their respective Rf values in the thin layer chromatography system of chloroform:ethanol (85:15v/v) were 0.48, 0.60, 0.74, 0.80. All the water was glass redistilled. All solvents and other chemicals were of analytical grade and were obtained from the British Drug Houses Ltd., Poole, Dorset. Silica gel Camag DSF - 5 + U.V. indicator was used for thin layer chromatography. The buffers used were 0.05M tris - hydrochloride (pH7.0 - 9.0), 0.067M Sorensen's phosphate (pH5.3 - 8.0), 0.1M Clark & Lub's borate (pH8.0 - 10.0) and tripotassium phosphate (pH7.5 - 11.0). Young adult male Sprague - Dawley rats were purchased from A. Tuck & Son Ltd., England and were in the range of 200-250g at the time of use. The scintillation fluid used for radioactive counting was NE 250, a dioxane-based scintillant purchased from Nuclear Enterprises, Sighthill, Edinburgh.

#### B. Washing of Glassware.

All glassware was cleaned by steeping in 2% RBS 25 (Chemical Concentrates Ltd., London, SW11 6RX) for 24 hours. This cleaned any residual steroid or radioactivity from the glass surface. RBS 25 was preferred to chromic acid because it is safer and easier to use. The cleaned glassware

was rinsed three times in tap water and twice in glass distilled water. The glassware was then put into a hot oven at 130°C to dry. After drying, the glassware was stored until further use.

C. Preparation of Homogenate and Incubation.

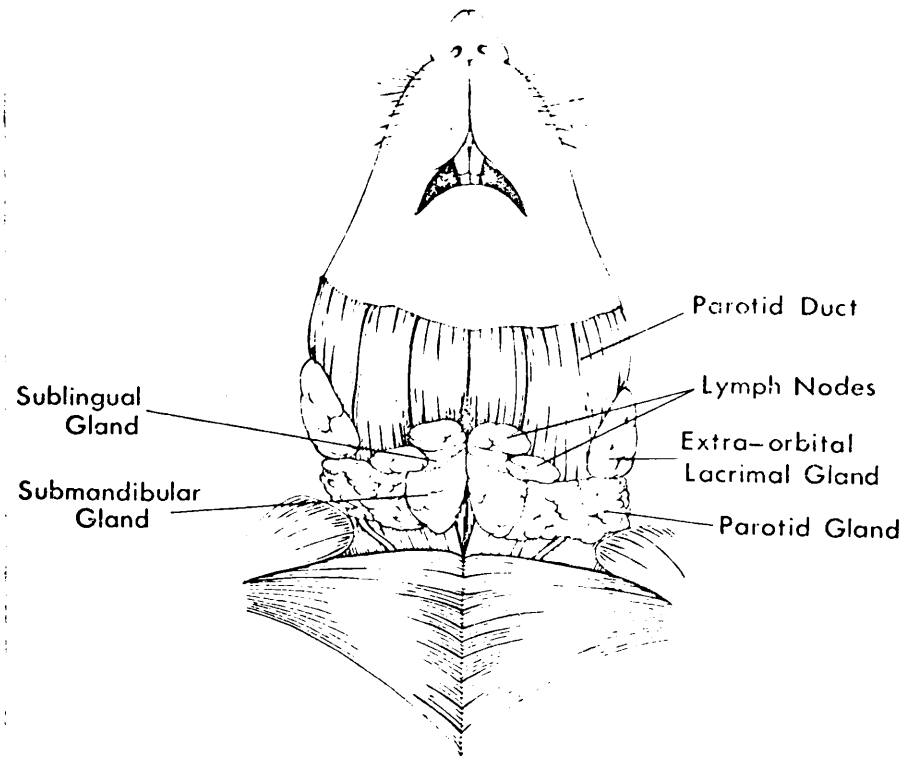
Mature Sprague - Dawley male rats (weight 250g), maintained on a commercial diet 41B, were killed by cervical dislocation and the sub-mandibular salivary glands (Fig. 2) were dissected out, chilled, weighed and homogenised with a teflon homogeniser - 100mg. tissue with 9.9ml of buffer. Cofactor (13.8μ moles of NAD or NADP) was added to the homogenate and the pH of the medium adjusted if necessary. The range of pH examined was pH5.0 - 11 but for optimal conditions of pH the NAD-linked 11β-HSD was prepared for assay at pH8.5 and the NADP-linked enzyme at pH7.6. The homogenate was then transferred to 50ml Erlenmeyer flasks containing cortisol-4-$^{14}$C (0.3-30n moles) dissolved in 0.05ml propylene glycol. A substrate concentration of 10n moles was used for the majority of the assays. The final volume of the assay was 11ml.

The flasks were incubated at 37°C in air in a shaking water bath for 0-64 minutes and the reaction was stopped by adding 2 volumes of acetone. To this mixture were added 1.7μ moles of both carrier cortisol and carrier cortisone in order that the amount of radioactivity incorporated into either the substrate or product could be assessed accurately. Control flasks were prepared by boiling the homogenate for 30 mins. and incubating as above.

D. Extraction Procedure.

The flask contents were allowed to equilibrate for 1 hr. and then filtered with washings of acetone:water mixture (2:1v/v). From the filtrate, acetone was evaporated under vacuum and the steroids were recovered by extracting twice with 3 volumes of benzene:chloroform (6:1v/v) and once with 3 volumes of ethyl acetate. The total recovery of the added radioactivity was in the range of 87-95% (Table 1). The combined organic extracts were dried over anhydrous sodium sulphate and taken to dryness.

Figure 2. Diagram of male rat salivary glands and adjacent structures.


Parotid Duct
Lymph Nodes
Sublingual Gland
Extra-orbital Lacrimal Gland
Submandibular Gland
Parotid Gland

| CPM in Sample | CPM after Extraction | % Recovery |
|---|---|---|
| 591,965 | 516,800 | 87.3 |
| 591,965 | 562,080 | 94.9 |
| 591,965 | 561,440 | 94.8 |
| 591,965 | 519,520 | 87.7 |
| 591,965 | 524,640 | 88.6 |

Table 1. Recovery of $^{14}C$ in solvent phase after extraction

(Solvent Extraction:-

2 x 3 vols. Benzene:Chloroform (6:1 v/v)
+ 1 x 3 vols. Ethyl Acetate)

E. Isolation and Identification of Radioactive Zones.

The metabolite (cortisone) was separated by chromatography on thin later plates coated with silica gel; the system was chloroform:ethanol (85:15v/v). Another three T.L.C. systems were examined and the Rf values found are shown in Table 2. Localisation of the steroids was obtained by using a U.V. light and a strip scanner (Nuclear Chicago) for the distribution of $^{14}C$-compounds. The two areas representing cortisol and cortisone were eluted separately with methanol.

The chromatographic mobilities of the two radioactive compounds were compared with those of reference steroids and found to correspond to cortisol (Rf 0.48) and cortisone (Rf 0.60). For positive identification of the radioactive cortisone zone this was subjected to reverse isotope dilution with non-radioactive cortisone and the specific activity remained constant after repeated crystallisations (Table 3). The acetate, prepared using acetic anhydride and pyridine (Bush, 1961), was found to have a constant specific activity. Oxidation of the radioactive cortisone zone and carrier cortisone by potassium chromate (Bush, 1961) both resulted in the formation of adrenosterone, as identified by chromatographic mobility of the reference adrenosterone (Rf 0.80).

F. Analytical Measurement.

Measurement of $^{14}C$-steroids was carried out in a Nuclear Chicago liquid scintillation counter with a 65-69% efficiency. Quenching corrections were made by employing the external standard method but all counts recorded showed maximum efficiency and negligible quenching.

The mass of the steroids was quantitated in a Unicam S.P. 500 spectrophotometer using the specific absorbances of the steroids, the blank being an equivalent methanolic elution from a silica gel plate run in the same T.L.C. system, and the calculation of percentage transfer of cortisol to cortisone was produced from the reverse isotope dilution formula:-

| System | Adsorbent | Rf of Cortisol | Rf of Cortisone |
|---|---|---|---|
| Benzene:Methanol 9:1 v/v | Silica Gel | 0.16 | 0.20 |
| Chloroform:Ethanol 95:5 v/v | Silica Gel | 0.10 | 0.22 |
| *Chloroform:Ethanol 85:15 v/v | Silica Gel | 0.48 | *0.60 |
| Chloroform:Acetone 8:2 v/v | Silica Gel | 0.12 | 0.23 |

* Preferred System

Table 2. Thin layer chromatography systems for the separation of cortisol and cortisone.

| Steroids | | Specific Activity (dpm/μ mole) | |
|---|---|---|---|
| | | Crystals | Mother Liquor |
| Cortisone (from Methanol-Water) | 1st | 1006 | 1089 |
| | 2nd | 1001 | 1045 |
| | 3rd | 996 | 1017 |
| Cortisone Acetate (from Acetone-Ether) | 1st | 1113 | 1070 |
| | 2nd | 1100 | 1058 |
| | 3rd | 1058 | 1113 |

Table 3.

Recrystallisation to constant specific activity with authentic steroids of the metabolite, cortisone, and the metabolic derivative, cortisone acetate.

$$\% \text{ Conversion} = \frac{\text{Specific activity of recovered cortisone} \times \text{Initial weight of carrier cortisone}}{\text{Total counts put in originally (in the form of cortisol)}}$$

The labelled precursor (cortisol) was assumed to have negligible mass compared with the carrier.

The decrease in the percentage of the substrate could also be used as a measurement.

G. Units of 11β-HSD Activity.

One unit of 11β-HSD activity is defined as 1n mole of cortisol converted to cortisone per minute.

H. Spectrophotometric and Fluorometric Methods of 11β-HSD Analysis.

The activities are determined optically from the rate of reduction of NAD in the presence of the appropriate steroid.

The spectrophotometric reaction mixture contained in a final volume of 3ml:100μ moles of phosphate buffer pH9.7, 0.4μ moles of NAD, 100μg (275n moles) of cortisol dissolved in methanol and 100mg. of submandibular salivary gland in a 10% (w/v) homogenate in 0.25M Sucrose (B.D.H., Dorset). The increase in absorbance at 340nm and room temperature (20°C) was measured in cuvettes of 1.0cm. light path against a blank containing all the ingredients except the steroid.

The fluorometric assay was based on the method of Schoer & Glick (1967) who used an Aminco-Bowman Blank Subtract Spectrophotofluorometer for the analysis of 3β-HSD in rat adrenal. To alter the method for submandibular 11β-HSD assay, cortisol was used as substrate and homogenate was used in place of microtome sections. The reaction mixture contained 10μl of a 1% (w/v) submandibular salivary gland homogenate prepared in 0.15MKCl, 50μl of NAD - buffer mixture (20 volumes of 0.1M glycine buffer at pH9.6, 10 volumes of 0.76mM NAD and 20 volumes of distilled water - prepared fresh for use). The fluorescence (345nm excitation, 455nm fluorescence) of this was recorded at room temperature (20°C). To the mixture was added 1μl of 2mM cortisol in ethanol and the fluorescence was

read each minute at room temperature. A standard curve of concentration of $NADH_2$ solutions measured fluorometrically over the range $4.3x10^{-6}M$ to $3.1x10^{-5}M$ was plotted. The blanks used were a substrate blank, an enzyme differentiation blank where ethanol was added without steroid to see if there was alcohol dehydrogenase activity, and an enzyme blank.

Note: All blanks did not show any activity, and for the enzyme determinations the blank subtract apparatus was used to block out the particulate matter with a substrate blank.

2. Gas Liquid Chromatography for the Identification of Borate Buffer Products.

The steroid products were prepared for on-gauze gas liquid chromatography by the method of Menini & Norymberski (1965). Stainless steel Dixon gauzes were used and the steroids were separated using a Pye Gas Chromatograph (Model 64) equipped with hydrogen flame ionisation detectors and column composed of 1.4% OV-17 (Applied Science Laboratories, Pennsylvania).

The column temperature was 230°C and the nitrogen flow was 50ml/min. The incubation products were identified by comparison of their retention times with those of authentic compounds.

3. Incubations of Submandibular Salivary Gland with Cortisone and Corticosterone.

A. Cortisone.

Submandibular salivary gland homogenates were prepared and incubated as described on p. 27, replacing the steroid substrate with 8n moles of cortisone-4-$^{14}C$ and the cofactor with either $NADH_2$ (14μ moles) or $NADPH_2$ (14μ moles). Cortisone-4-$^{14}C$ (S.A. 57mC/mM) was purchased from the Radiochemical Centre, Amersham and the radiochemical purity ascertained in the system chloroform:ethanol (85:15v/v). Incubations were carried out at pH7.4 for 10, 20 and 30 mins. duration, and incubations of 7 mins. duration at pH values 7.4, 8.0 and 8.5 were also investigated. Control flasks containing homogenate that had been boiled for 30 mins. were also incubated. Carrier steroids of 1.7μ moles cortisone and 1.7μ moles

cortisol were added at the end of the incubations and the steroids extracted and separated.

B. Corticosterone.

Corticosterone-4-$^{14}$C (S.A. 61mC/mM) was purchased from the Radiochemical Centre, Amersham and 8n mole aliquots were incubated at pH8.0 with submandibular gland homogenate according to the assay on p.27. Both cofactors, NAD and NADP, were used for the incubations which lasted 30 mins. Non-radioactive corticosterone and 11-dehydrocorticosterone were run in the T.L.C. systems, chloroform:ethanol (7:3v/v), chloroform:acetone (8:2v/v), and cyclohexane:ethyl acetate (1:1v/v) in order to find the best system for separation. The chloroform:acetone (8:2v/v) system gave the best separation with Rf values for corticosterone and 11-dehydrocorticosterone of 0.24 and 0.33 respectively. The radioactive metabolites extracted from the above incubations were separated in this system.

4. 11β-HSD in Rat Kidney.

Adult male Sprague - Dawley rats (200-250g) were killed by cervical dislocation and from these, kidneys were excised, blotted and weighed. Kidney homogenate was prepared by 6 strokes at 1,000rpm with a teflon homogeniser in isotonic saline at a concentration of 10% (w/v).

In the assay for the enzyme 10n moles of cortisol-4-$^{14}$C or cortisone-4-$^{14}$C was used as substrate dissolved in 0.05ml propylene glycol. 100mg. wet weight gland equivalents of 10% (w/v) homogenate (i.e. 1ml), 8.95ml of 0.067M phosphate buffer and 1ml of cofactor mixture were combined to a final volume of 11ml. NAD (13.8μ moles) or NADP (13.8μ moles) was used for the cortisol incubation at pH value of pH8.5 and pH7.4 respectively. When cortisone was used as substrate 13.8μ moles of both reduced pyridine nucleotides were used at pH7.4. The remaining conditions of assay were as described by the reverse isotope dilution assay on p.27.

## RESULTS

1. 11β-HSD Assay with and without Carrier Steroids.

When no carrier steroids were used in the assay there was a difficulty in determining the specific activity of the separated material as when the amount of steroid recovered falls to a few micrograms the accuracy of the determination of 11β-HSD activity falls off sharply.

The addition of carrier steroids to make the method a reverse isotope dilution analysis was found to be a far better method of assay. The U.V. absorbing spots could be easily detected under U.V. light. Carrier steroids allow the specific activities of the isolated products to be measured accurately and the boiled homogenate incubations were in the range of 2.48 $\pm$ 1% conversion. This method proved reliable, sensitive and accurate.

2. 11β-HSD Assay by Spectrophotometric and Fluorometric Methods.

Spectrophotometric and fluorometric analyses were useless for the detection of 11β-HSD in submandibular salivary gland homogenate. There was no activity seen in the homogenate preparations using either of the two methods.

They were not sensitive enough assay methods for the detection of 11β-HSD in submandibular salivary gland.

3. Effect of Various Parameters on 11β-HSD Activity in SMSG Homogenate.

The 11β-HSD assay (p.27) was used, the parameters of activity being altered where shown.

A. Time of Incubation.

Figs. 3 and 4 show the progress curves of the 11β-HSD reactions using NAD and NADP as cofactors. The curves are in the general form of enzyme velocity reactions. The initial velocity is increasingly proportional; up to 3 minutes for the NAD-linked 11β-HSD at pH8.3, up to 9 minutes at pH7.3 and up to 25 minutes for the NADP-linked enzyme at pH7.3, and after these times the velocity falls with time. It can be seen that the

Figure 3. Effect of time of incubation on NAD-linked 11β-HSD activity in submandibular gland homogenate.

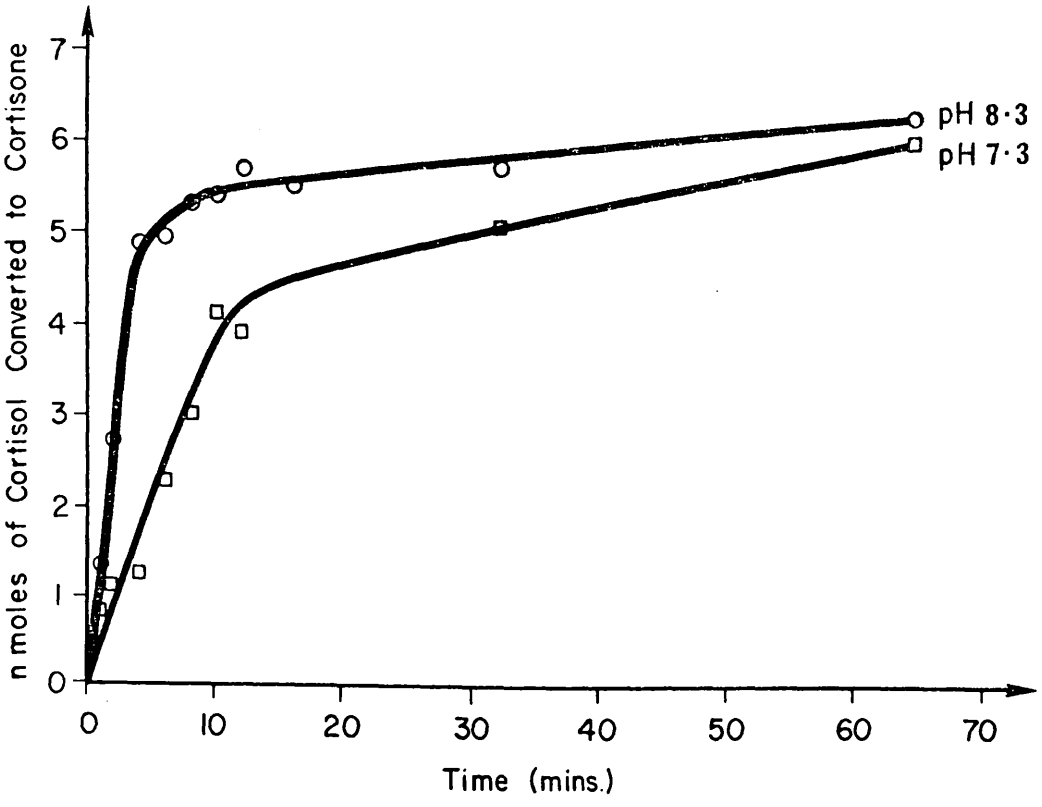
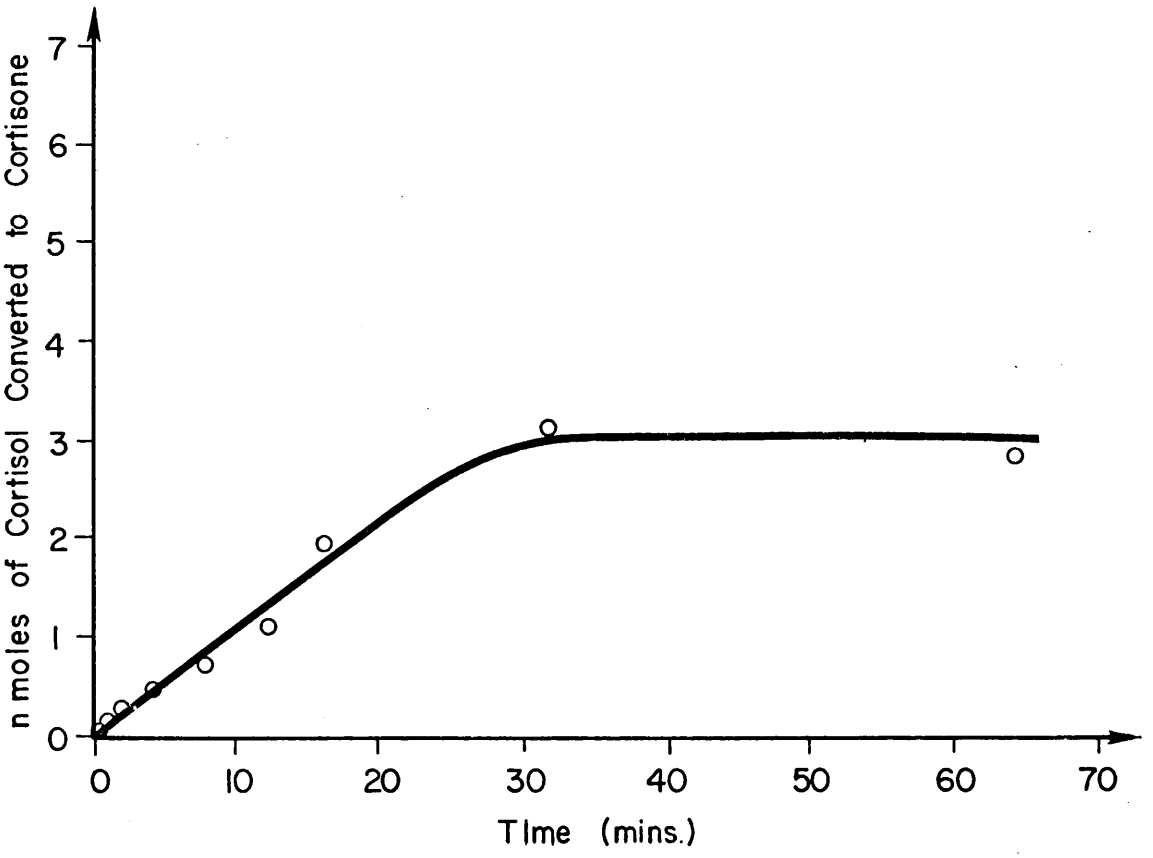

n moles of Cortisol Converted to Cortisone
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
Time (mins.)
pH 8·3
pH 7·3

Figure 4. Effect of time of incubation on NADP-linked 11β-HSD activity in submandibular gland homogenate.


n moles of Cortisol Converted to Cortisone
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
Time (mins.)

initial velocity is (approx. 1.0 unit) greater at pH8.3 for the NAD-linked 11β-HSD and therefore this pH must be nearer the optimum than pH7.3. Therefore an appropriate time of incubation for the initial rate of reaction of NAD-linked 11β-HSD to be proportional would be 2 minutes. An appropriate time of incubation for the initial rate of reaction of the NADP-linked 11β-HSD would be 7 minutes.

B. pH and Various Buffers.

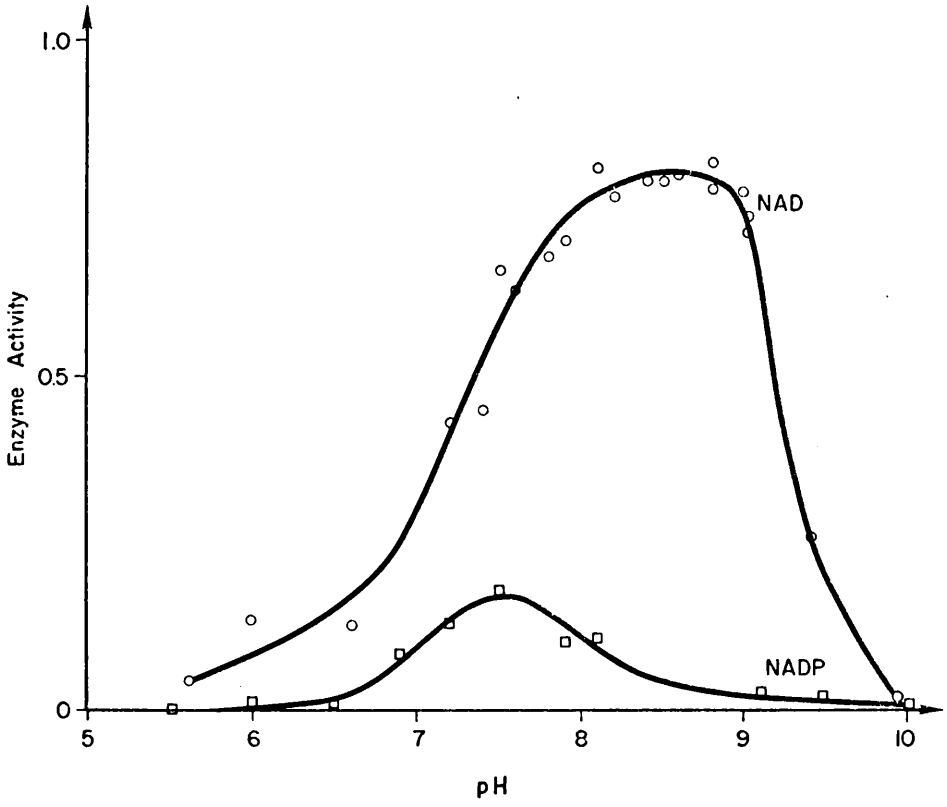
Using borate buffer (pH8 - 10) a chemical oxidation of both cortisol and cortisone occurred. There was a radioactive area of lower polarity (over Rf 0.70) when an incubation was carried out. Analysis of this area by gas liquid chromatography showed that 11β-hydroxyandrostenedione and adrenosterone had been formed. Cholesterol was also present in this area. The cortisol was probably oxidised to the 11β-hydroxyandrostenedione and the cortisone oxidised to the adrenosterone. Gas liquid chromatography can degrade cortisol and cortisone to these products but since the less polar area was used for G.L.C. determination then this factor can be discounted.

A similar problem arose using tris-hydrochloride buffer (pH7 - 9) where a chemical reaction between the steroids and the buffer took place. When cortisone was incubated with tris-hydrochloride buffer alone for 2 hrs. a radioactive peak of Rf 0.35 was formed, and when cortisol was incubated a peak of Rf 0.25 was formed.

No chemical reaction occurred between the phosphate buffers (pH5.3 - 11) and the steroids. The phosphate buffers were therefore considered suitable for the 11β-HSD assay and neither of the other buffers was considered of use.

pH curves of the NAD-and NADP-linked 11β-HSD's were established by incubating cortisol at different pH values. Care had to be taken to measure the pH of incubation mixture before and after incubation as it can alter significantly. The pH curves are shown in Fig. 5 and these show

Figure 5. Effect of pH on 11β-HSD activity in submandibular gland homogenate.


1.0
0.5
0
Enzyme Activity
NAD
NADP
5
6
7
8
9
10
pH

that the optimal pH of the NAD-linked 11β-HSD is between pH8.1 and pH8.9 and the optimal pH for the NADP-linked 11β-HSD is pH7.6.

C. Cofactor Concentration.

Differing cofactor concentrations of NAD and NADP were incubated in 11β-HSD assays and the results are graphed of enzyme activity against cofactor concentration in Fig. 6. pH8.5 was used for the NAD incubations and pH7.6 for the NADP incubations.

It is seen from the curves that 13.8μ moles (1.26mM) of cofactor is a sufficient amount to add to the assay system in order not to become a limiting factor in the reaction.

The substrate concentration for the assays was 0.6μM and the saturating amount of cofactor needed was 1.26mM which is 2,000 times the substrate concentration which shows that the enzyme system is very crude.

When no cofactor was used in an incubation at pH7.4 only a 2% conversion of cortisol to cortisone was observed which shows the need for pyridine nucleotide cofactors to give a large enough and, therefore, more reliable conversion.

D. Temperature.

11β-HSD assays were carried out for the NAD- and NADP-linked 11β-HSD's, varying the temperature of assay. Graphs were drawn of these parameters against enzyme activity and are shown in Fig. 7. The optimal temperature of the enzyme in the presence of both cofactors is 37°C: however, the importance taken from this result is discussed on p.39.

E. Substrate Concentration.

With other variables (e.g. pH, temperature, cofactors) and the amount of enzyme being constant as for the 11β-HSD assay conditions on p.27, the velocity of 11β-HSD activity was measured with increasing concentrations of substrate.

A direct plot of velocity against substrate concentrations is of little value in determining a true Vmax, which is the maximal velocity of the

Figure 6. Effect of cofactor concentration on 11β-HSD activity in submandibular gland homogenate.

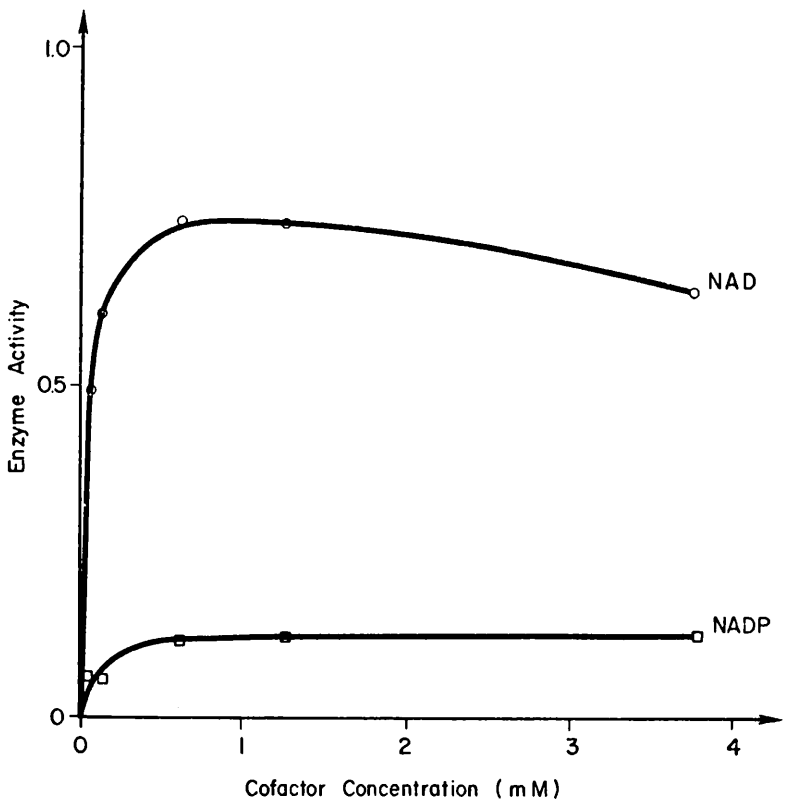
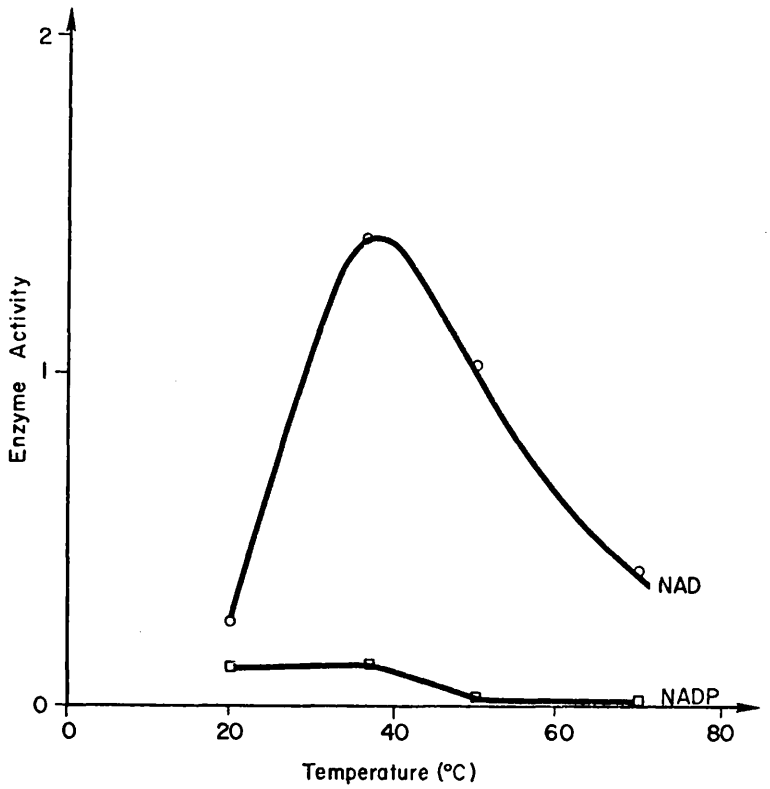

1.0
0.5
0
Enzyme Activity
NAD
NADP
0
1
2
3
4
Cofactor Concentration (mM)

Figure 7. Effect of temperature on 11ß-HSD activity in submandibular gland homogenate.


2
1
0
Enzyme Activity
NAD
NADP
0
20
40
60
80
Temperature (°C)

reaction for the parameters given, and therefore a true Km or "Michaelis Constant" cannot be determined properly. The "Michaelis Constant" of an enzyme is numerically equal to the substrate concentration at which the velocity of the reaction (v) equals $\frac{1}{2}$ of the maximal velocity (Vmax) or the substrate concentration which half saturates the enzyme. The Km is characteristic for an enzyme and substrate. Nevertheless true Km and Vmax values can be obtained by reciprocal plots of v and s (substrate concentration) according to Lineweaver & Burk (1934).

The results of the present investigation are shown in Figs. 8 and 9. The straight lines were obtained by computing the data by linear regression on the Olivetti Programma 101-.

The Km values for the NAD- and NADP-linked 11β-HSD's were 0.48μM and 0.95μM respectively and the corresponding Vmax's were 1.27n moles/min. and 0.37n moles/min. respectively.

From the values given it seems that the cofactor preference of the submandibular 11β-HSD is for NAD rather than NADP which seems to conflict with reports given by previous authors (see p. 2 ) for other tissues.

Very high substrate concentrations were found to be impracticable because a value of 10xKm inhibited the reaction.

F. Tissue ('Enzyme') Concentration.

Different aliquots of submandibular salivary gland were assayed for NAD-linked 11β-HSD activity and the result of the investigation is shown in Fig. 10, a graph of enzyme activity versus 'enzyme' concentration. The line drawn is a straight line demonstrating that enzyme activity is increasingly proportional to the 'enzyme' concentration for the 11β-HSD assay given (p.27).

There is no falling off from the linear relationship due to a limitation in the capacity of the method of estimation, e.g. insufficient substrate. The result shows that two molecules of 11β-HSD acting independently in solution will transform twice as much cortisol in a given time (2 mins.)

Figure 8. Lineweaver-Burk plot of NAD-linked 11β-HSD in submandibular gland homogenate.

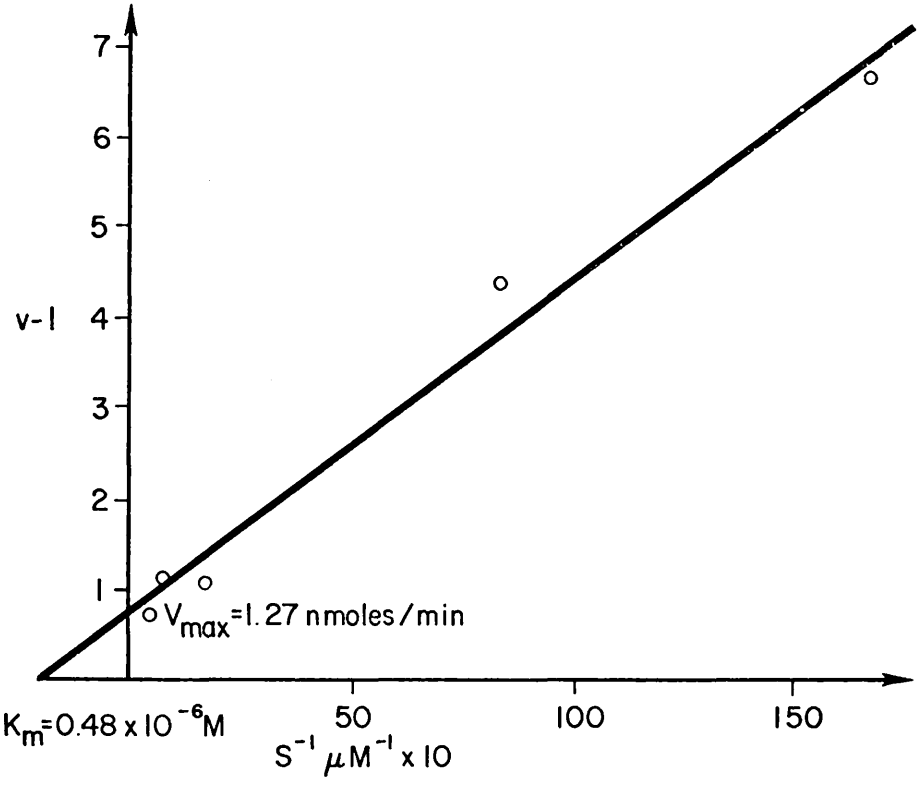

7
6
5
v-1 4
3
2
1
$V_{max}$=1.27 nmoles/min
$K_m$=0.48 x 10$^{-6}$M
50
100
150
$S^{-1}\ \mu M^{-1}$ x 10

Figure 9. Lineweaver-Burk plot of NADP-linked 11β-HSD in submandibular gland homogenate.

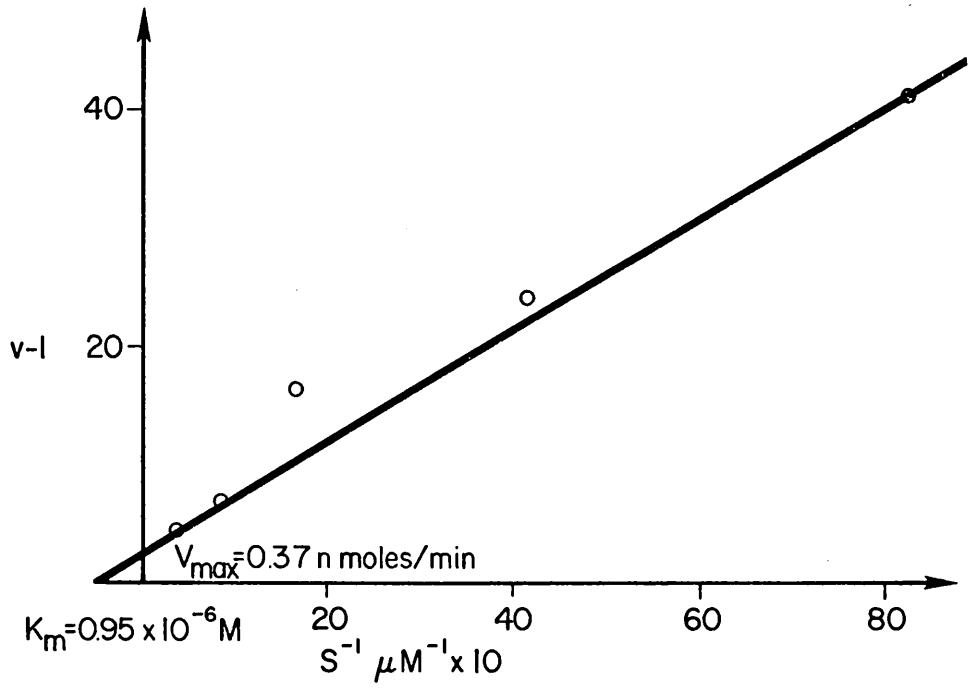
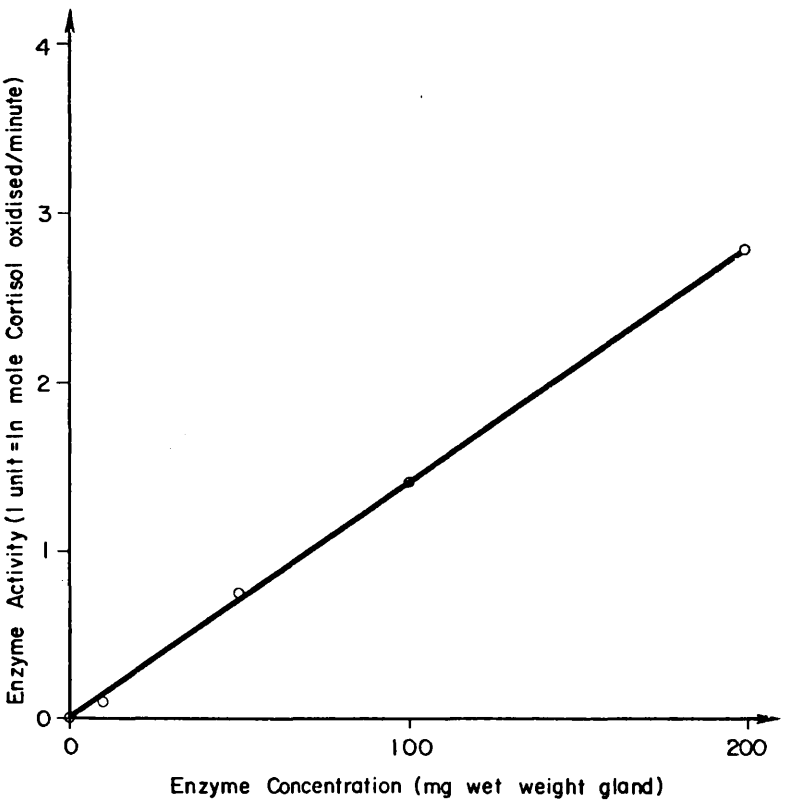

40
v-1
20
$V_{max}$=0.37 n moles/min
$K_m$=0.95 x $10^{-6}$M
20
40
60
80
$S^{-1}$ $\mu M^{-1}$ x 10

Figure 10. Effect of tissue ('enzyme') concentration on 11β-HSD activity in submandibular gland homogenate.


Enzyme Activity (1 unit = 1n mole Cortisol oxidised/minute)
4
3
2
1
0
0
100
200
Enzyme Concentration (mg wet weight gland)

than one molecule. This verifies the validity of the enzyme assay.

4. Metabolism of Steroid Substrates.

The effect of cortisone as substrate is shown in Table 4. There was little metabolism of cortisone showing that the 11β-HSD of rat submandibular salivary gland is not fully reversible. The reduction of cortisone is small compared with the oxidation of cortisol.

Corticosterone showed a 30% conversion to 11-dehydrocorticosterone (identification by Rf values) with both cofactors, NAD and NADP. An identical incubation with cortisol-4-$^{14}$C and NAD showed a 76.5% conversion to cortisone.

5. Metabolism of Cortisol and Cortisone in Rat Kidney Homogenate.

The results of this investigation are shown in Table 5. With cortisol as substrate, there is both NAD- and NADP-linked 11β-HSD activity. This appears to be of a level higher for the kidney NADP-linked 11β-HSD (0.84 units) than for the submandibular gland enzyme (0.37 units) but is the same level of activity for the kidney and submandibular NAD-linked 11β-HSD. It is not possible to say whether the oxidation of the 11β-hydroxy group of cortisol is equally effective with either NAD or NADP as a different pH has been used for each assay.

With cortisone as substrate, there is very slight 11β-HSD activity, the conversion to cortisol being small (0.09 units) and so the enzyme cannot be fully reversible. There is a large conversion to a more polar metabolite (Rf 0.40) and the possible explanation of this metabolite is discussed on p. 41 .

| pH | Incubation Time (mins.) | Cofactor | Yield of Cortisol (%) |
|---|---|---|---|
| 7.4 | 10 | $NADH_2$ | 4.8 |
| 7.4 | 20 | $NADH_2$ | 3.1 |
| 7.4 | 30 | $NADH_2$ | 4.9 |
| 7.4 | 10 | $NADPH_2$ | 7.1 |
| 7.4 | 20 | $NADPH_2$ | 5.7 |
| 7.4 | 30 | $NADPH_2$ | 6.3 |
| 7.4 | 7 | $NADH_2$ | 3.5 |
| 8.0 | 7 | $NADH_2$ | 2.8 |
| 8.5 | 7 | $NADH_2$ | 2.2 |
| 7.4 | 7 | $NADPH_2$ | 3.4 |
| 8.0 | 7 | $NADPH_2$ | 3.9 |
| 8.5 | 7 | $NADPH_2$ | 3.4 |
| Control incubations average 2.5% | | | |

Table 4. The effect of time of incubation and pH on the reduction of cortisone to cortisol by rat submandibular salivary gland *in vitro*.

| Substrate | pH | Cofactor | No. of Experiments | 11β-HSD activity (enzyme units) |
|---|---|---|---|---|
| Cortisol | 8.5 | NAD | 2 | 1.23 |
| Cortisol | 7.4 | NADP | 2 | 0.84 |
| *Cortisone | 7.4 | $NADH_2$ + $NADPH_2$ | 2 | 0.09 |

*Major conversion in this case to an unknown of approximately 30% of the substrate.

Table 5. Metabolism of cortisol and cortisone by kidney.

## DISCUSSION

It can be seen from the results of 11β-HSD assays in rat submandibular salivary gland homogenates that the reverse isotope dilution assay is the preferred analysis method. The homogenate contains too much particulate matter for the enzymes to be assayed by a spectrophotometric or fluorometric method. This is unfortunate because these other methods would have been less time consuming than the isotope method.

In the study of an enzyme reaction, the rate of the enzyme reaction should always be taken as the measurement of the initial rate, and this rate is obtained from the slope of the tangent to the reaction curve at zero time. It is only at the initial point that conditions are accurately known and limiting factors have not yet acted, e.g. the product of the reaction may inhibit the enzyme, the enzyme may be inactivated at the temperature or pH of the reaction, or the degree of saturation of the enzyme with substrate may fall because of the fall in substrate concentration as the reaction proceeds. The enzyme activity values in this report have been calculated from the measurement of the initial rate.

The effect of pH on enzymes, like all pH effects, is due to changes in the state of ionisation of the components of the system as the pH changes. Either the free enzyme, the enzyme-substrate complex or the substrate may undergo such changes. Since proteins and enzymes contain many ionisable groups, they exist in a whole series of different states of ionisation and the distribution of the total enzyme among the various ionic forms depends on pH and the ionisation constants of the various groups, and usually the catalytic activity is confirmed to a relatively small range of pH which is the optimal pH. The present study has revealed two optimal pH values, one with NAD as cofactor, the other with NADP as cofactor. This difference may be due to the nature of the cofactor used or there may be two separate enzyme systems, but the former proposition seems the more likely.

There is little mentioned in the literature on the optimal pH of 11β-HSD in the various tissues as most workers carried out their investigations at pH7.4. The histochemical method has a pH optimum of 7.0 - 7.2 (Baillie et al., 1965c) whereas biochemical studies showed the pH optimum at pH9 - 10 (Meigs & Engel, 1961; Koerner & Hellman, 1964; Bush et al., 1969; Koerner, 1969). Histochemically, however, incubations at pH values greater than pH8 result in complications because of the so-called 'nothing dehydrogenase' (Pearse, 1960).

Cofactors are organic substances of relatively low molecular weights (as compared to enzyme proteins) and in the case of NAD and NADP it is the specific function of hydrogen transfer with which they are important. The amount of cofactor needed in the 11β-HSD assay for submandibular salivary gland exceeds the cortisol substrate by 2,000 times and it is possible that there are enzymes in the homogenate which are degrading the cofactor, e.g. coenzyme nucleotidase, as well as enzymes still using intrinsic substrates. Koerner (1969) reported that $NADPH_2$ was cleaved by dinucleotide nucleotidohydrolase which has been demonstrated in rat liver microsomes (Jacobson & Kaplan, 1957) and is known to act on $NADPH_2$. NAD is the preferred cofactor in submandibular gland and the cofactor preference of 11β-HSD in various tissues has been discussed in the General Introduction to this thesis. Filsell & Jarrett (1965) found a high $(NAD.NADH_2)/(NADP.NADPH_2)$ ratio of 10:1 in sheep parotid gland and propose that salivary gland prefers oxidative metabolism that leads to production of ATP as a source of energy for cation secretion.

The finding of the optimal temperature to be 37°C for the enzyme reactions has been dependent on the time during which the 11β-HSD and cortisol are allowed to interact. As a general rule the shorter the reaction time the higher will be the optimal temperature whereas at a lower temperature, there the enzyme is more stable, the reaction will be slow but the final yield of product may be greater. Although the effects

of temperature on the activity of enzymes are very complex and are interrelated with other variables such as pH, buffer system and substrate concentration they present themselves as two forces acting simultaneously but in opposite directions. With increasing temperature the activity increases and at the same time inactivation (= denaturation of the enzyme protein) is accelerated. The higher the temperature, the more will inactivation dominate. This is the behaviour shown by both NAD- and NADP-linked 11β-HSD's. At 37°C the degrees of inactivation for 2 or 7 mins. incubations are not as great as at 50°C and 70°C. At 20°C the enzyme is not as active as at 37°C for the incubation times chosen.

The "Michaelis Constant" is specific for an enzyme and substrate used and the Km values shown in this present study of 0.48μM and 0.95μM for the oxidation of cortisol, using NAD and NADP respectively, differ significantly from the value of 20μM found by Bush et al. (1968) for washed microsomes and acetone-dried powders of washed microsomes of rat liver under the conditions of spectrophotometric assay. Their study confirmed the finding of Koerner & Hellman (1964) who found a Km of 30μM with acetone-dried powders of whole rat liver homogenate acting on cortisol. The probable reason for the difference in the Km values of the present study from these with rat liver is that 11β-HSD in submandibular salivary gland has a different function from that in the liver.

The enzyme reaction is irreversible in the submandibular gland. Cortisone is not metabolised and one of the possible functions of salivary gland may be to limit the amount of active steroid (cortisol) present in the gland, cortisone being potentially less active (Burton, 1965). The liver enzyme is reversible (Hurlock & Talalay, 1959) but the kidney enzyme is not fully reversible (Mahesh & Ulrich, 1960).

Corticosterone, the naturally secreted adrenocorticoid in the rat, showed 11β-HSD activity when incubated with submandibular gland but the reaction was slower than with cortisol as substrate. Therefore the enzyme in the

rat salivary gland utilises 11β-, 17-hydroxylated steroids more readily. This agrees with histochemical findings (Dr M. M. Ferguson, Glasgow Dental Hospital, personal communication).

Kidney has previously been reported as a site for 11β-HSD activity (Mahesh & Ulrich, 1960; Baillie et al., 1965a). It, too, is an important gland concerned with water and electrolyte transport. The oxidation of cortisol to cortisone in the kidney appears to be the most important step for 11β-HSD whereas the product formed by the reduction of cortisone was probably caused by the existence of 20-reductase or 20β-HSD in rat kidney. Mahesh & Ulrich (1960) have identified this product as 20-dihydrocortisone.

SUMMARY

1. A reverse isotope dilution assay using cortisol-4-$^{14}$C as substrate was used to measure 11β-HSD activity in male rat submandibular salivary gland homogenate, spectrophotometric and fluorometric methods being unsuitable. The metabolite of the reaction was identified as cortisone by formation of derivatives and recrystallisation to constant specific activity.

2. The kinetic aspects of 11β-HSD activity in submandibular gland homogenate were investigated. A variety of assay parameters were investigated to find the optimal conditions of enzyme activity. Nicotinamide adenine dinucleotide was the preferred cofactor to nicotinamide adenine dinucleotide phosphate with optimal pH conditions for the cofactor-linked enzyme being pH8.1 - 8.9 and pH7.6 respectively. A phosphate buffer was used to control pH as borate and tris buffers showed chemical reactions with the steroids. In the final assay volume of 11ml both cofactors were in saturing amounts at 1.25mM for 100mg. of tissue ('enzyme'). The optimal temperature for the NAD- and NADP-linked 11β-HSD was 37°C for incubations of 2 mins. and 7 mins. respectively (first order kinetics). Km and Vmax for the NAD-linked 11β-HSD were 0.48μM and 1.27n moles/min., the NADP-linked 11β-HSD values being 0.95μM and 0.37n moles/min. The substrate concentration chosen for enzyme assay was approximately 2xKm of the NAD-linked 11β-HSD (10n moles). Verification of the enzyme assay for 100mg. gland homogenates was shown by increasingly proportional enzyme activity with 'enzyme' (gland) concentration around this value.

3. Other steroid substrates were incubated with submandibular gland. Cortisone-4-$^{14}$C could not be shown to metabolise although reduced cofactors and a variety of pH values and incubation periods were used. This showed that the enzyme reaction is irreversible. Corticosterone, the naturally secreted adrenocorticoid in the rat, was metabolised by the submandibular gland 11β-HSD.

4. 11β-HSD activity was investigated in rat kidney homogenate. Cortisol was converted to cortisone, but cortisone was converted to a product other than cortisol indicating the difference between kidney and salivary gland metabolism of cortisone.

# PART II

## 11β-HYDROXYSTEROID DEHYDROGENASE IN SUBCELLULAR COMPONENTS OF MALE RAT SUBMANDIBULAR SALIVARY GLAND

# PART II

## 11β-HYDROXYSTEROID DEHYDROGENASE IN SUBCELLULAR COMPONENTS OF MALE RAT SUBMANDIBULAR SALIVARY GLAND

## INTRODUCTION

### 1. Isolation of Subcellular Components.

The living cell is by no means a simple sac, but is in reality a system exhibiting complex structures when viewed under the microscope either in the living state or after fixation. The localisation of enzymes within the cell has been studied by two main methods: a) histochemical method:- Ferguson (1967) and Ferguson et al. (1970) have dealt with the histochemistry of 11β-HSD in salivary gland; b) separation of particulate fractions from disintegrated cells. Because of the isolation of 11β-HSD in liver microsomes (Hurlock & Talalay, 1959; Bush, 1968; Koerner, 1969), whereas Mahesh & Ulrich (1960) found the highest activity in kidney homogenate was associated with the ultracentrifugal fraction containing nuclei and cell debris, it was decided to do biochemical studies on cell fractions of the rat submandibular salivary gland.

All fractionation experiments proceed in three steps: 1) homogenisation; 2) fractionation; 3) analysis of fraction for enzyme. By subjecting tissues to conditions of high shear in the appropriate media it is possible to retain the morphological and cytological properties of the particulate components of the cell. This cell-free preparation is called a homogenate.

The homogenate can then be centrifuged and various cellular components isolated. The nuclei sediment first, followed by the mitochondria. High speed centrifugation (100,000g or more) causes a fraction made up of granules, called the microsomal fraction, to sediment. The standard scheme of fractionation did not develop until the work of Dounce (1943), Claude (1946a, b) and Hogeboom et al. (1948).

In the analysis of the tissue fractions first we must identify the fraction by examination of unfixed and unstained material wherever possible, supplemented by observations employing staining methods and examination in

the electron microscope. Secondly, there is the essential requirement of a balance sheet when studying the enzyme associated with the subcellular elements (Schneider & Hogeboom, 1951). The amount of enzyme activity in each subcellular fraction is expressed as a percentage of the amount in the original homogenate. This provides an indispensible test of the validity of results.

A. Centrifugation.

Fractionation of cell components by means of centrifugation is possible because particles which differ in size (or shape) and density will sediment at different rates when under a centrifugal force. Centrifugation has received extensive theoretical treatment. Particle behaviour in a centrifugal field and their sedimentation rates have been reviewed by several authors (Svedberg & Pedersen, 1940; Ogston, 1956; Schachman, 1959).

There are two main types of centrifugation:

(1) Differential Centrifugation.

This type of centrifugation depends on the various structural components of a homogenate sedimenting at different rates, according to the size and density of the particles. The method is most successful when the differences in the sedimentation rates are large. By suitable choice of speeds the sedimented fractions can be arranged to contain, successively, whole cells and cell debris, nuclei, mitochondria etc., and microsomes, the final supernatant fluid containing soluble constituents. Several articles have dealt with the theoretical and practical aspects of this technique (Pickels, 1943; De Duve & Berthet, 1954; Allfrey, 1959). There are limitations of the technique, however. In differential centrifugation, fractions are collected consecutively at the bottom of the centrifuge tubes but, in practice, the species purity is usually lower than predicted. This is due to wall effects where the particles adhere to the wall of the centrifuge tube and set up convection currents as they travel down the wall with

a loss of resolution in the predicted purity of the fraction. In a similar manner, thermal convection currents can be set up at high centrifugal speed, but refrigeration of the centrifuge head largely eliminates this problem.

(2) Density Gradient Centrifugation (first used by Brakke, 1951).

In this method separations are achieved by virtue of difference in the density of the particles. A mixture of particles can be separated into several fractions which differ in specific gravity by centrifuging them through a density gradient until each species finds its own density level. The technique can be used in 'cleaning up' nuclear fractions by layering the nuclear suspension over a medium of higher density which acts as a barrier to particles of low specific gravity and permits the denser nuclei to pass to the bottom of the tube.

The uses of density gradients have been detailed in several reviews (Anderson, 1956; De Duve et al., 1959; Beaufrey & Berthet, 1963). This technique, also, has limitations. These include wall effects, difficulty in stabilising the density gradient and small rotor capacities. Several of these difficulties have been overcome by the advent of a new system for density gradient centrifugation, the zonal centrifuge (Anderson, 1966a, b; Birnie, 1969; Reid, 1971) in which the following ideal conditions exist:

a. minimal wall effects

b. much greater rotor capacities

c. a stabilised gradient which is rapidly introduced into, and recovered from, the rotor with minimum disturbance and loss of resolution

d. a sharp initial zone

e. a variety of rotors, some of which are capable of high speeds

B. Isolation of Nuclei.

Many methods have been proposed for the isolation of nuclei but there are deficiencies in most of the methods, e.g. low yields, denaturation, contamination by other cell components and morphological alterations (Dounce, 1955; Roodyn, 1969). There is no ideal method for isolating intact, uncontaminated nuclei suitable for metabolic and chemical studies.

Citric acid containing solutions have been among the most popular for the isolation of nuclei (Dounce, 1943). This method is good if purity and ease of preparation of the fraction are important but a number of enzymes are inactivated by this method.

Another method for the isolation of nuclei involves the use of organic solvents. This method was originally proposed by Behrens (1932). The method was used for the study of soluble enzymes of the nucleus and the enzymology of the nucleus has been surveyed in great detail by Georgiev (1967) who discussed the advantages and disadvantages of using organic solvents for isolating nuclei. There is still considerable doubt about the significance of studies on nuclei prepared in organic solvents because this treatment has considerable effect on the morphology of other cytoplasmic particles.

Most metabolic studies on nuclei have been with nuclei isolated by the techniques of Hogeboom et al. (1952) or Stirpe & Aldridge (1961). These methods involve a layering technique where homogenates in 0.25M Sucrose - Salt solution are layered over 0.34M Sucrose - Salt solution and centrifuged at 600g/10 mins. The pellet is then resuspended in 0.25M Sucrose - Salt solution, relayered and centrifuged twice more for purification of the nuclear fraction. Dense sucrose methods (Chaveau et al., 1956; Maggio et al., 1963) have been used to isolated nuclei for metabolic studies. Due to the high density of the medium, 2.2M, only nuclei sediment at high speed centrifugation, 40,000g, and all the cytoplasmic contaminants float. These methods yield nuclei of high purity. A drawback, however, of using

dense sucrose is that many enzyme systems are inactivated by its use. If the preservation of morphology is essential, more complex salt media as in the method of Maver et al. (1952) may be required.

There have been an increasing number of papers in which various detergents have been used for isolating nuclei (Hubert et al., 1962; Rappoport et al., 1963; Holtzman et al., 1966). The nuclei isolated appear to be pure but a drawback of using detergents is that hydrolytic enzymes are released by their use.

C. Isolation of Mitochondria.

Warburg demonstrated in 1913 that cellular respiration is associated with sedimenting particles. This cellular respiration is caused by mitochondria. However, it was not until the work of Schneider & Potter (1949) and further research that it was found that mitochondria are the main sites of cellular oxidations and phosphorylations. De Duve et al. (1955) have shown that the 'mitochondrial' fraction contains, in addition to true mitochondria, particles of a second kind to which the name lysosomes has been given. Estabrook & Pullman (1967) have an extensive article on the preparation of mitochondria from a wide variety of cells, tissues and organisms.

In the method of Schneider & Hogeboom (1950) the 0.25M Sucrose supernatant from the nuclear fraction of rat liver homogenate is collected and spun twice for 10 minutes at 5,000g, the pellet is resuspended and spun twice for 10 minutes at 24,000g. Microscopic examination of this fraction showed that it contained mitochondria alone.

C. Isolation of Microsomes.

The term 'microsomes' does not refer to a discrete cytoplasmic organelle. It denotes the particulate fraction that sediments upon centrifugation at speeds exceeding 100,000g (De Duve, 1964; Reid, 1967). The term 'microsomes' refers to a mixture of membraneous elements and ribosomes.

The most successful procedure for isolating microsomal fractions is that of differential centrifugation by a method devised by Palade & Siekevitz (1956). Reid (1967) has written an historical account of the problem. The method involves the prior separation of nuclei and mitochondria from the tissue homogenates by low speed centrifugation followed by ultracentrifugation of the supernatant at 100,000g or more for 1 - 2 hrs.

Many important discoveries have been made from the studies on microsomes, e.g. identification of the ribosomes as the main centres of protein synthesis (Littlefield et al., 1955; Palade & Siekevitz, 1956).

## MATERIAL AND METHODS

### 1. Preliminary Fractionation Technique.

Unfasted male Sprague - Dawley rats weighing 200-250g, fed on commercial diet 41B, were used. The rats were killed by neck dislocation, and the submandibular glands removed and placed on ice. The glands were trimmed of fat and weighed. Scissors were used to chop the glands finely and a 10% (w/v) homogenate in 0.25M-0.88M Sucrose was prepared with 6 strokes of a teflon - glass homogeniser at 1,000rpm. A homogenate prepared in 0.88M Sucrose/0.02% STI (Soybean Trypsin Inhibitor from Sigma, London) retained activity for 24 hours whereas without STI lost 11β-HSD activity in 4 hours.

Subcellular particles were prepared as follows:- 20ml of homogenate was pipetted into 2x10ml graduated centrifuge tubes and centrifuged at 700g for 10 mins. to sediment the 'nuclear' fractions which were suspended in 2x10ml 0.88M Sucrose. The supernatants were transferred to 25ml polycarbonate tubes and centrifuged at 15,000g for 20 mins. in an MSE Superspeed 50 Ultracentrifuge. The sediment in this fraction was designated as the 'mitochondrial' fraction.

The sediments were resuspended in 2x10ml 0.88M Sucrose and the supernatants from this fraction were then put in other polycarbonate tubes and spun at 105,000g for 1 hour in the ultracentrifuge. The sediment from this spin resulted in a 'microsomal' particle deposit. This deposit was also made up to 2x10ml volume. The three fractions were processed and photographed by the E/M (electron microscopy) technique described on p. 53. The supernatant of the 105,000g/hour fraction was made up to 2x10ml volume and designated as the soluble fraction. 1ml aliquots (100mg. homogenate equivalents) of all the fractions were taken for 11β-HSD assay (p. 54). Protein estimations were carried out according to the method on p. 52.

2. Revised Fractionation Technique.

There are several drawbacks in the preliminary fractionation technique and these were cleared up by this revised fractionation technique (Fig. 11). Submandibular glands of male rats were chopped finely and weighed. The homogenate was prepared by 6 strokes with a tightly fitting teflon pestle at 1,000rpm in 0.88M Sucrose, 1.5 M$CaCl_2$, 0.02% STI at a 10% concentration (w/v). It was then filtered through 4 layers of surgical gauze. This eliminated whole cells, cellular and gland debris, and precipitated protein.

A looser fitting pestle produced too many whole cells and gland debris and the use of an Ultra-Turrax homogeniser disintegrated the subcellular structures. A nuclear fraction was produced by centrifugation at 600g for 10 mins., the zymogenic and mitochondrial fraction by centrifugation at 4,000g for 10 mins., the microsomal fraction at 75,000g for 30 mins. and the supernatant from this being the soluble fraction. Electron micrographs were taken of the three fractions by the method described on p. 53 and 11β-HSD activity assayed by the modification on p. 54. The procedure for protein estimation was after Lowry et al. (1951) (p. 52).

3. Nuclear Isolation and Nuclear Membrane Solubilisation.

Submandibular salivary gland nuclei were prepared according to the technique illustrated in Fig. 12. A 10% (w/v) homogenate was prepared by homogenising freshly chopped submandibular gland in 0.25M Sucrose, $5x10^{-3}$ $MgCl_2$ and 0.02% STI with 6 strokes of a teflon-glass homogeniser at 1,000rpm, followed by filtration through 4 layers surgical gauze and re-constitution to a 10% (w/v) concentration. A crude nuclear fraction was then prepared by centrifuging the homogenate at 600g for 10 mins., the operating temperature being $0^{o}$-$2^{o}$C. A portion of the crude nuclear pellet was kept for 11β-HSD assay. The supernatant was discarded and the pellet rehomogenised in 1.8M Sucrose, $5x10^{-3}$M $MgCl_2$ and 0.02% STI. This mixture was spun at 40,000g for 1 hour in an MSE Superspeed 50 Ultracentrifuge. The pellet obtained in this spin was washed three times with 0.25M Sucrose

Figure 11. Differential centrifugation of SMSG subcellular particles.

10% (w/v) Homogenised gland in 10ml 0.88M Sucrose, 1.5mM $CaCl_2$, 0.02% STI

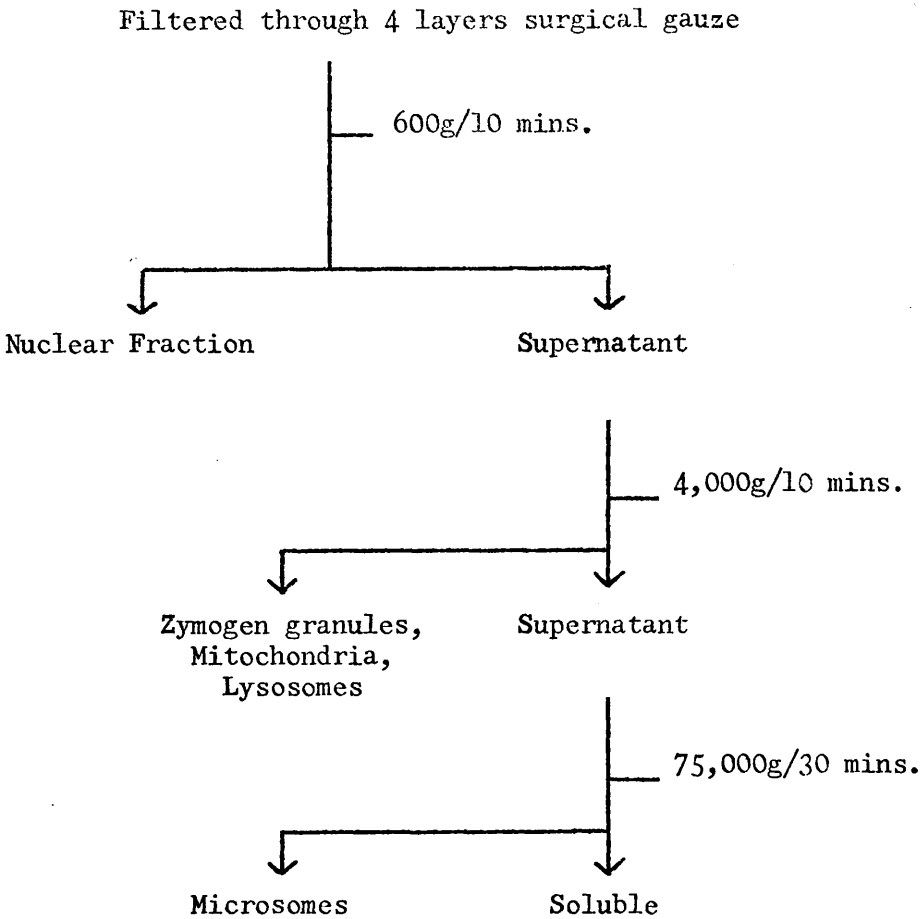
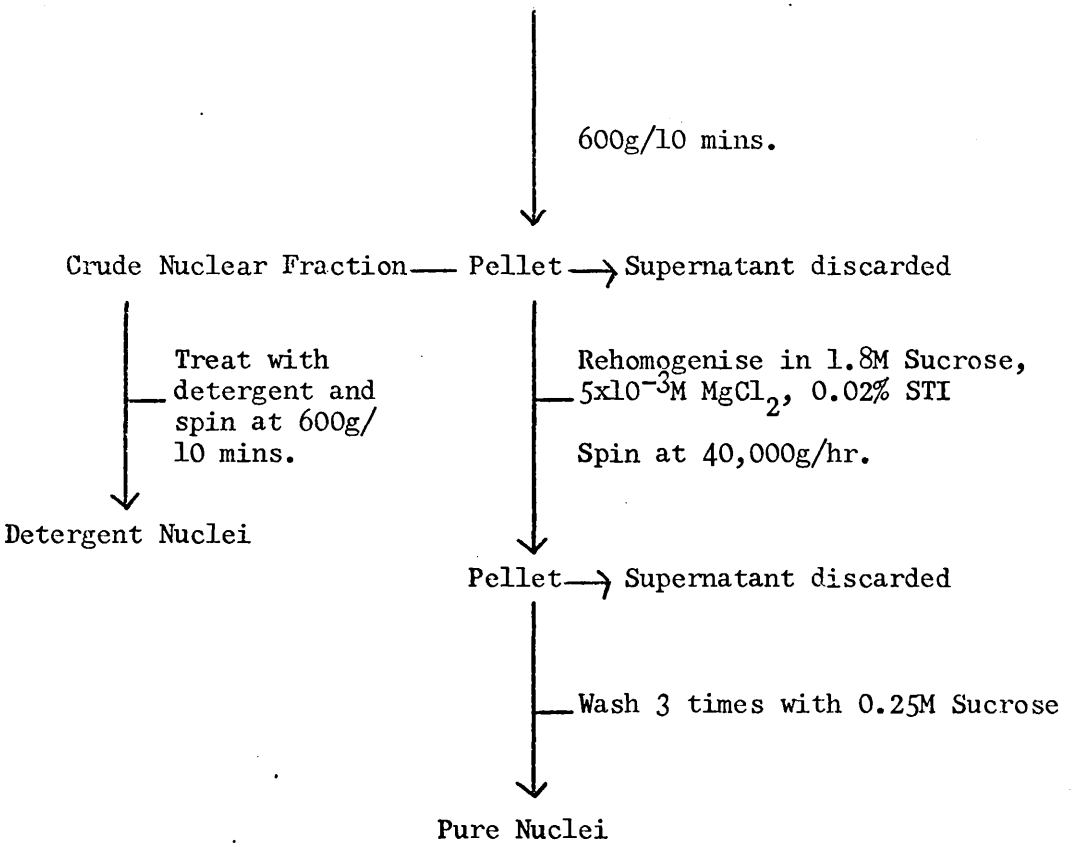


Figure 12. Method used for isolation of SMSG nuclei.

10% (w/v) homogenate in 0.25M Sucrose, $5 \times 10^{-3}$M $MgCl_2$, 0.02% STI

Filtered through 4 layers surgical gauze



and suspended as a 10% (w/v) homogenate equivalent. Samples of the pure nuclei obtained were processed for electron microscopy according to the method described (p. 53). Aliquots were taken for 11β-HSD assay (p. 54).

The solubilisation of the nuclear membrane could be obtained by treating the crude nuclear fraction with detergent, Triton x-100 (a non-ionic detergent). To the crude nuclear fraction a Triton x-100 solution was added in the concentration of 0.1% and mixed for 1 min. by a vortex mixer. This mixture was spun at 600g for 10 mins. and the detergent nuclei pellet obtained. Sodium deoxycholate and Tween 80 were also used as detergents, the same effect being noted as with Triton x-100.

Use of a hypotonic medium, such as water, as used by Neville (1960) to break the nuclear fraction into membraneous components was used to investigate the possibility of 11β-HSD activity in the membraneous components of the nuclear fraction.

4. Isolation of Salivary Gland Tubules by Collagenase.

A modification of the procedure of Burg & Orloff (1962) for kidney tubules was used as follows: 1g of submandibular salivary gland was chopped finely and immersed in 4 volumes of isotonic saline. 0.1% Soybean Trypsin Inhibitor and 0.1% collagenase (Sigma, London) were added to the mixture and incubated at room temperature. This mixture was passed through gauze and the tubules centrifuged at 50g for 90 seconds. The tubules were identified by light microscopy and 500mg. gland equivalents were incubated with cortisol-4-$^{14}$C for 15 mins. in order to estimate 11β-HSD activity.

5. Assay for Protein.

The measurement of protein in the homogenate and subcellular fractions was performed using the method of Lowry et al. (1951). They have suggested that the application of the copper-Folin reaction includes the measurement of protein during enzyme fractionations. It is 10 or 20 times more sensitive than measurement of the ultraviolet absorption at 280nm

and is much more specific and much less liable to disturbance by turbidities. It has the advantage of simplicity and sensitivity in use.

Method: All the chemicals were purchased from B.D.H., Dorset. Reagent A, 2% $Na_2 CO_3$ in 0.10N Na OH. Reagent B, 0.5% Cu $SO_4.5H_2O$ in 1% sodium tartrate. Reagent C, alkaline copper solution. Mix 50ml of Reagent A with 1ml of Reagent B. Discard after 1 day. Reagent D not used. Reagent E, diluted Folin reagent. Titrate Folin - Ciocalteu phenol reagent with NaOH to a phenolphthalein end-point. The titration carried out made it 3.2N and the Folin reagent was diluted accordingly to make it 1N in acid. The standard protein solution prepared was 1mg./ml of crystalline bovine serum albumin. This was checked against a human serum albumin equivalent.

To a sample of 5 to 100µg of protein in 100µl or less in a 10ml test tube, 1ml of Reagent C was added, mixed and allowed to stand for 10 mins. or longer at room temperature. 0.1ml of Reagent E was added very rapidly and mixed immediately. After 30 mins. or longer, 2ml of distilled water was added and the sample was read in an S.P. 500 spectrophotometer at 500nm. The concentration of the protein in the samples was calculated from a standard curve constructed using known amounts of protein, 10µg, 25µg, 50µg, 100µg.

6. Processing Cycle for Electron Microscopy.

All the chemicals were purchased from B.D.H., Dorset unless otherwise stated. 2% Glutaraldehyde (Taab Laboratories, Reading) in 0.2M sodium cacodylate buffer pH7.2, osmotic pressure 300 mOsm. 1% Osmium tetroxide (Taab Laboratories, Reading) in 0.2M sodium cacodylate buffer with 5.5% sucrose added pH7.2, osmotic pressure approximately 250 mOsm.

Processing Sequence: Glutaraldehyde fixation of subcellular fraction pellets for 2 hrs. Wash in buffer for 1 hr. (0.2M sodium cacodylate pH7.2). Postfix in osmium for $\frac{1}{2}$ hr.

Rinse in distilled water.

Dehydration in 50% alcohol for 5 mins., 75% alcohol for 5 mins., 95% alcohol for 5 mins., absolute alcohol, three changes of 10, 10 and 20 mins. each.

Soaking Mixture:- 50% Propylene oxide with 50% Araldite for 30 mins. then add Araldite to give a 66/34 mixture, for 1 to 4 hrs. Araldite 100% leave at room temperature for 5 to 8 hrs. Embed and incubate for 48 hrs. at 60°C. (Araldite - Durkapan, manufacturer Fluka, Basle.)

Examination: 90nm sections were prepared on an MT 2B Sorvall Ultramicrotome and mounted on Formvar coated grids stabilised with carbon coating. The sections were stained in saturated uranyl acetate for 15 mins., washed and stained for 4 mins. in lead citrate (Reynolds, 1963). The sections were then examined in an EM 6B electron microscope.

7. Modification of 11β-HSD Assay.

Subcellular fractionation depends on various concentrations of sucrose. The subcellular fractions were made up of 0.25-0.88M Sucrose and sometimes isotonic saline in order to maintain structural integrity. However, the homogenate was made up as 10% (w/v) for the fractionation experiments compared with 1% (w/v) for the kinetic experiments. Therefore 100mg. equivalents in sucrose of tissue homogenate or subcellular fractions were added to the 11β-HSD assay (p. 27) and the volume of phosphate buffer altered accordingly. The final volume in the assay consisted of 1ml of 100mg. tissue equivalent in sucrose or saline, 8.95ml of 0.067M phosphate buffer, 0.05ml of substrate dissolved in propylene glycol and 1ml of NAD (13.8μ moles) in final volume of 11ml.

## RESULTS

### 1. Inactivation of 11β-HSD in Submandibular Salivary Gland Homogenate.

#### A. Effect of Temperature.

It is of interest to see what conditions a) freezing (-15°C); b) refrigeration (0-5°C); c) room temperature (20°C) impose on NAD-linked 11β-HSD activity after 3 days at these temperatures in a sucrose homogenate. Assays were performed for 11β-HSD activity (pp. 27 and 54) on these three parameters. Freezing (-15°C) retains almost total activity after 3 days but at 5°C and 20°C no activity is retained after 3 days. An incubation of homogenate kept frozen 10 days revealed a total loss of activity.

#### B. Effect of Time.

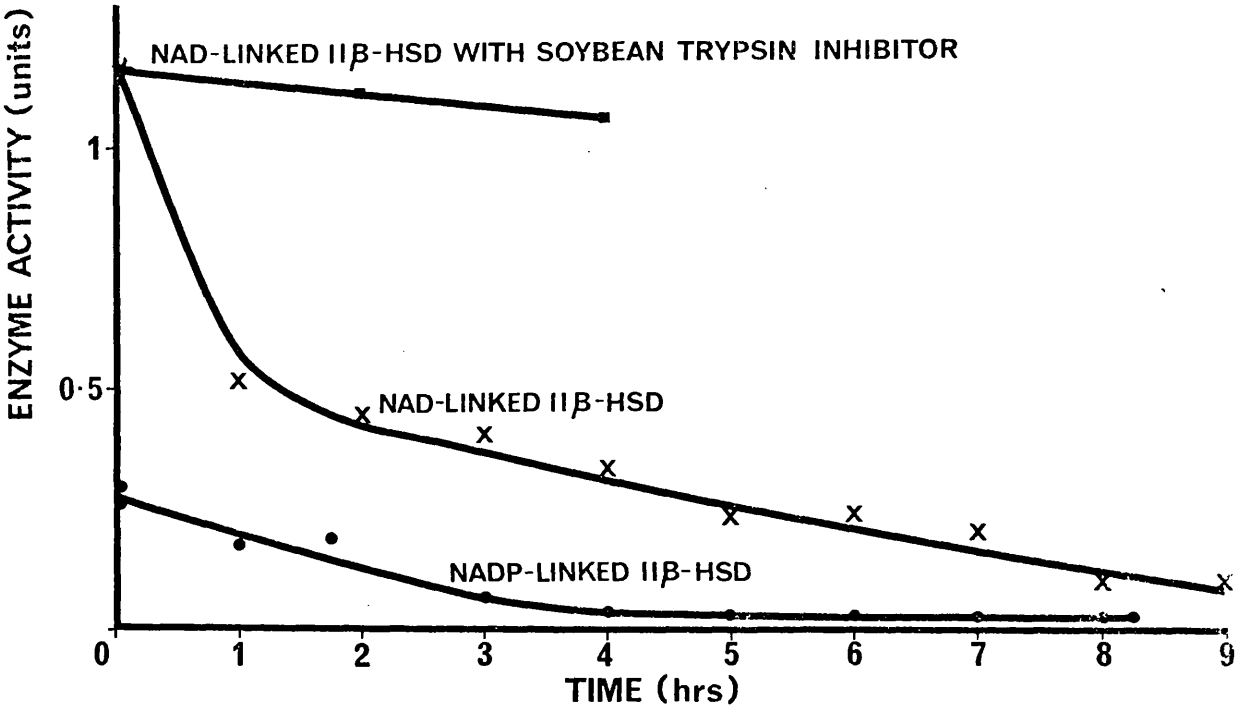
In initial experiments for the assay of 11β-HSD activity in subcellular fractions of submandibular gland there was a total loss of enzyme activity within 8 hrs. of the excision of the gland, even though the homogenate and subcellular fractions were kept on ice at 0°C during this time. It was essential therefore to follow the rate of enzyme denaturation in gland homogenate at 0°C. 11β-HSD assays were performed on sucrose homogenates of rat submandibular gland which were kept on ice at 0°C for 0 mins., 1 hr., 2 hrs., 3 hrs., 5 hrs., 6 hrs., 7 hrs., 8 hrs., 9 hrs. for both NAD- and NADP-linked 11β-HSD. The enzyme activity was plotted against time of homogenates at 0°C and the resultant graphs are shown in Fig. 13. The 11β-HSD is inactivated within 8 hrs. of preparing the homogenate. Fractionation processes usually take about 3 hrs. and the maximum retention of activity is preferable. The use of 0.02% Soybean Trypsin Inhibitor gave a better retention of 11β-HSD activity in the homogenate (Fig. 13).

### 2. 11β-HSD in Subcellular Fractions.

#### A. Preliminary Fractionation.

The main 11β-HSD enzyme activity was located in the crude nuclear fraction. A balance sheet of the 11β-HSD activities recovered in the fractions of this technique is shown in Table 6. The electron micrographs

Figure 13. Inactivation of 11ß-HSD in submandibular gland homogenate at 0°C.


NAD-LINKED 11β-HSD WITH SOYBEAN TRYPSIN INHIBITOR
NAD-LINKED 11β-HSD
NADP-LINKED 11β-HSD
ENZYME ACTIVITY (units)
1
0·5
0
1
2
3
4
5
6
7
8
9
TIME (hrs)

| Fraction | Activity (Units) | % Activity | mg Protein/ 100mg gland | Specific Activity | Relative Specific Activity |
|---|---|---|---|---|---|
| Homogenate | 1.275 | 100 | 13.583 | 0.0938 | 1 |
| 700g/10 mins. Crude Nuclear Fraction | 0.885 | 69.4 | 5.463 | 0.1619 | 1.726 |
| 15,000g/20 mins. Zymogen Granule Mitochondrial Microsomal Lysosomal Fraction | 0.229 | 11.9 | 3.17 | 0.072 | 0.7675 |
| 105,000g/hr. Ribosomal Fraction | 0.113 | 5.9 | 1.34 | 0.0842 | 0.8976 |
| Soluble | 0.131 | 10.3 | 4.17 | 0.0314 | 0.3344 |

Table 6. 11β-HSD in SMSG subcellular particles isolated by initial differential centrifugation technique in 0.88M Sucrose/0.02% STI (1n mole conversion of cortisol to cortisone/minute = 1 unit of 11β-HSD activity).

of the fractions are shown in Figs. 14-16. The nuclear fraction is very crude, composed mainly of nuclei, but also containing erythrocytes, unbroken cells and collagen. The 'mitochondrial' fraction is heavily contaminated with microsomal particles and lysosomes whereas the 'microsomal' fraction is a pure fraction of ribosomes.

B. Revised Fractionation.

The main 11β-HSD enzyme activity was located in the crude nuclear fraction and Table 7 shows the balance sheet of the 11β-HSD activity in the fractions. The nuclear fraction does not contain whole cells in this case as these were retained by the surgical gauze (Fig. 17). The mitochondrial fraction (Fig. 18) only contains secretory granules and mitochondria and shows no contamination by microsomal particles which are seen in the microsomal fraction (Fig. 19). The relative importance of mitochondria or microsomes in 11β-HSD metabolism in submandibular gland can therefore be discounted as there is little activity in these particles.

C. Isolation of Nuclei and Nuclear Solubilisation.

The crude nuclear fraction obtained in the revised fractionation technique can not be considered a pure preparation of nuclei because of the contaminating cellular debris. No 11β-HSD activity was observed in the solubilisation method of Neville (1960), the 11β-HSD not even being present in a lysed homogenate.

A nuclear fraction formed by passing crude nuclei through a 1.8M Sucrose can be considered to be pure morphologically as shown by electron microscopy (Fig. 20). The nuclei would not sediment through a 2.2M Sucrose. The detergent treated nuclei show a pure nucleoprotein fraction (Fig. 21), the nuclear membranes having been solubilised by the detergent.

The relative specific activity of the 11β-HSD was present in higher amounts in the three nuclear fractions than in the filtered homogenate (Table 8). The crude nuclei showed the highest relative specific activity, followed by the pure nuclear fraction and lastly the detergent treated

Figure 14. Electron-micrograph of a nucleus (N) with adjoining whole cell (A) and collagen (C).

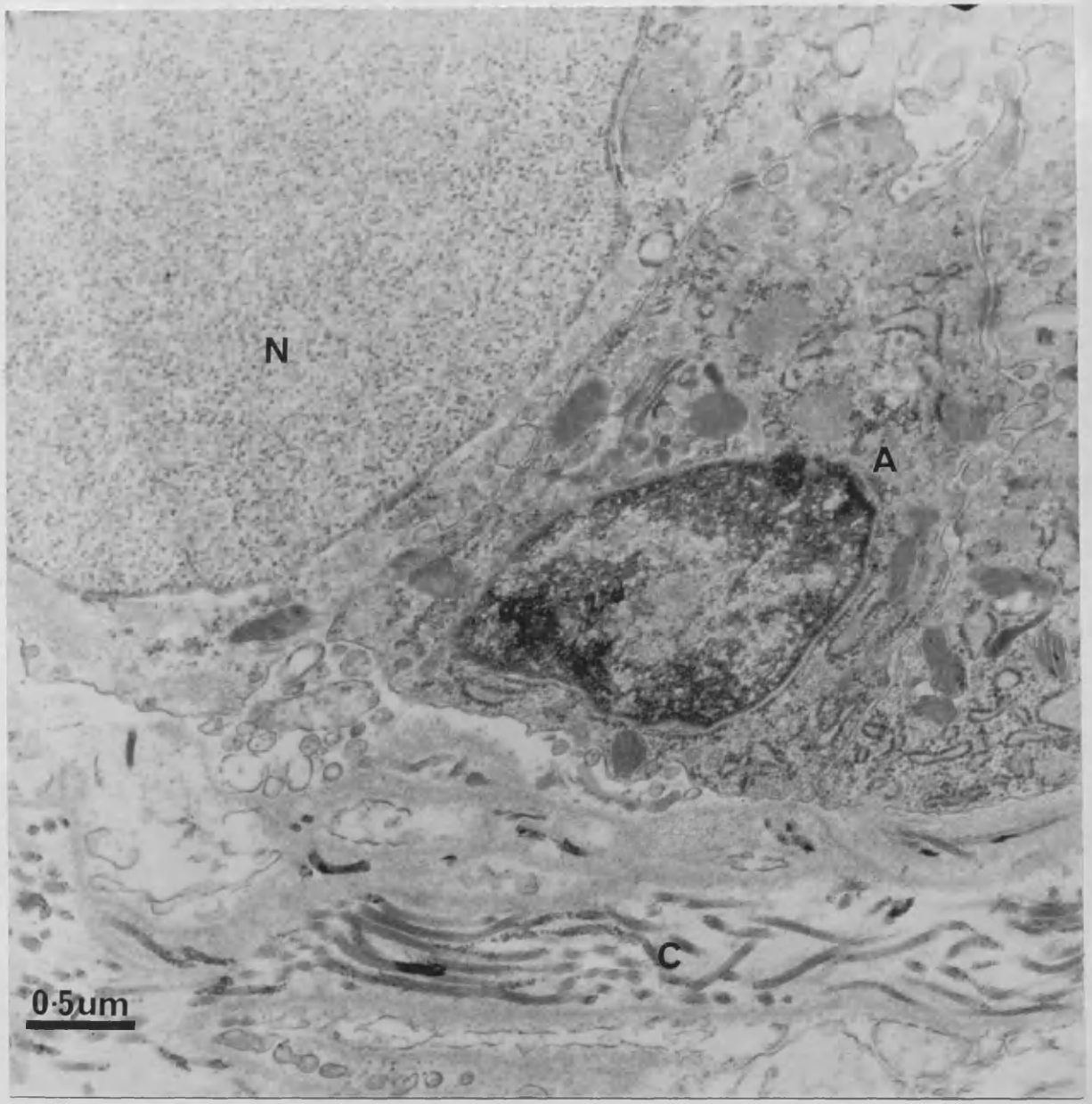

N
A
C
0·5um

Figure 15. 'Mitochondrial' (M) fraction prepared at 15,000g for 20 minutes showing gross contamination by microsomal particles (Mc).

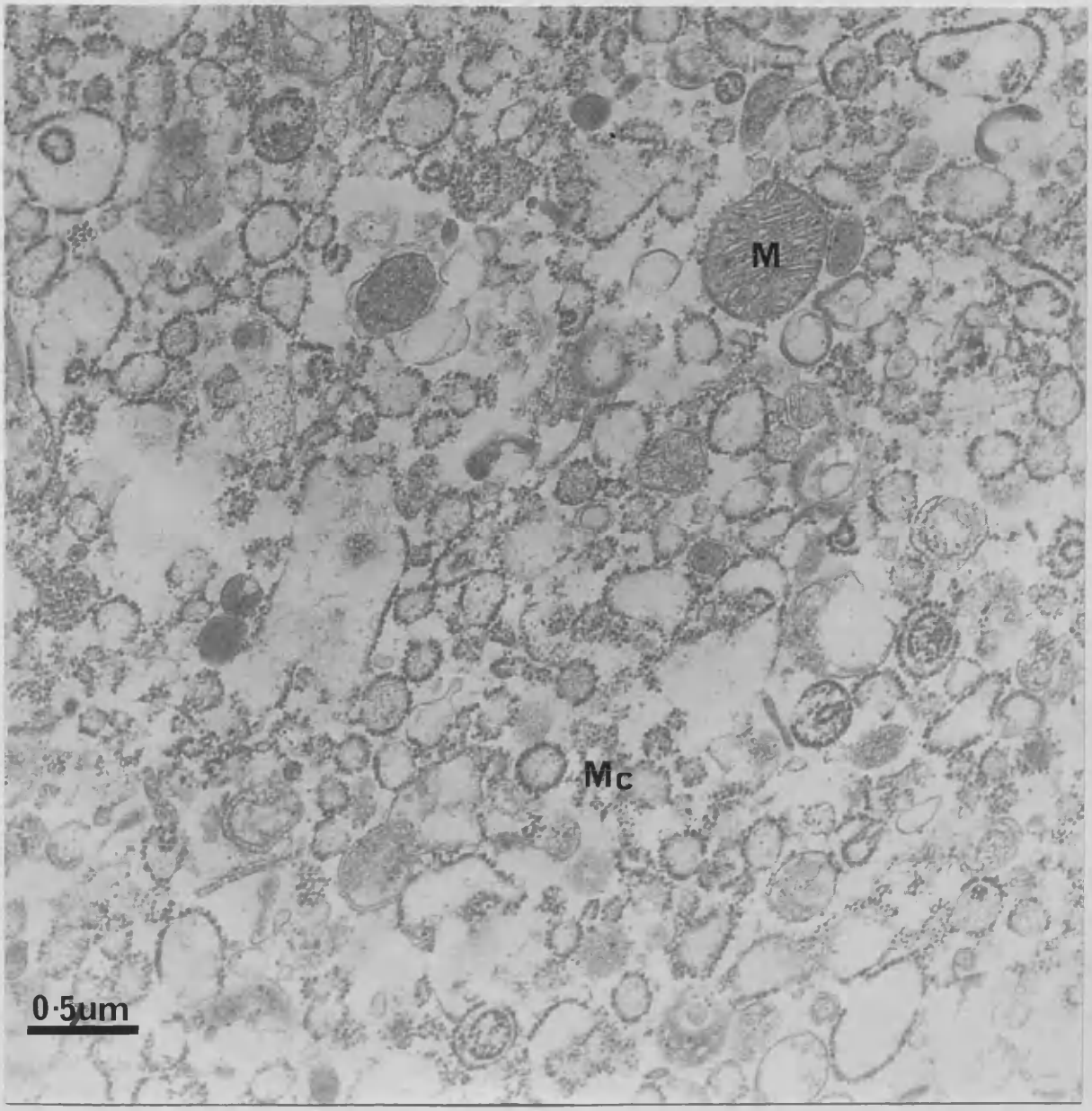
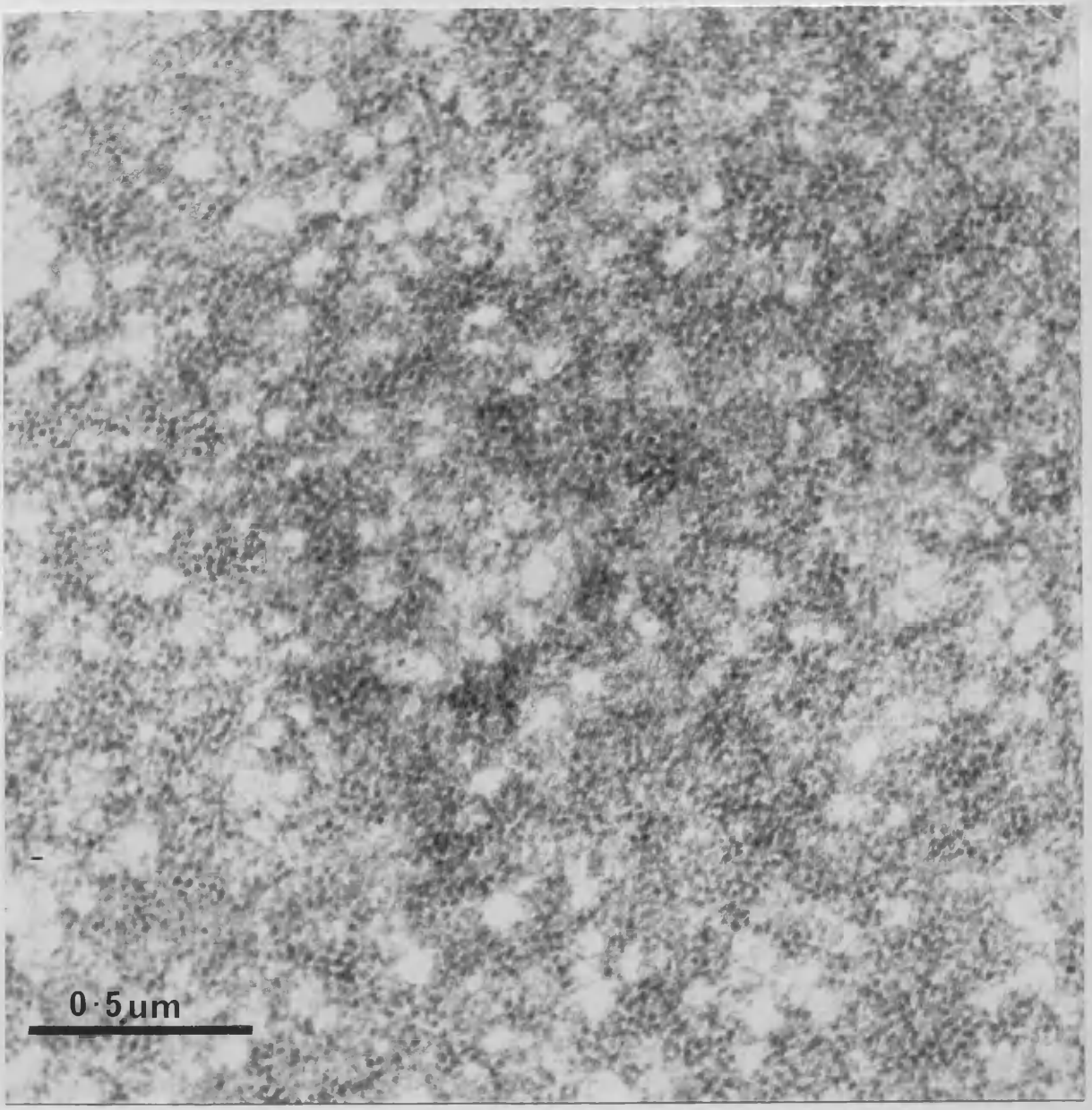

M
Mc
0·5um

Figure 16. Ribosomes isolated as the 'Microsomal' fraction from the preliminary fractionation technique.


0·5um

| Fraction | Activity (Units) | % Activity | mg Protein/ 100mg Gland | Specific Activity | Relative Specific Activity |
|---|---|---|---|---|---|
| Filtered Homogenate Through 4 Layers Surgical Gauze | 1.0921 | 100 | 13.0 | 0.0840 | 1.00 |
| 600g/10 mins. Nuclear Fraction | 0.6065 | 55.5 | 4.1 | 0.1479 | 1.76 |
| 4,000g/10 mins. Zymogen Granule Mitochondrial Fraction | 0.1820 | 16.7 | 2.5 | 0.0728 | 0.87 |
| 75,00g/30 mins. Microsomal Fraction | 0.1213 | 11.1 | 2.0 | 0.0606 | 0.72 |
| Soluble Fraction | 0.1213 | 11.1 | 4.2 | 0.0280 | 0.33 |

Table 7. 11β-HSD activity in SMSG subcellular particles isolated by revised differential centrifugation method in 0.88M Sucrose, 1.5mM $CaCl_2$, 0.02% STI.

Figure 17. Crude nucleus (N) isolated by revised fractionation technique showing cellular debris contamination.

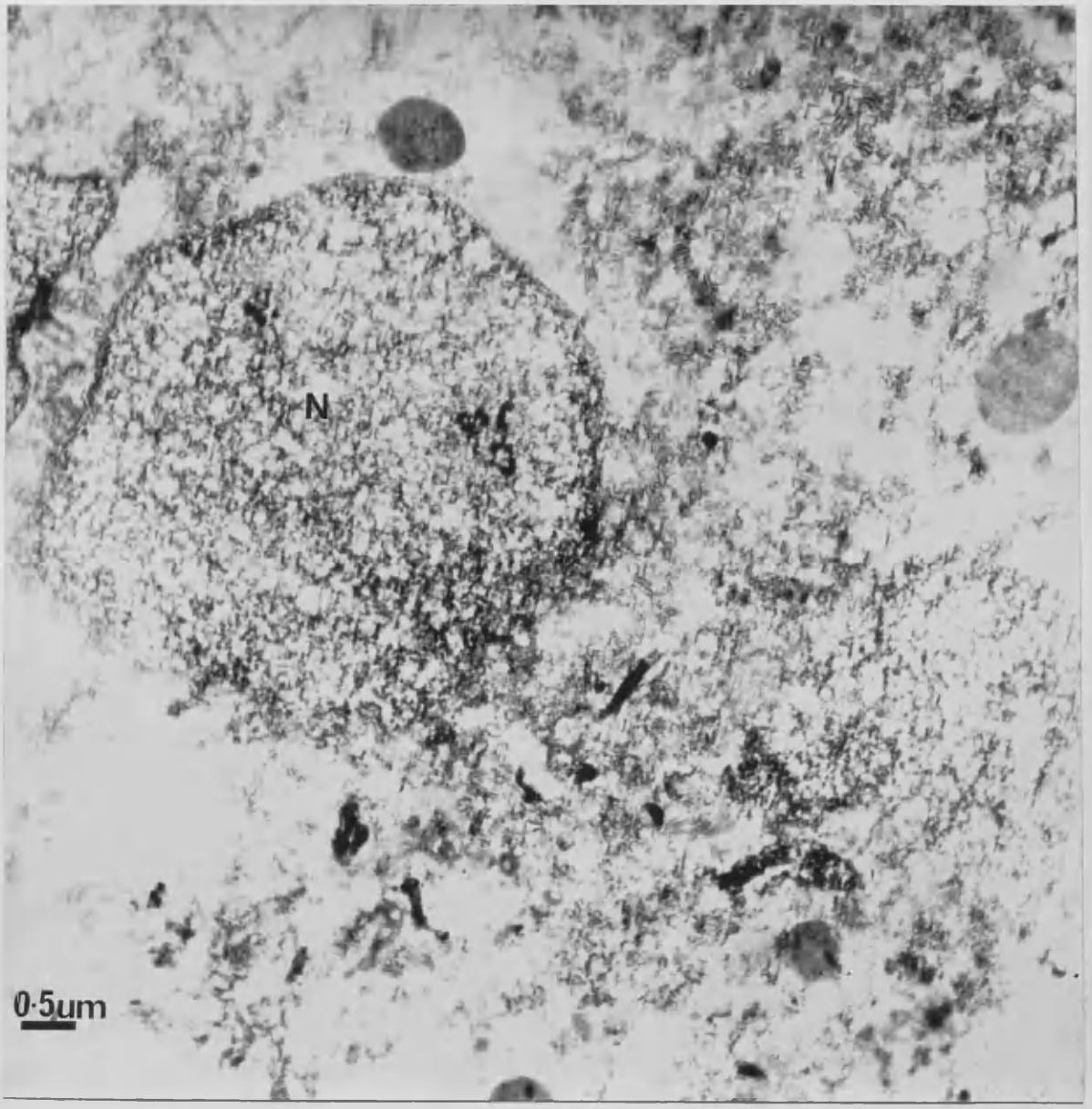

N
0·5um

Figure 18. Mitochondria (M) and Zymogen Granules (Z) isolated at 4,000g for 10 minutes.

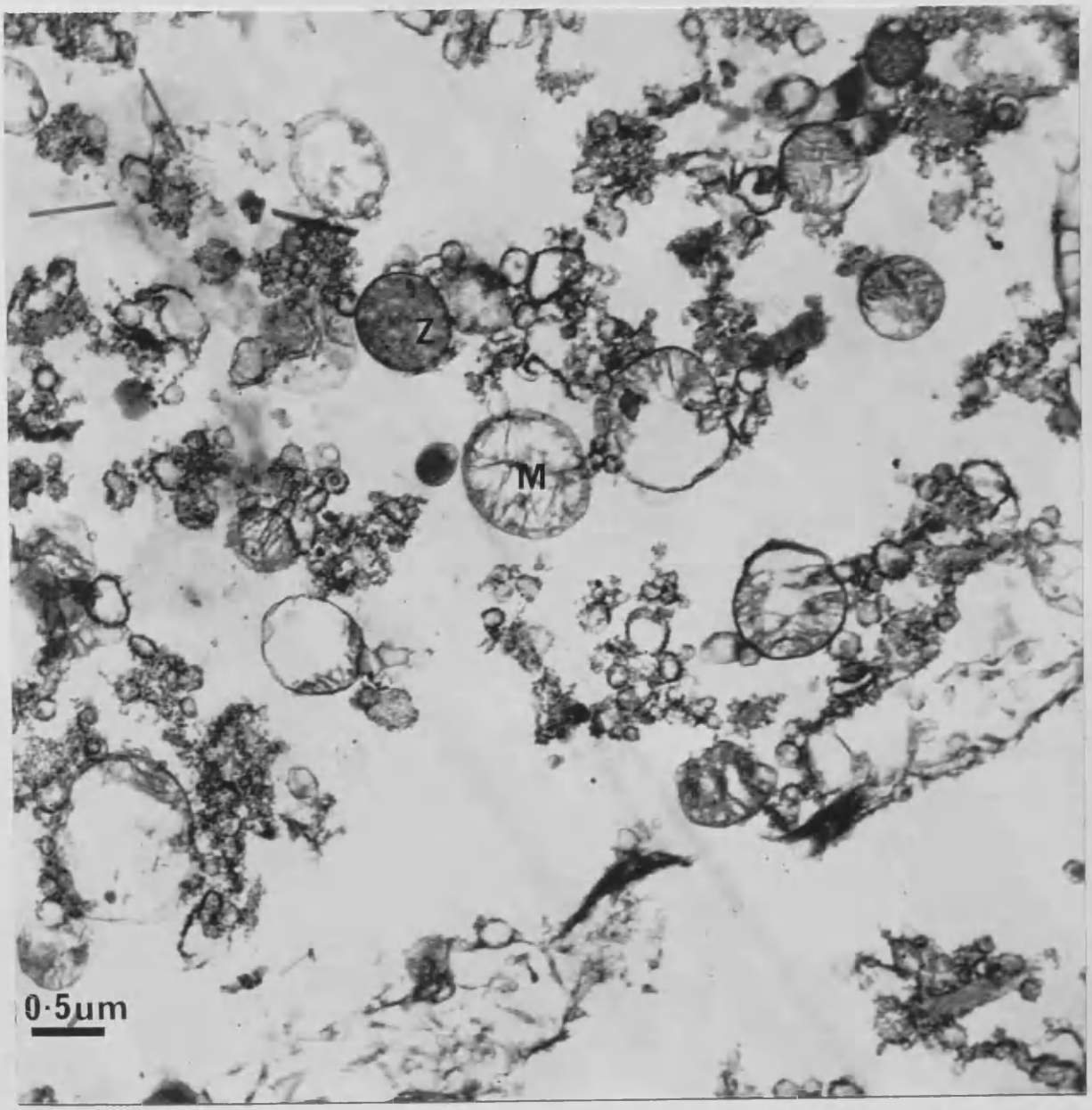

Z
M
0·5µm

Figure 19. Microsomal fraction isolated at 75,000g for 30 minutes.

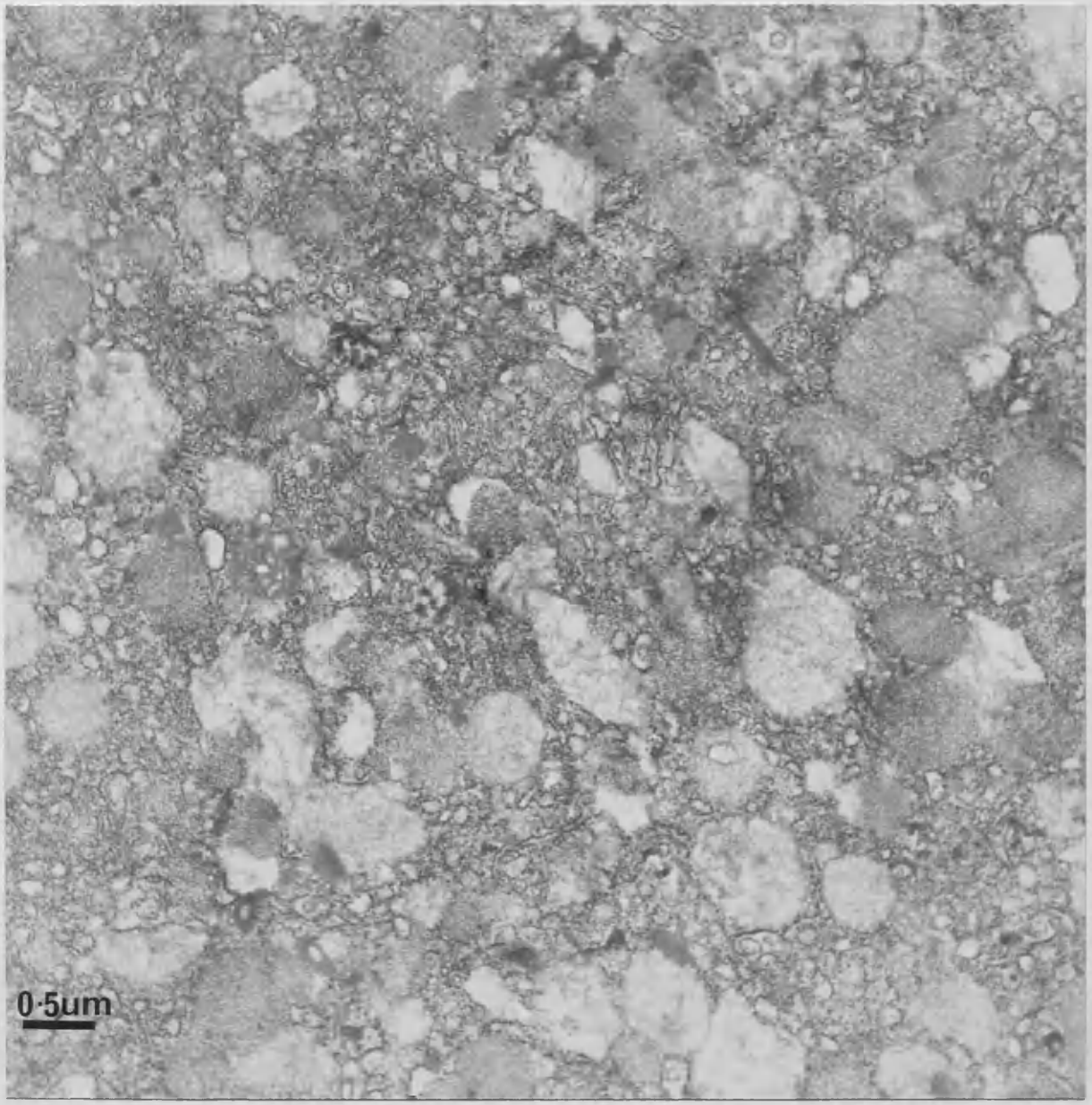

0·5um

Figure 20. Pure Nuclei (N) isolated through a 1.8M Sucrose solution.

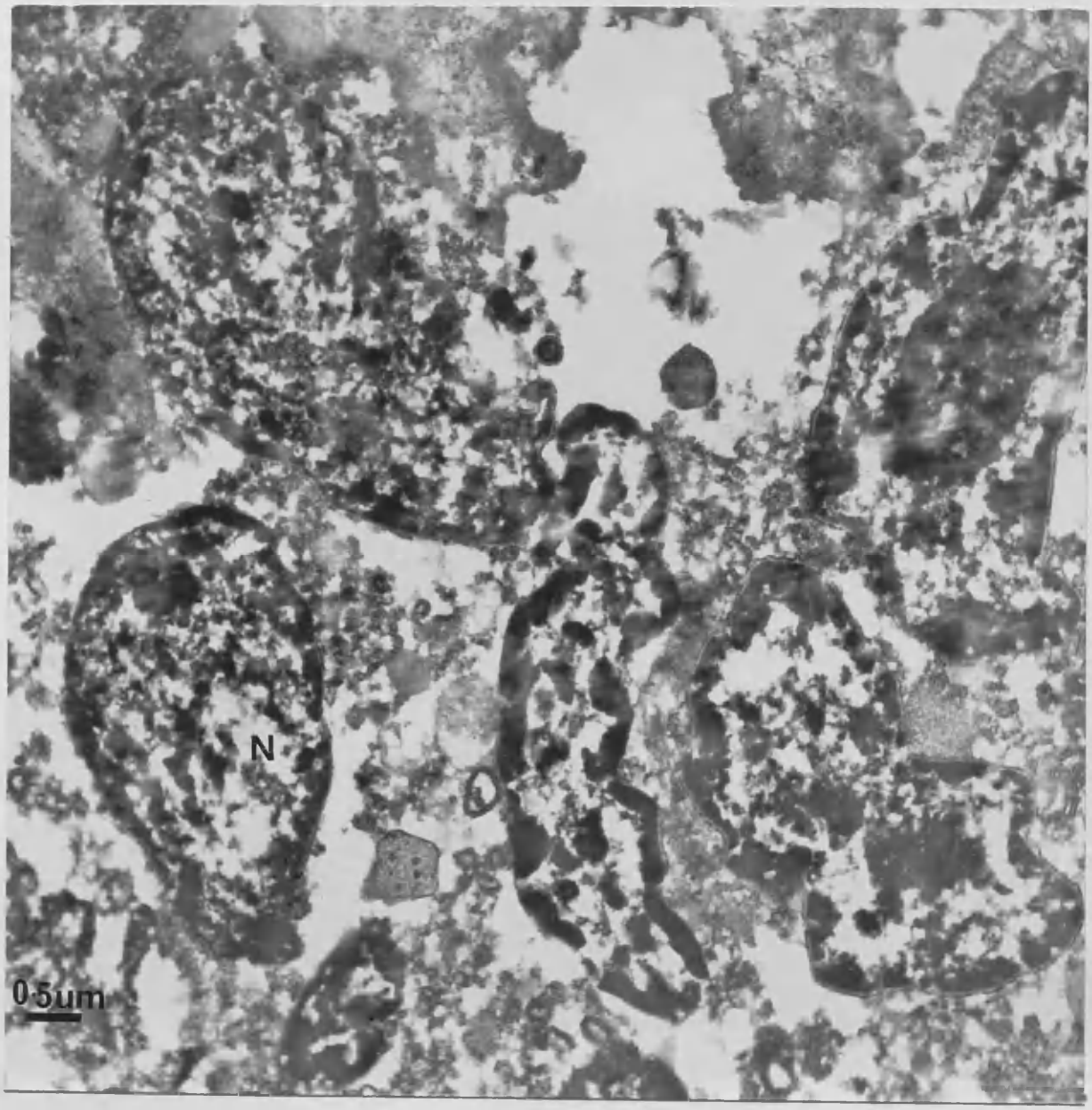
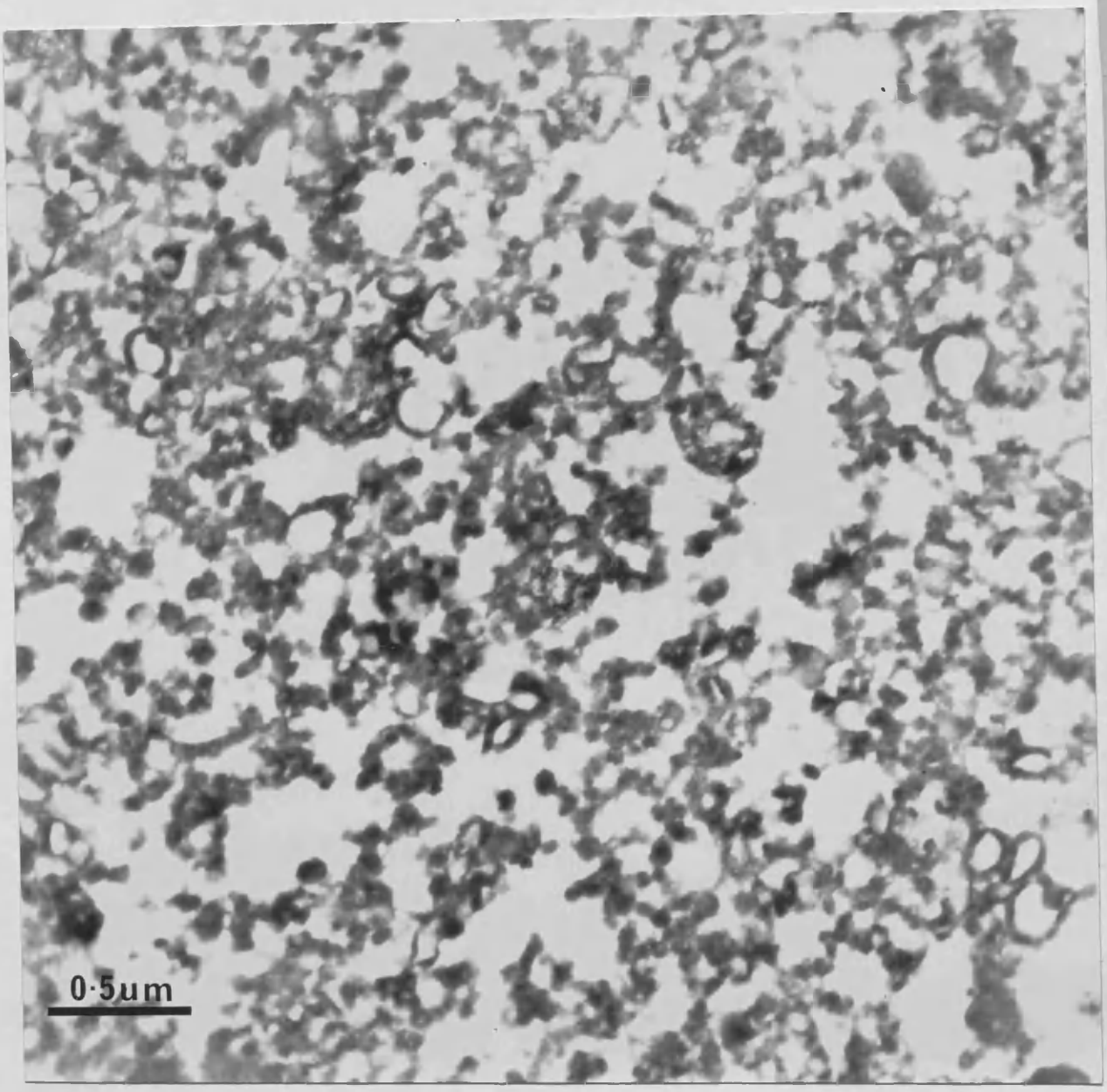

N
0.5um

Figure 21. Nucleoprotein isolated from a detergent treated crude nuclear fraction.


0·5um

| Fraction | Activity | % Activity | mg Protein/ 100mg Gland | Specific Activity | Relative Specific Activity |
|---|---|---|---|---|---|
| Homogenate | 1.1322 | 100 | 13.5 | 0.0838 | 1 |
| Crude Nuclear Fraction | 0.805 | 71.1 | 5.9 | 0.1364 | 1.628 |
| Detergent Nuclei | 0.3129 | 27.6 | 3.2 | 0.0977 | 1.166 |
| Pure Nuclei | 0.50 | 44.2 | 4.0 | 0.1250 | 1.49 |

Table 8. 11β-HSD activity in SMSG nuclei isolated as in Fig. 12.

nuclei. The major activity of 11β-HSD in submandibular salivary gland is therefore located in the nucleus.

The activity in the nucleoprotein isolated by detergent could possibly be higher than it was but there may have been slight enzyme denaturation using the detergent method. Since the enzyme's relative specific activity in nuclei is not all that much greater than in homogenate there is probably also a minor interaction with other cell components in the neo-nuclear area which might be related to the possibility that the enzyme is involved in genome expression (see Discussion p. 60).

3. 11β-HSD in Submandibular Gland Tubules.

The submandibular gland tubules were isolated by the use of the enzyme, collagenase. This degraded the layer of collagen which surrounds the salivary gland ducts and collagen probably accounts for the toughness of the tissue.

The activity of the 11β-HSD in the isolated tubules was minimal compared with the activity which would have been found in the submandibular homogenate.

Therefore, although duct cells exhibit the histochemical localisation of 11β-HSD (Fig. 22) the isolation of tubules does not lead to a more purified preparation of 11β-HSD than the gland homogenate.

## DISCUSSION

Proteases and nucleases are released by homogenisation (Robinovitch et al., 1969) and this accounts for the fast degradation of 11β-HSD in the homogenate which was slowed down by the addition of a protease inhibitor, Soybean Trypsin Inhibitor (Fig. 13). Hirose et al. (1971) examined the hydrolytic enzymes of the salivary glands. They found that rat submaxillary gland has high protease activity at pH7.5 and they separated five protease fractions with different properties from the gland.

In order to preserve structure of cell components any pressure during homogenisation has to be avoided. Feinstein & Schramm (1970) used a very loose fitting pestle (0.8mm clearance) for the preparation of rat parotid gland mitochondria. However, Horak & Pritchard (1971) could not apply the procedure to submandibular gland tissue. The present study has shown that the use of the tighter fitting pestle is preferred to the use of the looser fitting pestle as with the latter there is too much wastage of tissue in the filtration of the homogenate.

Salivary gland is a very tough tissue with a high content of calcium which reportedly disrupts salivary mitochondria (Feinstein & Schramm, 1970) and makes the isolation of intact, functioning mitochondria rather difficult. The present morphological study of the mitochondrial fractions (Figs. 15 and 18) shows the mitochondria appearing to be slightly broken up, but not to a significant extent because there is quite a high content of intact mitochondria in Fig. 18. Horak & Pritchard (1971) in the preparation of submandibular gland mitochondria used a modification of the method of Chance & Hagihara (1963) where a brief digestion of the tissue with a bacterial protease precedes homogenisation.

The use of collagenase in the present study to break down the tissue with the intention of isolating salivary ducts and tubules with an 11β-HSD was not successful. Burg & Orloff (1962) had isolated kidney tubules for the purpose of studying reabsorption phenomena. They had injected

collagenase solution (0.375%) into the renal arteries of excised rabbit kidneys and incubated the diced cortex obtained from these kidneys in the collagenase solution for 45 mins. at room temperature. The mixture was passed through surgical gauze and the tubules were centrifuged at 50g for 90 seconds. The absence of 11β-HSD in the collagenase treated salivary glands can be accounted for in three ways.

1. The collagenase solution may have contained contaminating proteases which were not inactivated by the Soybean Trypsin Inhibitor and therefore inactivated the 11β-HSD.

2. Collagenase has been reported as being an 'antimorphogenetic' agent for salivary epithelium (Grobstein & Cohen, 1965) and this may have a detrimental effect on 11β-HSD metabolism.

3. Osinski (1960) reported that the 11β-HSD of human placenta was bound to connective tissue and Berliner & Dougherty (1961) found that fibroblasts contained the enzyme and therefore the use of collagenase may have disrupted the enzyme-tissue link.

The preliminary fractionation technique was a universal technique used for liver, first brought about by Schneider & Hogeboom (1950), whereas the revised fractionation technique used similar centrifugal speeds to those used by Pritchard et al. (1971) to isolate the mitochondrial and zymogen granule fraction and the microsomal fraction of rat submandibular salivary glands. The liver is different from submandibular gland in the composition of cells, the liver being relatively easy to homogenise whereas the submandibular gland is not.

There is no standardisation of technique for the isolation of nuclei with the liver and the comparison of the prostate and submandibular glands in structure and toughness of tissue is more appropriate. For this reason the submandibular gland nuclei were isolated by a method similar to Grant et al. (1970) (after Sierralta & Minguell, 1970) for the isolation of prostatic nuclei, but a few modifications were made. The nuclei were

found to be the major site of 11β-HSD activity. Mahesh & Ulrich (1960) found the enzyme present in nuclear particles and microsomes but could not find the enzyme present in pure nuclei.

Lang (1971) has reported that for the steroid hormones, ecdysone and cortisol, a primary action in the nucleus is evident. By some not yet understood mechanism the RNA polymerase activity is stimulated. In consequence of this there is an increase in RNA synthesis and a part of the newly synthesised RNA can be regarded as an m RNA (messenger ribonucleic acid) which carries the information for the synthesis of specific proteins. It can be assumed that this m RNA controls enzyme synthesis in the cytoplasm. Grant (1969) has, however, doubts about the intervention of steroid hormones in the processes of translation of the genetic message. It can only be proposed that 11β-HSD may have an effect on the genetic expression of submandibular gland cell processes but further extensive work would have to be undertaken to substantiate this view.

SUMMARY

1. 11β-HSD was studied in intracellular components of the rat submandibular salivary gland, using NAD as cofactor. The relative difficulty in obtaining pure subcellular particles has been overcome and 11β-HSD was found to have its main activity in the crude nuclear fraction of the gland. Nuclei were isolated using 1.8M Sucrose and these were found to retain the major enzyme activity. Attempts to solubilise the nuclear enzyme using detergent and hypotonic solutions were unsuccessful. The enzyme in the salivary gland may influence nucleic acid metabolism but further work will have to be undertaken to investigate this possibility.

2. Rat submandibular gland is a tissue of differentiated cell types. It was therefore considered important to investigate the activity of 11β-HSD in separated tubules using the enzyme, collagenase, to break up the gland. However, little activity was recovered in the tubules probably because the collagenase used included a number of protease and peptidase impurities.

# PART III

## ENZYME HISTOCHEMISTRY IN SUBMANDIBULAR GLAND

# PART III

## ENZYME HISTOCHEMISTRY IN SUBMANDIBULAR GLAND

### INTRODUCTION

1. Salivary Gland Enzymes.

Amylase is the only enzyme which plays an important part in digestion. It is produced by the salivary glands, mainly the parotid gland. Amylase is a combination of two enzymes; $\alpha$-amylase which hydrolyses dextrans and lowers saliva viscosity, and $\beta$-amylase which breaks down large carbohydrates into smaller fractions, usually maltose. This enzyme consists of about 12% of the total amount of organic matter in saliva. Among other carbohydrases produced by the salivary glands are $\beta$-glucuronidase (Chauncey, 1961) and $\beta$-galactosidase found in rat submaxillary gland by Sukeno et al. (1972).

Alkaline phosphatase is present in salivary glands and histochemically the enzyme has been found irregularly by various workers (Noback & Montagna, 1947; Junqueira et al., 1949; Hill & Bourne, 1954). Acid phosphatase can be identified in both duct and acinar cells. Aliesterases hydrolyse esters of short chain fatty acids and these were found in the duct cells of different glands (Hill & Bourne, 1954). Lipases which attack the glycerides of long chain fatty acids were found by Martin (1953) in the interlobular ducts of dog submaxillary glands.

Cholinesterase is found mainly in the nerve fibres of the salivary gland. The density of stained nerve fibres which surrounds the main ducts was much less in the sublingual than in the submaxillary gland (Snell & Garrett, 1957).

Amino peptidase has been demonstrated in the interlobular ducts of the human parotid and submaxillary glands (Burstone & Folk, 1956). Transferring enzymes catalyse reactions in which a chemical group is transferred from one compound to another. Peroxidase, which is present in the salivary glands, contains iron and requires hydrogen peroxide as its hydrogen acceptor. Succinic dehydrogenase has been found in high con-

centrations in the duct cells of the rat, guinea pig and rabbit with much less enzyme in the acinar cells (Padykula, 1952; Hill & Bourne, 1954). Pritchard et al. (1971) found fatty acid CoA synthetase activity and highly active palmityl-CoA-carnitine transferase in the mitochondrial and microsomal fractions of rat submandibular glands. Their study indicated that SMSG subcellular fractions possessed a capacity for rapid lipid formation. They used a variety of enzyme markers for their subcellular fractions, namely: succinic dehydrogenase for mitochondria; glucose-6-phosphatase for microsomes; 5′-nucleotidase for plasma membrane; p-nitrophenylphosphatase for plasma membrane and nuclei; β-glucuronidase for lysosomes; adenyl cyclase and galactosidase for plasma membrane. Recently, Sukeno et al. (1972) have made an enzymic characterisation of golgi - rich fractions from rat submaxillary and sublingual glands. Each fraction was characterised by enzyme markers, including three glycosyltransferases: galactosyltransferase, N-acetylglucosaminyltransferase, and N-acetylgalactosaminyltransferase. $NADPH_2$ diaphorase, an enzyme bound to smooth membrane was not detected either in the homogenate or in the subcellular fractions. Horak & Pritchard (1971) reported on the preparation of coupled mitochondria from submandibular gland and their capacity for fatty acid oxidation. Filsell & Jarrett (1965) have discovered a highly active $Na^+,K^+$-ATPase enzyme system in the parotid gland and support the contention that active cation transport does occur in salivary glands and may be involved in the secretory process.

Other enzymes present in salivary glands are lysozyme, whose concentration in saliva is 8 times higher than in blood serum, carbonic anhydrase and aldolase. Sreebny & Meyer (1964) have reported the presence of a protease present in the convoluted tubules of the rat submaxillary gland. Junqueira et al. (1964) also noted the presence of protease in rat submaxillary gland.

Of the steroid enzymes found in salivary glands, Ferguson (1967) has

reported 11β-HSD present in the cells of the striated, intralobular and interlobular ducts of rat, mouse and rabbit parotid and submandibular glands. No other hydroxysteroid dehydrogenases were found. Rosner et al. (1965), however, reported biochemically that rat submaxillary gland showed, 17β-hydroxylase, $\Delta^5$-3β-HSD and $C_{17-20}$ desmolase activity. The parotid glands and pancreas did not share these enzymic activities. Katkov et al. (1972) found $\Delta^5$-3β-HSD-isomerase activity in boar salivary glands. Rosner et al. (1969) also demonstrated that the submaxillary glands of rat had all the enzymic mechanisms required to convert acetate to cholesterol in vitro and a $C_{20-22}$ desmolase activity was observed. Recently, Cardinali et al. (1971) have shown that rabbit and human submaxillary glands have 17β- and 20β-steroid reductase activity and Baldi & Charreau (1972) have reported a 17β-HSD in rat submaxillary gland.

2. Advantages of Histochemistry.

The undeniable advantage that tissue histochemical methods offer over biochemical ones employing cell suspensions, homogenates or fractions is that they allow an appreciation of the chemical characteristics of individual cells and of the heterogeneity of cell populations. Biochemical methods yield only a statistical average of the chemical nature of a population of cells or of a given subcellular organelle or particulate fraction. Biochemical analyses of whole organ homogenates give poor estimates of the enzyme activity of a single cell type because the cellular population may change considerably under different experiments. However, the accuracy of histochemical localisation of a substance or enzyme in cells and tissues hinges on various factors. In addition to the problems of preserving fine structure and chemical identity of intracellular substances, accurate localisation depends on the distribution and nature of the substance to be studied, the non-specific binding of the histochemical reagent, the characteristics of the histochemical capture reaction and, finally, the solubility of the reaction end product.

Because of some of the advantages of histochemical over biochemical methods of cell localisation of enzymes it was decided to investigate the histochemical position of several enzymes including 11β-HSD in male rat submandibular salivary gland.

## MATERIAL AND METHODS

Submandibular salivary glands were excised from adult male Sprague - Dawley rats after killing by cervical dislocation and immediately frozen in pulverised solid carbon dioxide. Each gland was sectioned at 10μm in a cryostat and the tissue sections mounted on to glass coverslips. For the localisation of hydroxysteroid dehydrogenase activity sections were incubated separately at 37°C for up to 3 hours, according to the method described by Baillie et al. (1966b), in a phosphate buffered medium (pH7.4) containing NAD, Nitro B.T. (Nitro Blue Tetrazolium) and substrate. The steroid substrates used for the localisation of 11β-HSD activity were cortisol, prednisolone (Sigma, London) and 5α-dihydrocortisol (Mann Research Laboratories Inc., New York), each dissolved in dimethyl formamide.

Succinic, α-glycerophosphate, lactic, malic, glucose-6-phosphate and β-hydroxybutyrate dehydrogenases were demonstrated by adding the respective substrate to the phosphate buffer medium with Nitro B.T. and NAD or NADP where necessary. Control sections were incubated concurrently in a medium without substrate and upon completion of the incubation the sections were washed in water and mounted with an aqueous mountant (Hydromount). The remaining histochemical methods for enzyme localisation were taken from Pearse (1960):

The histochemical localisation of acid phosphatase in the submandibular salivary gland was exhibited by the lead nitrate method of Gomori (1950) and the Naphthol AS-MX phosphate method of Burstone (1958c); alkaline phosphatase was located by the Naphthol AS-BI phosphate method of Burstone (1958a, b); 5′-nucleotidase localisation was by the method of Pearse & Reis (1952); the calcium method of Padykula & Herman (1955) was used for adenosine triphosphatase; the Chiquoine method (1953, 1955) was used for glucose-6-phosphatase; and the utilisation of esterase by the submandibular salivary glands was shown by the Naphthol AS acetate method after Gomori (1952a, b).

## RESULTS

No formazan was deposited in the control sections for dehydrogenase activity. The results are summarised in Table 9 and Figs. 22-38. 11β-HSD activity was not located in any single component of the salivary duct system but was evident in striated, lobular and interlobular excretory ducts. The dehydrogenase enzymes were localised mainly in the duct epithelium. The phosphatases were localised in both the acinar and duct systems except for alkaline phosphatase which was localised mainly in the acinar tissue.

| Enzyme | Arteriole | Ducts and Tubules |
|---|---|---|
| Acid Phosphatase | | |
| 1. Lead Nitrate Method | + | + |
| 2. Naphthol AS-MX Phosphate Method | + | ++ |
| Alkaline Phosphatase | + | - |
| 5'-Nucleotidase | - | ± (deposit at blood vessel) |
| Adenosine Triphosphatase | + | + |
| Glucose-6-Phosphatase | + | + |
| Esterase | + | ++ |
| Succinic Dehydrogenase | + | ++ |
| α-Glycerophosphate Dehydrogenase | - | ++ |
| Lactic Dehydrogenase | + | ++ |
| Malic Dehydrogenase | + | ++ |
| Glucose-6-Phosphate Dehydrogenase | - | ++ |
| β-Hydroxybutyrate Dehydrogenase | - | ++ |
| 11β-Hydroxysteroid Dehydrogenase | | |
| 1. Cortisol as substrate | - | ++ |
| 2. Prednisolone as substrate | - | ++ |
| 3. 5α-Dihydrocortisol as substrate | - | ++ |

- \- enzyme activity absent
- ± weak enzyme activity present
- \+ enzyme activity present
- ++ strong enzyme activity present

Table 9. Histochemical distribution of enzymes in male rat submandibular gland.

Figure 22. (x800) 11β-Hydroxysteroid dehydrogenase in male rat submandibular gland (cortisol as substrate).

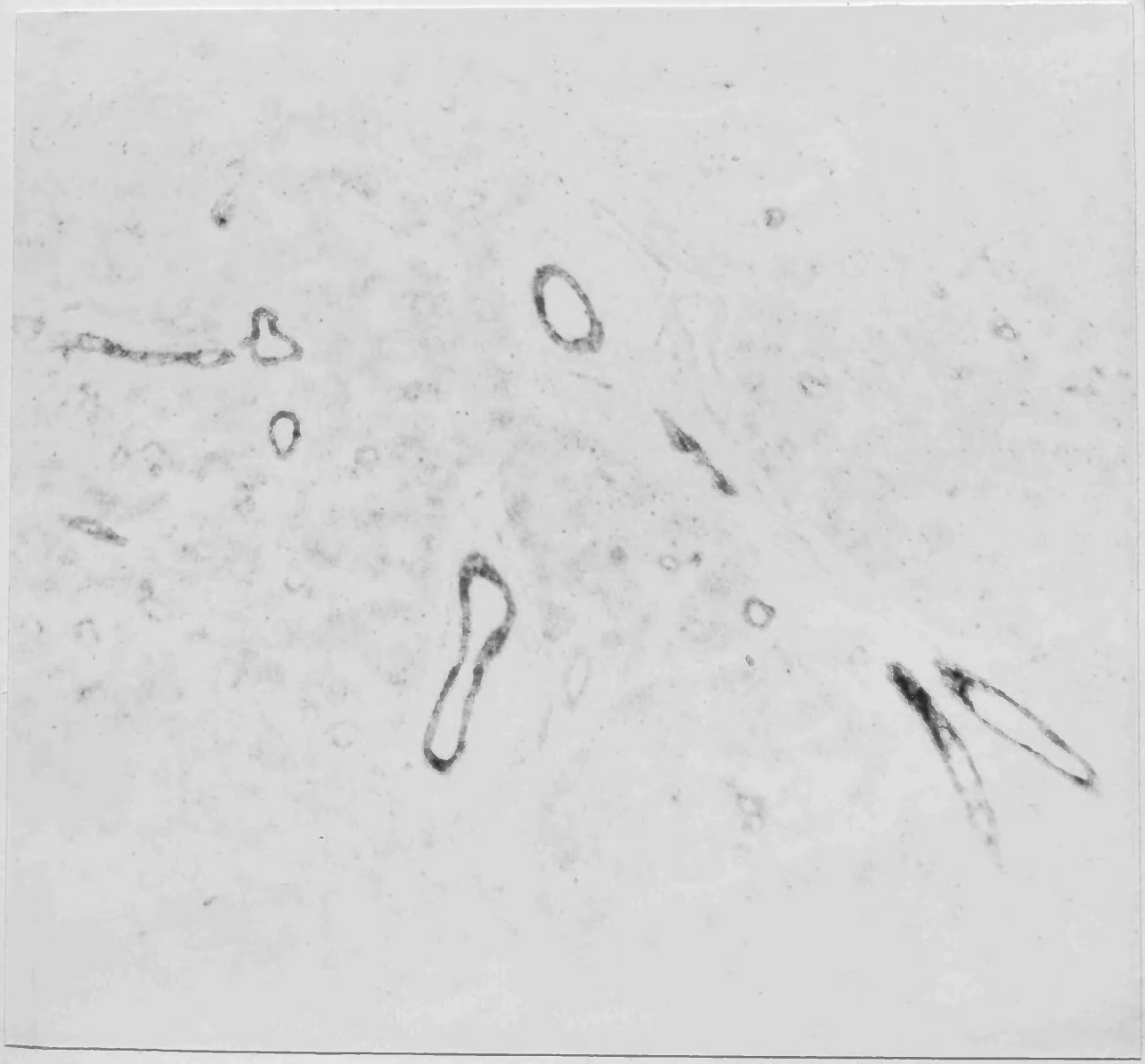


Figure 23. (x2000) Acid phosphatase in male rat submandibular gland (lead nitrate method).

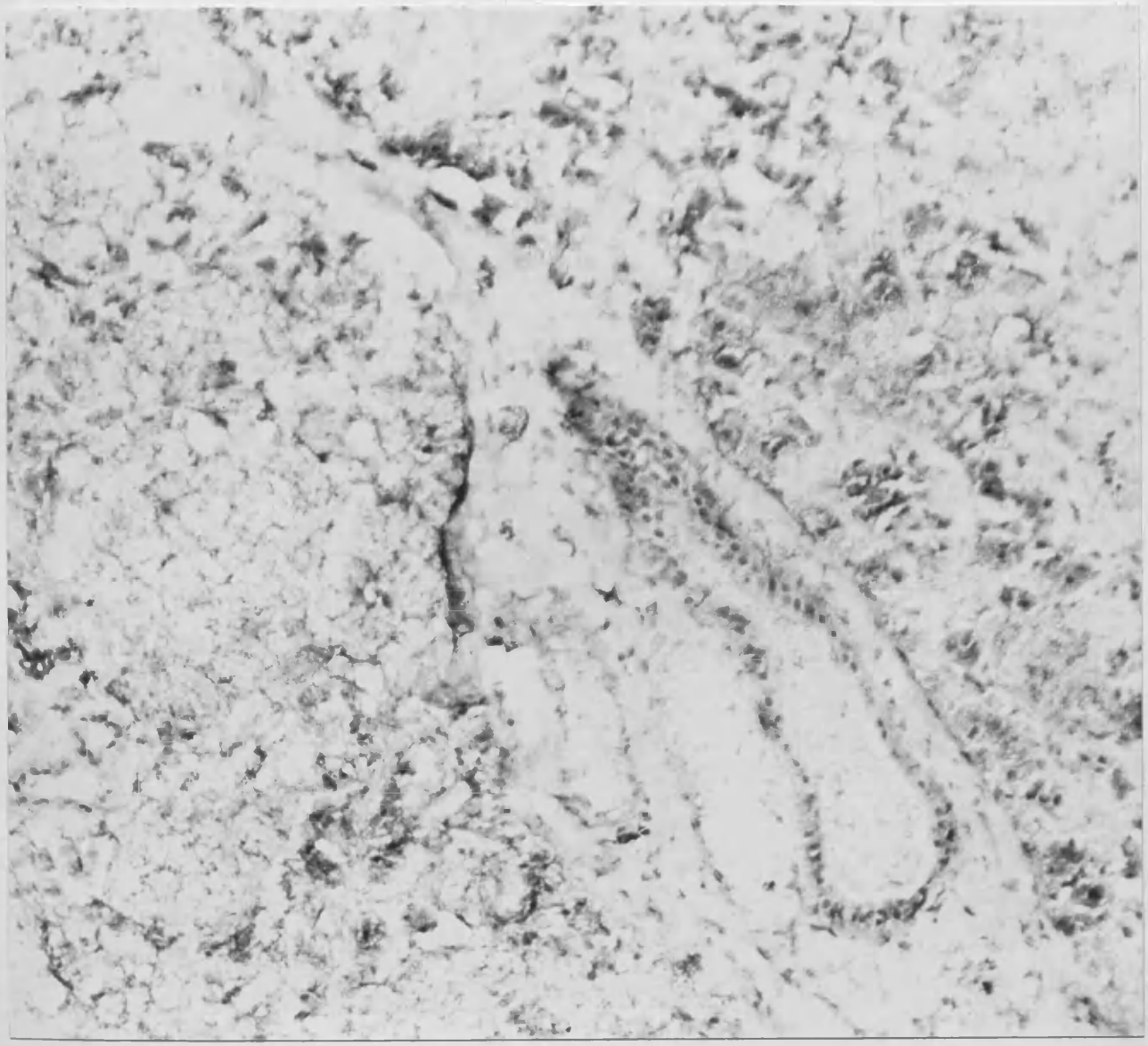


Figure 24. (x1900) Acid phosphatase in male rat submandibular gland (Naphthol AS-MX phosphate method).

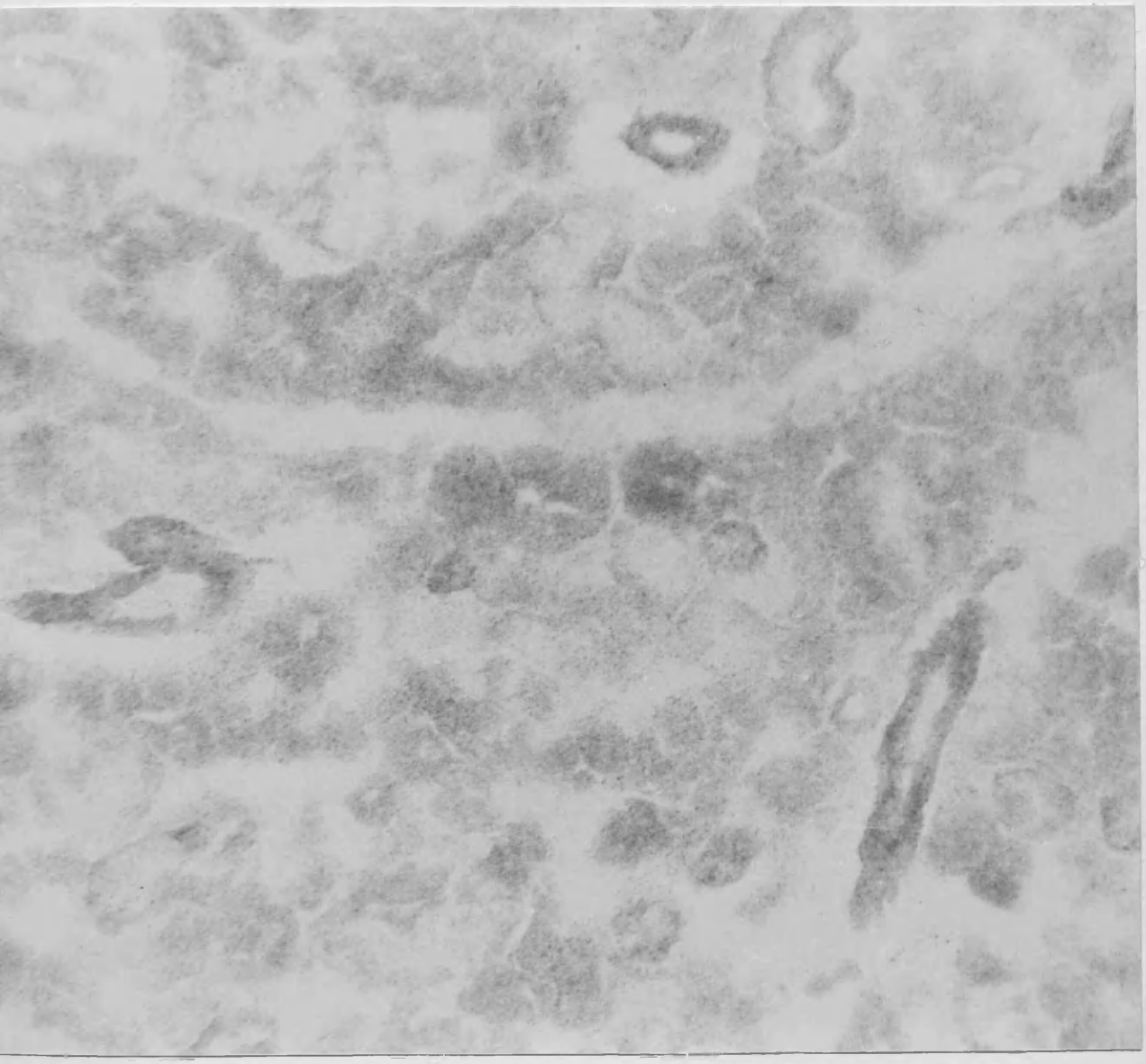


Figure 25. (x2000) Alkaline phosphatase in male rat submandibular gland.

Figure 26. (x1400) 5′-Nucleotidase in male rat submandibular gland.

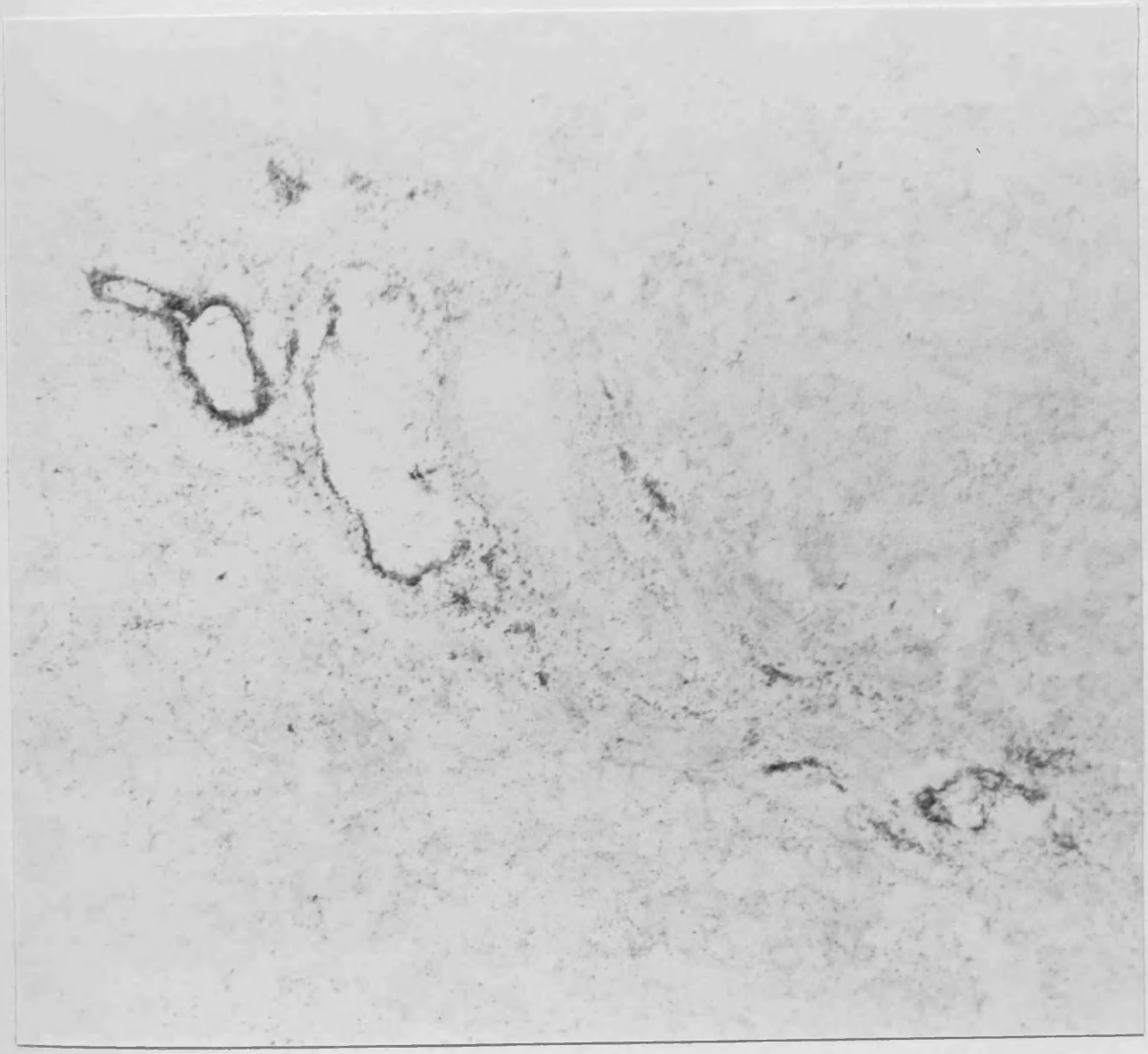


Figure 27. (x1000) Adenosine triphosphatase in male rat submandibular gland.

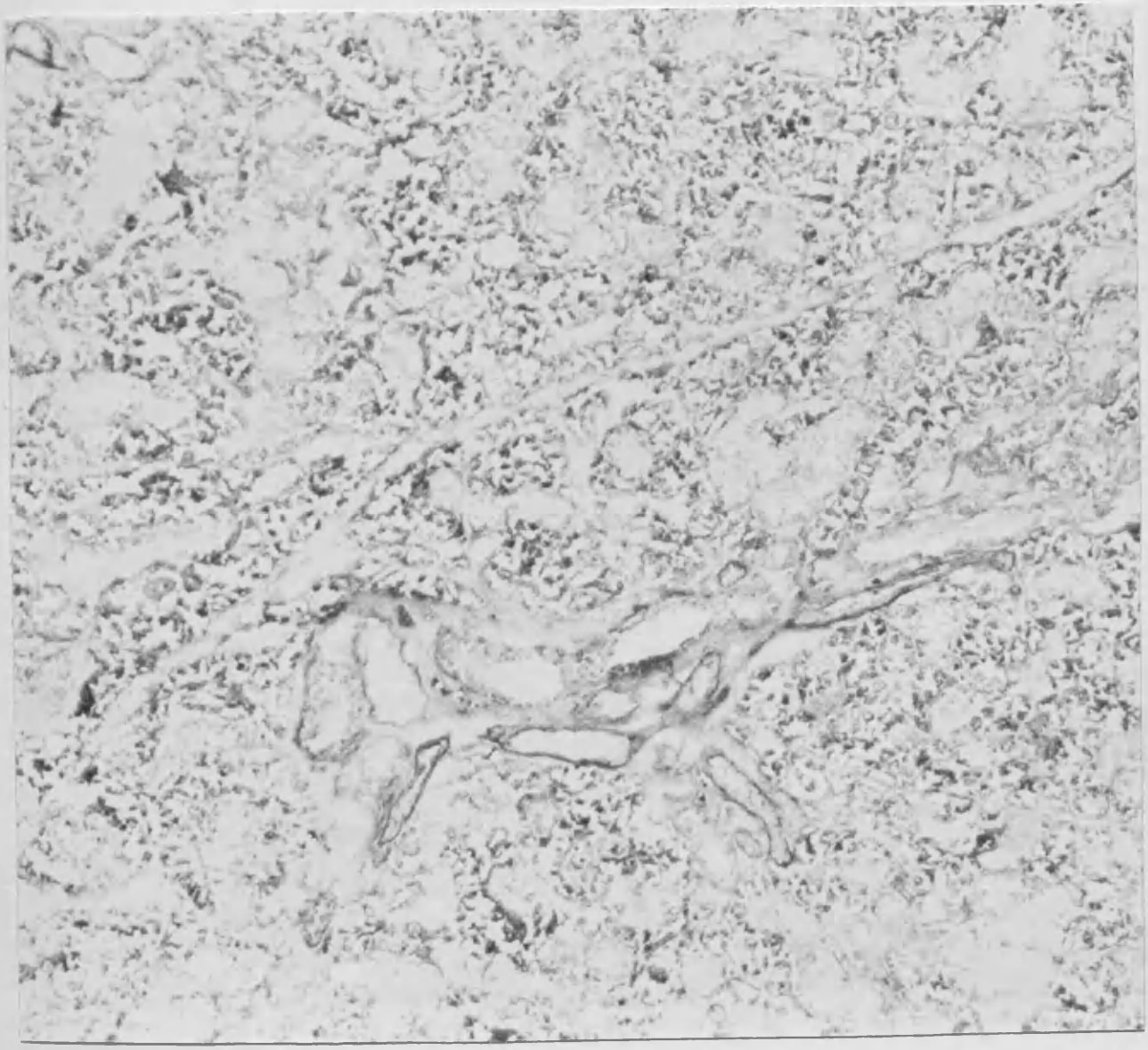


Figure 28. (x9000) Glucose-6-phosphatase in male rat submandibular gland.

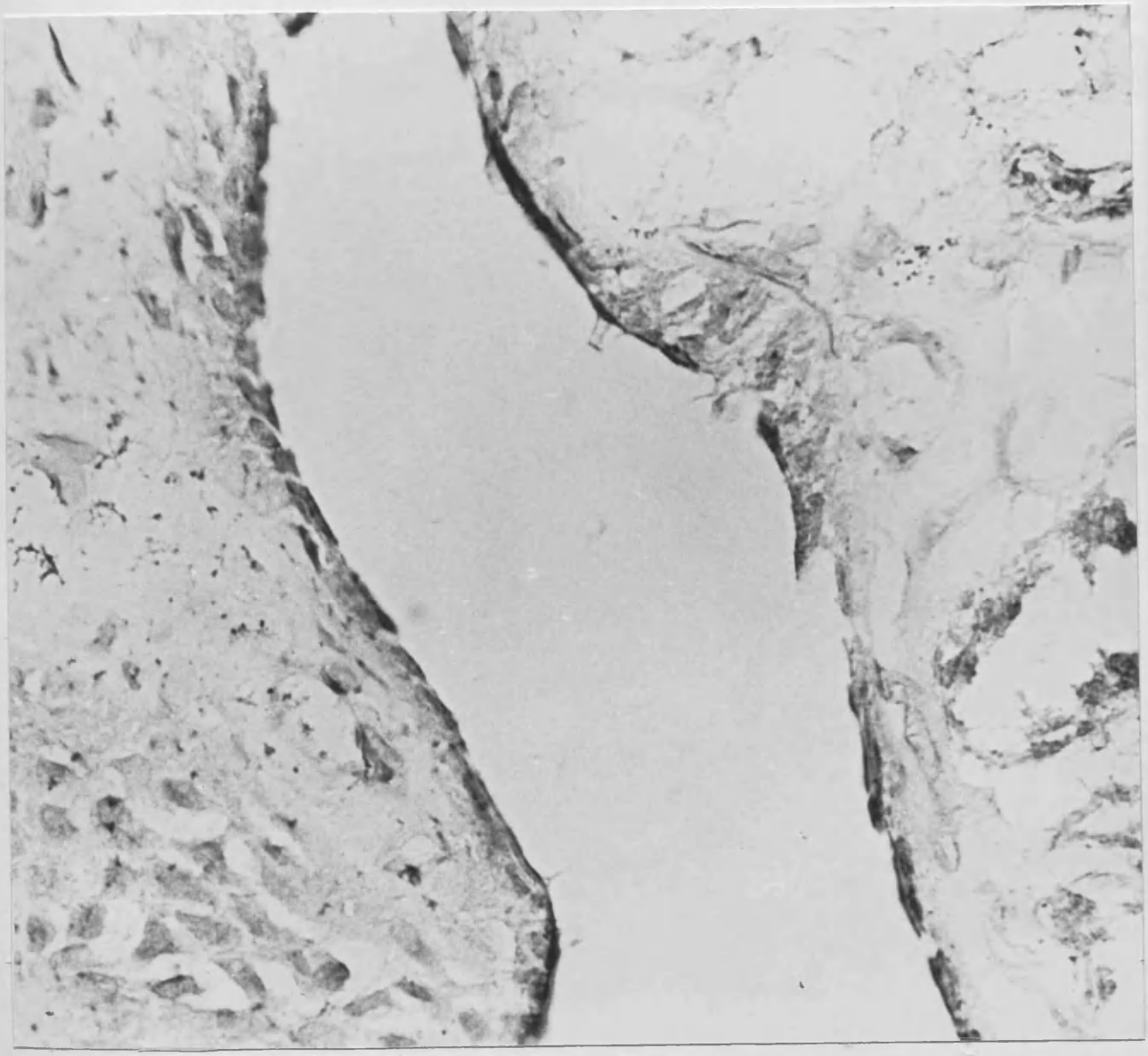


Figure 29. (x1900) Esterase in male rat submandibular gland.

Figure 30. (x900) Succinic dehydrogenase in male rat submandibular gland.

Figure 31. (x8400) α-Glycerophosphate dehydrogenase in male rat submandibular gland.

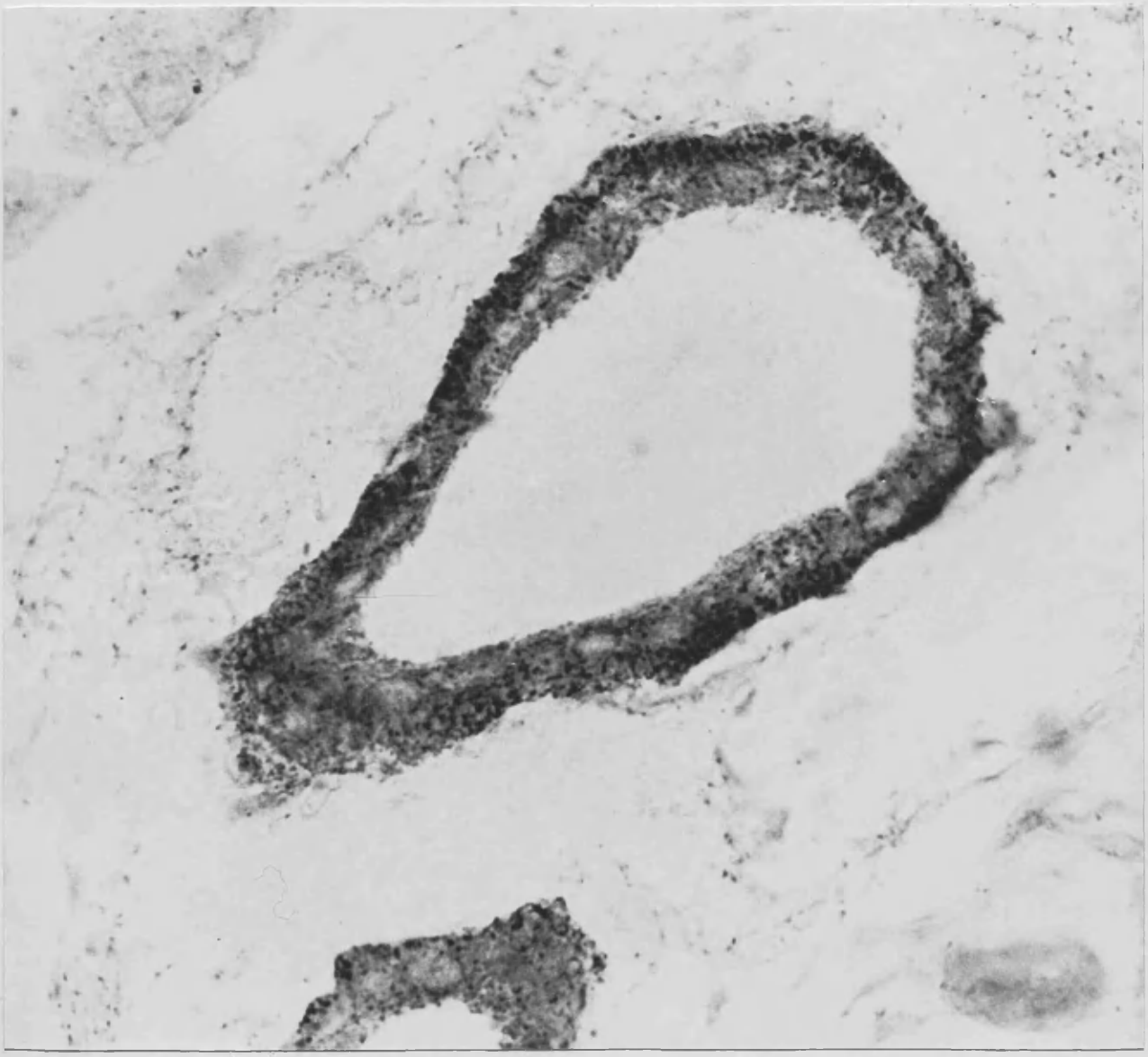


Figure 32. (x8800) Lactic dehydrogenase in male rat submandibular gland.

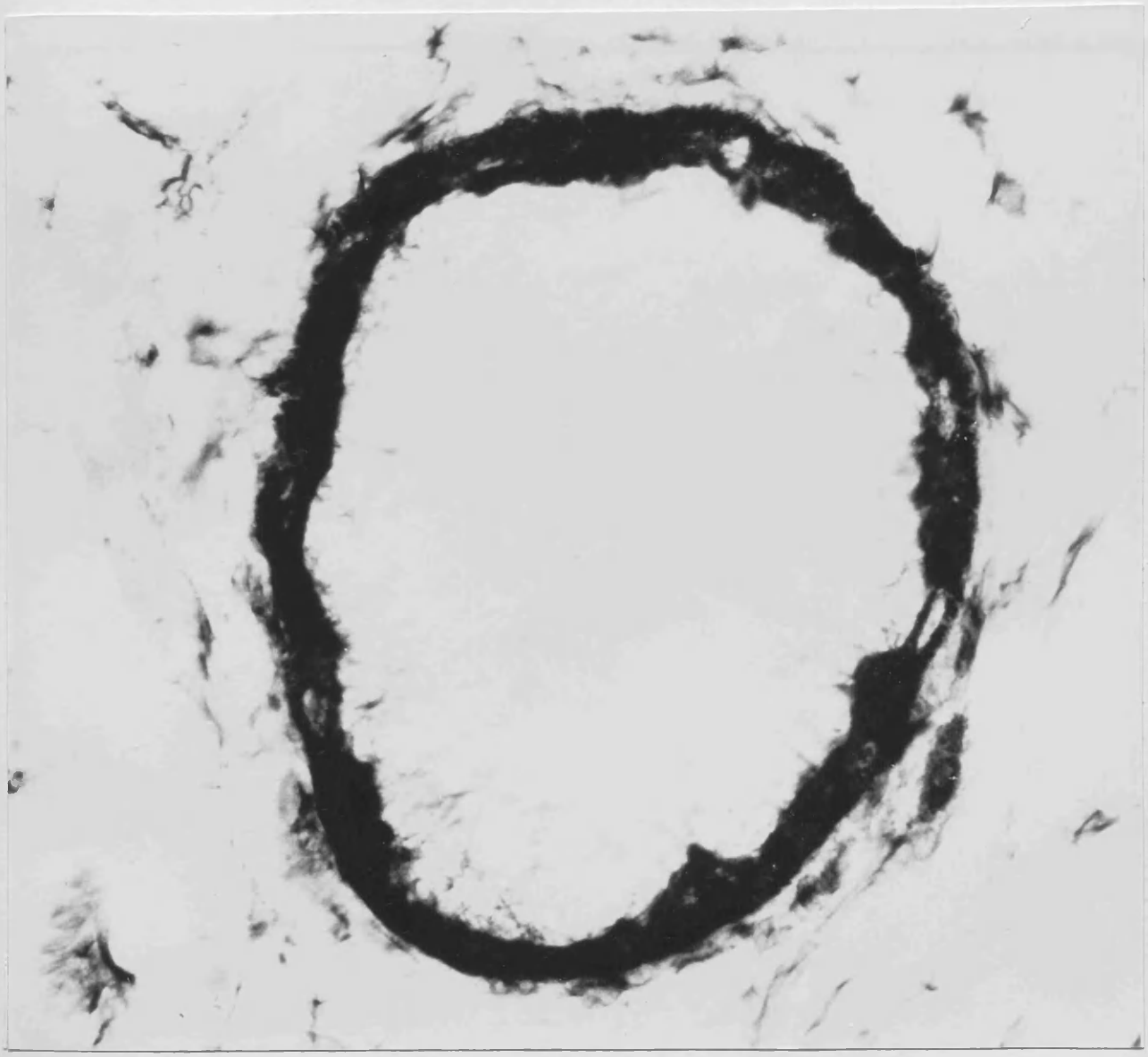


Figure 33. (x9200) Malic dehydrogenase in male rat submandibular gland.

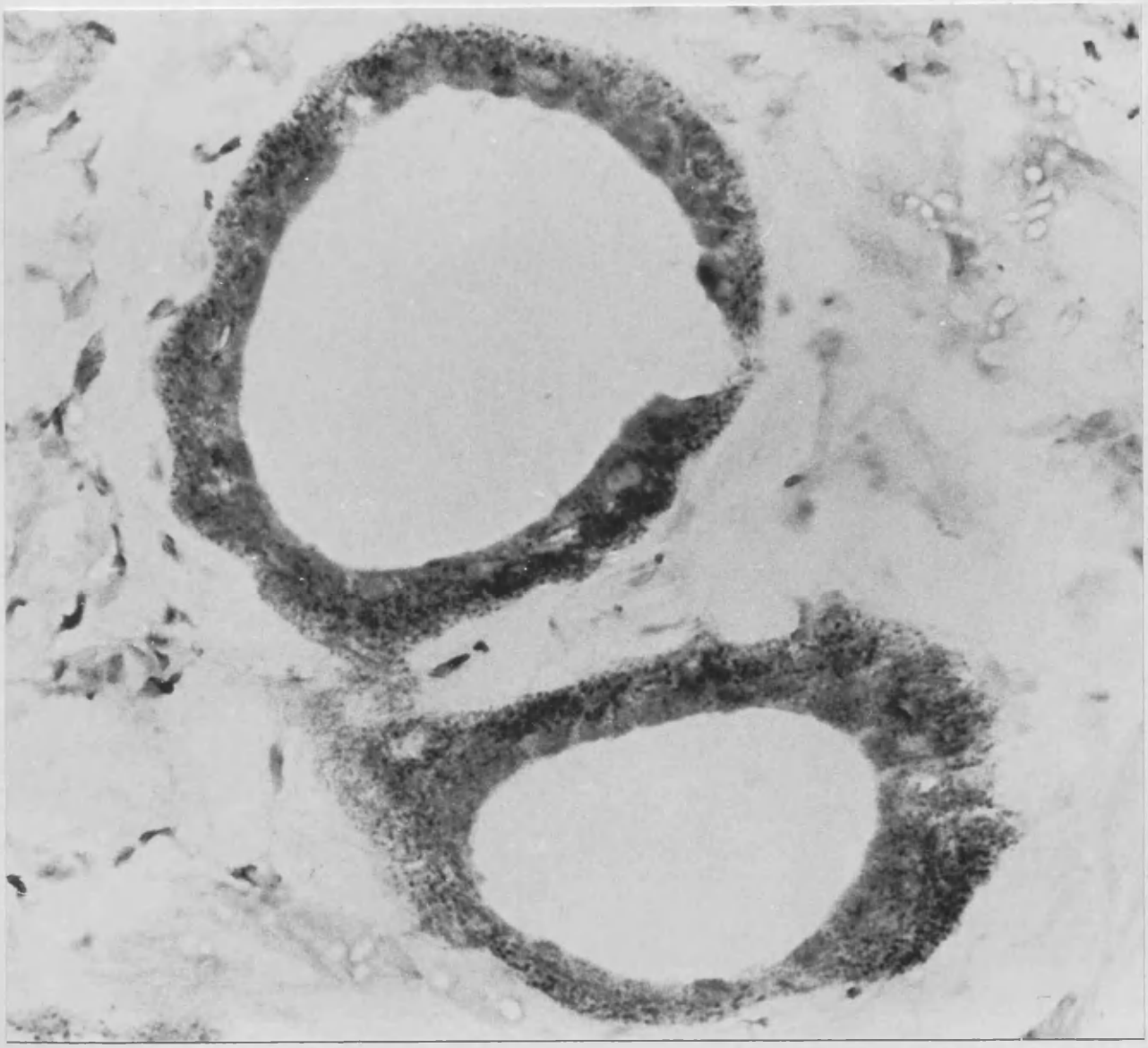


Figure 34. (x8200) Glucose-6-phosphate dehydrogenase in male rat submandibular gland.

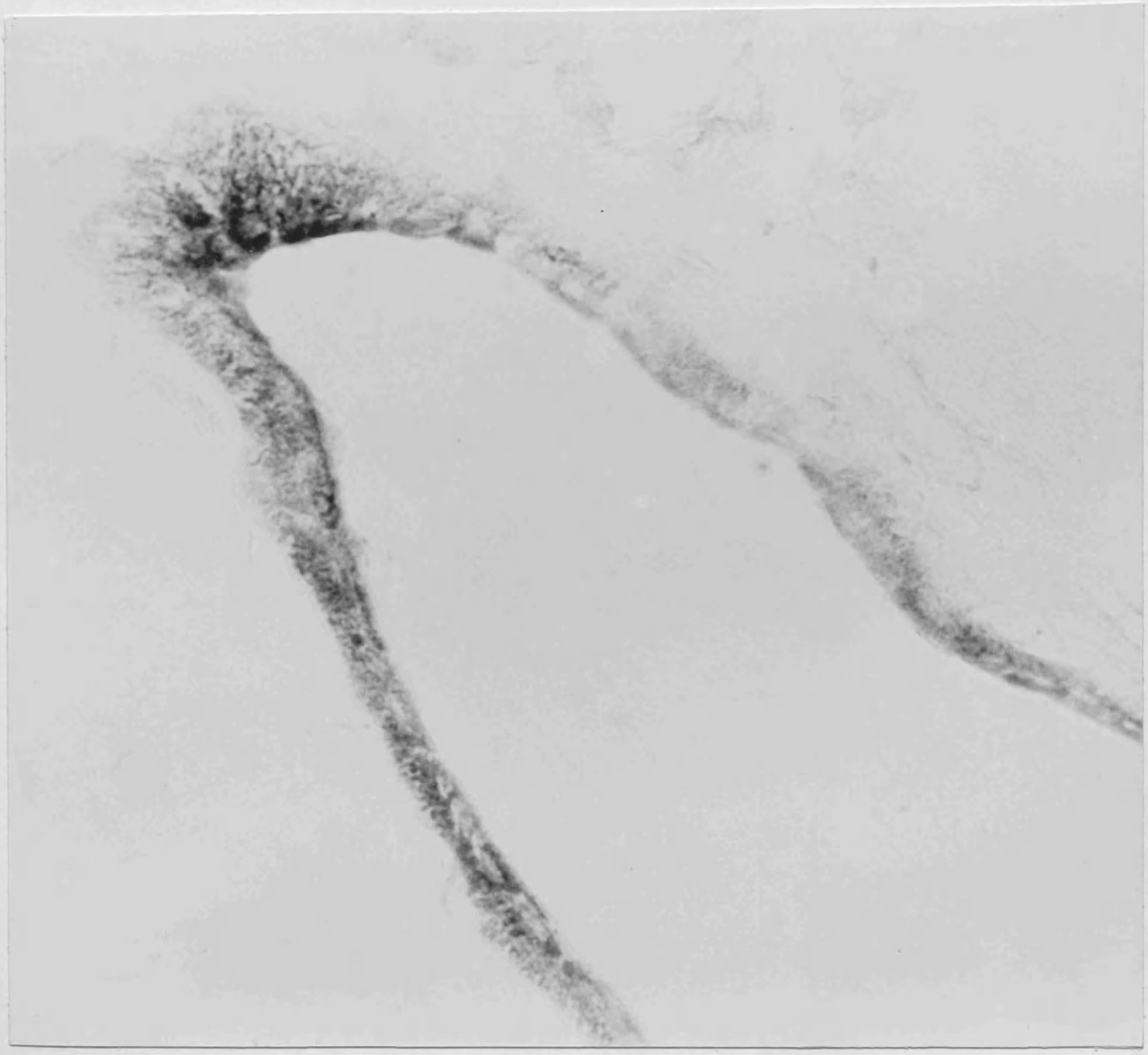


Figure 35. (x7900) β-Hydroxybutyrate dehydrogenase in male rat submandibular gland.

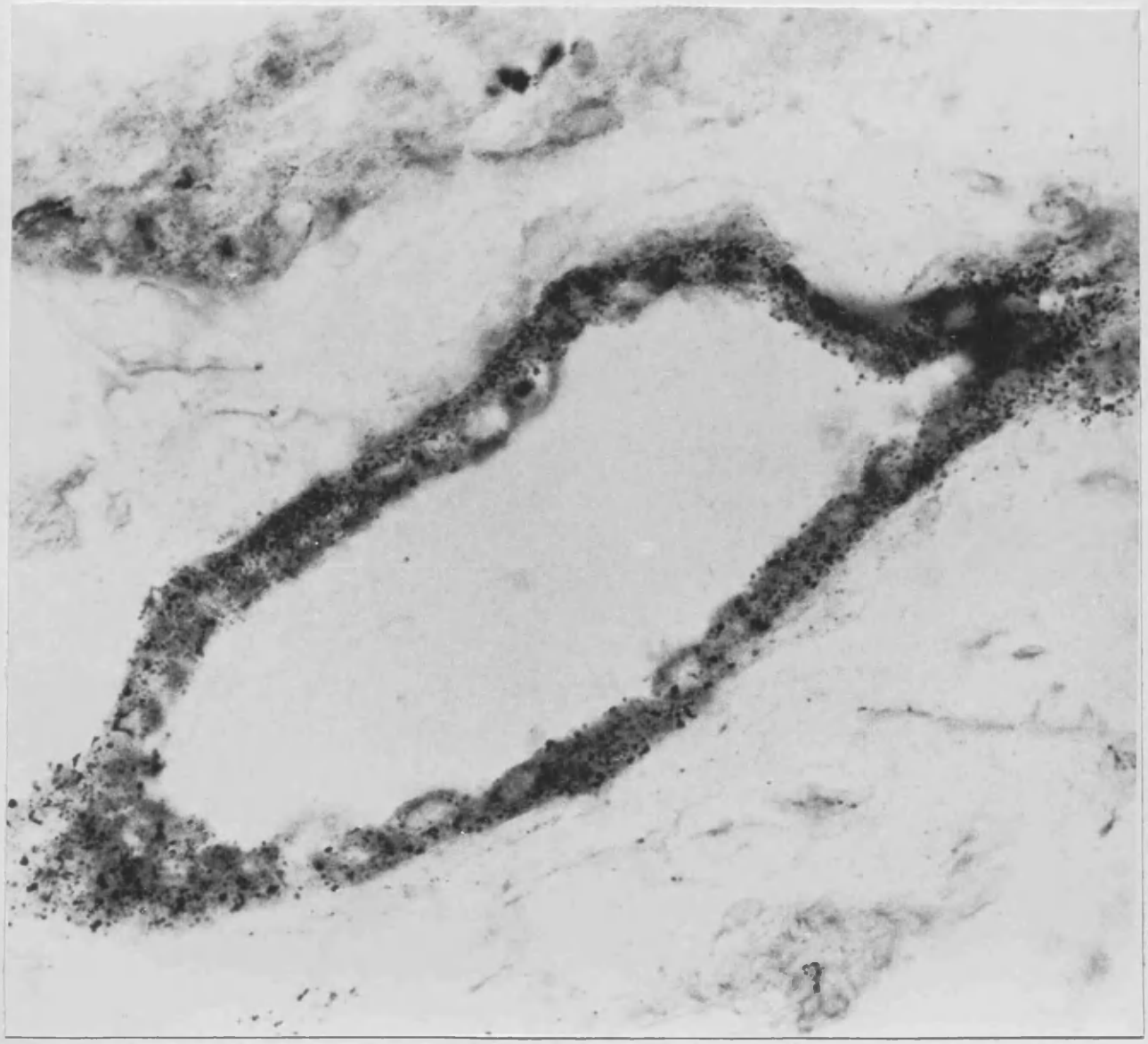


Figure 36. (x11,400) 11β-Hydroxysteroid dehydrogenase in male rat submandibular gland (cortisol as substrate).

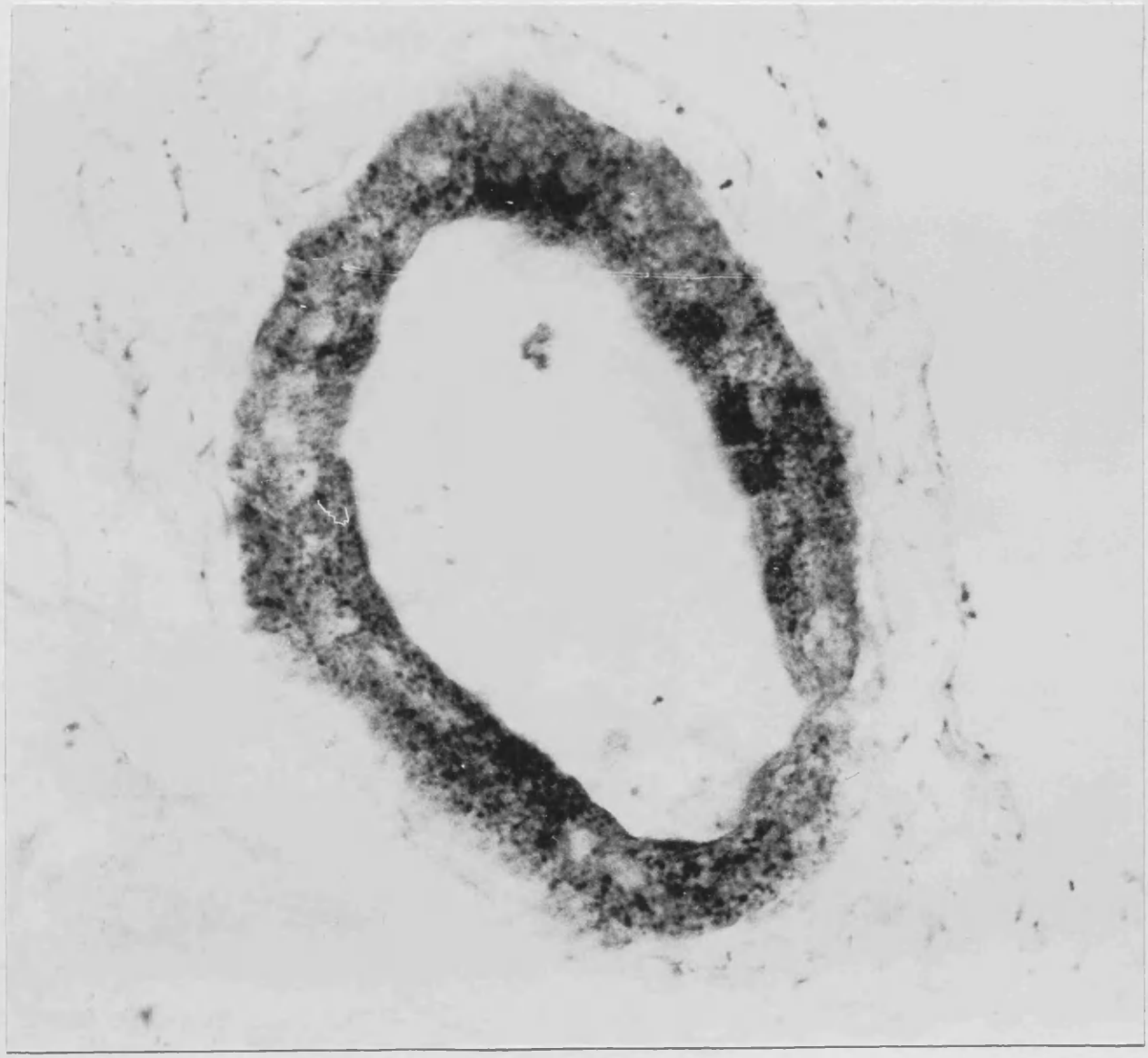


Figure 37. (x10,200) 11β-Hydroxysteroid dehydrogenase in male rat submandibular gland (5α-dihydrocortisol as substrate).

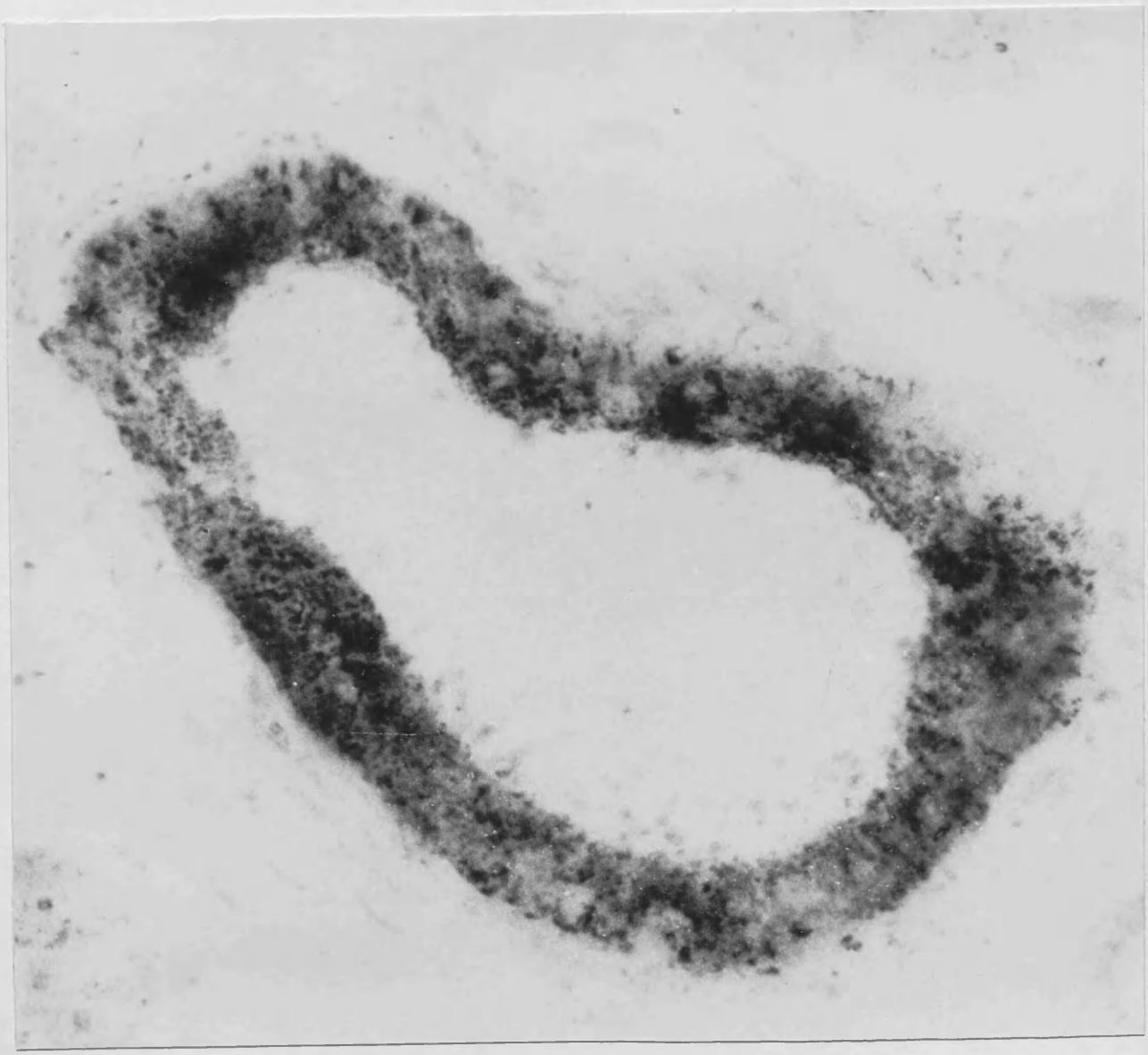
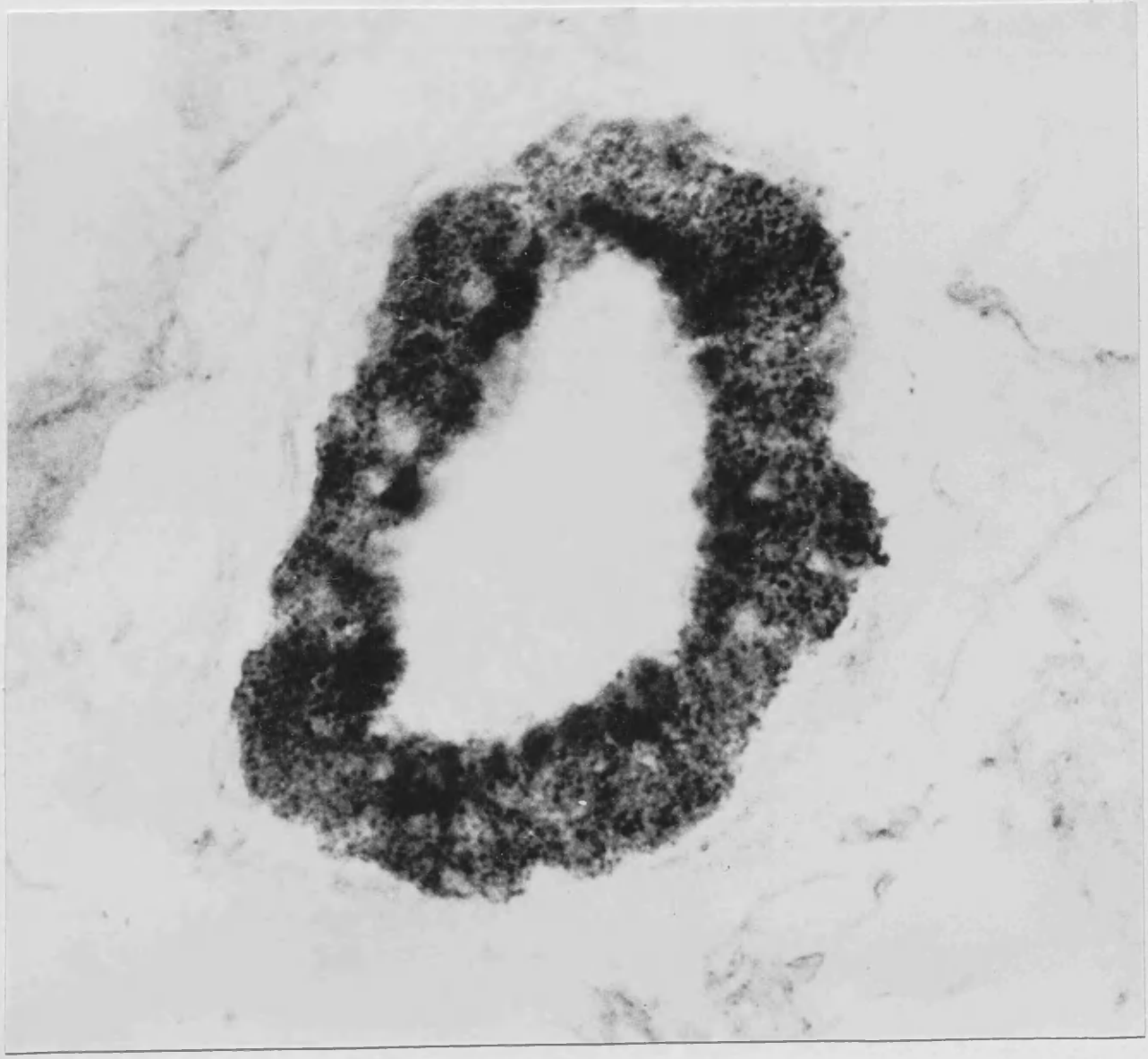


Figure 38. (x12,200) 11β-Hydroxysteroid dehydrogenase in male rat submandibular gland (prednisolone as substrate).



## DISCUSSION

The presence of the dehydrogenases in the salivary duct epithelium gives proof of the concept of a highly active metabolic duct epithelium.

A high level of succinic dehydrogenase activity has been localised in salivary ducts of several species (Hill & Bourne, 1954; Nachlas et al., 1957; Dewey, 1958) which is indicative of a high metabolic rate and it is noted that Telkka et al. (1954) found a diminution in the activity of this enzyme, histochemically, following adrenalectomy which could be restored on administration of cortisone (Telkka & Kuusisto, 1955). Succinic and malic dehydrogenases are important in the Tricarboxylic Acid Cycle which is involved in the central metabolism of the cell. Glucose-6-phosphate dehydrogenase is involved in the Hexose Monophosphate Shunt activity which can lead to synthesis of ATP and Nucleotides. α-Glycerophosphate and lactic dehydrogenase are involved in early and late glycolysis respectively resulting in the synthesis of ATP. β-Hydroxybutyrate dehydrogenase is involved in the oxidation of fatty acids leading to active acetate synthesis.

The duct epithelium seems therefore to be active for the transport of metabolites into and out of these cells. The existence of 11β-HSD activity using ring A analogues of cortisol, 5α-dihydrocortisol and prednisolone, shows the variation in structures of these substrates does not significantly alter 11β-HSD activity. These analogues retain a flat ring A/B junction and verify the work of Bush (1956) and Bush et al. (1968).

## SUMMARY

Histochemical studies were performed with a variety of enzymes on male rat submandibular gland. The presence of the anaplerotic system enzymes and 11β-hydroxysteroid dehydrogenase concentrated in the duct epithelium gives proof of the concept of a highly active metabolic duct epithelium.

# PART IV

# AN ATTEMPT TO PRODUCE 11β-HYDROXYSTEROID DEHYDROGENASE IN CULTURES FROM THE SUBMANDIBULAR GLAND OF THE ADULT RAT IN VITRO

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## AN ATTEMPT TO PRODUCE 11β-HYDROXYSTEROID DEHYDROGENASE IN CULTURES FROM THE SUBMANDIBULAR GLAND OF THE ADULT RAT IN VITRO

### INTRODUCTION

There are three main types of culture methods: a) tissue culture, b) organ culture and c) cell culture. In tissue culture and organ culture very small fragments of tissue are placed in medium and allowed to develop. In tissue culture no special precautions are taken and the tissue fragments soon become disorganised because of the motility of cells. On the other hand, organ culture incorporates special measures to prevent disorganisation of the tissue. In cell culture, the tissue is intentionally disorganised to produce individual cells, usually by digestion with trypsin.

These cultivation techniques are now widely used in current research in the biological sciences. Comprehensive accounts of the development and exercise of various procedures of cell and tissue culture are recorded by Paul (1970).

The organ culture of the submandibular gland of the adult rat has been reported by Trowell (1959) and Tapp (1967). They showed that the cultures underwent a series of characteristic changes. The former paper reported that pieces of the submandibular gland taken from 60g rats (3-4 weeks old) usually remained healthy for up to six days when they were cultured in a special medium, T8, and in specially designed culture chambers. Tapp (1967), however, chose to use adult rats (12 weeks old). Charreau & Villee (1968) have reported on the metabolism of progesterone by rat submaxillary gland explants maintained in organ culture.

However, little has been reported on the maintenance of cell or tissue cultures from the submandibular salivary gland. The histochemical localisation of 11β-hydroxysteroid dehydrogenase was found in the duct cells (Fig. 22) and it was wondered whether the enzyme might be found in cells or tissue cultured from submandibular gland.

## MATERIAL AND METHODS

### 1. General Preparative Procedures.

#### A. Preparation of Glassware.

Medicine bottles, pipettes, universal containers, and bottle tops were treated according to the following procedures:

All glassware is first rinsed in tap-water followed by an overnight soak in 2% RBS 25 (Chemical Concentrates Ltd., London). After a second tap-water rinse the glassware is rinsed twice in distilled water and then dried completely in a hot oven. Pipettes were rinsed in an automatic, syphonic rinser.

The pipettes were sterilised at 160°C for two hours and all other glassware was steam sterilised at 15 lbs./in$^2$ pressure for 20 minutes.

Bottle tops and liners were treated in the same way as the glassware with sterilisation in a pressure cooker.

#### B. Growth Medium.

Ham's F12 synthetic defined medium was prepared in the following manner. All chemicals, where required, were bought from Flow Laboratories, Irvine, Scotland unless otherwise stated.

20% Hams:

| | |
|---|---|
| 20ml | foetal calf serum |
| 8ml | Ham's F12 without sodium bicarbonate |
| 72ml | sterile distilled water prepared in laboratory |
| 2ml | Hepes buffer |
| 0.6ml | sodium bicarbonate (5.6%) |
| 0.5ml | glutamine |
| 1.0ml | penicillin/streptomycin |

#### C. Balanced Salt Solution.

Hank's balanced salt solution was prepared in the following manner:

10ml Hank's (Flow Laboratories)

90ml sterile distilled water

0.6ml sodium bicarbonate (5.6%)

1.0ml penicillin/streptomycin

D. Trypsin-Collagenase Solution.

(1) Trypsin: 2% in BSS - DVC (Balanced salt solution minus divalent cations)

(2) Collagenase (highly purified ex Worthington & Co.:-

0.02mg./ml BSS - DVC)

(3) Foetal bovine serum - 11% in BSS - DVC

Combine before use:-

2 parts trypsin solution

4 parts collagenase solution

2.5 parts foetal bovine serum

E. Trypsin- Versene Solution.

For 100ml of stock versene buffer:

8g sodium chloride (B.D.H.)

0.4g potassium chloride (B.D.H.)

0.2g versene (B.D.H.)

0.02g phenol red (B.D.H.)

This stock is diluted with 900ml of distilled water and distributed in 18ml amounts in universal bottles and sterilised by autoclaving. These bottles are labelled versene buffer solution.

The trypsin-versene stock solution is prepared by diluting 100ml of versene buffer stock with 1,900ml distilled water and 5g trypsin dissolved in this solution which is sterilised by millipore filter. For use 2ml of trypsin-versene stock is added to one universal bottle containing the versene buffer solution and the pH adjusted to pH7.6 with sodium bicarbonate ready for use.

F. Aseptic Conditions.

The antibiotic incorporation into the balanced salt solution and growth medium prevents contamination of the gland by micro-organisms. Further precautions were taken by carrying out growth medium dispensing procedures in a laminar air flow cabinet. Aliquoting of the fluid was performed by sterile disposable (Gillette) syringes and needles. All prepared fluids were filtered through a millipore filter to dispose of any contaminants in the fluid.

2. Cell Cultivation Procedure.

Adult male (200-250g) Sprague - Dawley rats fed on diet 41B were killed by cervical dislocation and the submandibular glands removed. The glands were put in a universal container containing balanced salt solution. The medium was kept at 37°C until ready for use. The tissue was then chopped finely and incubated in the trypsin-collagenase solution for 2 hrs. in a shaking water bath at 37°C. A cell suspension was left, aliquots having been taken for counting in a haemocytometer, and these cells were spun at 1,000g for 5 minutes to remove the disaggregating enzyme solution. The pellet of cells was then suspended in the growth medium and portions were inoculated into the medicine bottles, the total volume being 5ml.

Specimens were taken for staining with 0.1% trypan blue to ascertain the viability of the cells, the majority of the cells being viable by this criterion. The cell cultures were allowed to grow and multiply at 37°C and any flasks requiring changing were trypsinised with trypsin-versene solution and re-inoculated into further flasks with the growth medium. Microscopical examination of the cell cultures was performed by use of a Nikkon inverted light microscope. 11β-hydroxysteroid dehydrogenase examination of the cells was carried out by incubating $1x10^6$ cells (removed from the flasks by a rubber policeman and centrifuged) in the assay (p. 27) instead of the 100mg. gland homogenate.

3. Tissue (Explant) Cultivation Procedure.

The submandibular glands were removed and processed as previously described under the cell cultivation procedure up to the trypsin-collagenase treatment where, instead of this, the chopped tissue (explants) were placed into the growth medium in the medicine bottles. Further experimentation found that the use of disposable plastic petri dishes was preferable to glass medicine bottles. Contamination from the air was prevented during the 37°C incubation by sealing the joins on the petri plate with sellotape. The medium was changed every 7 days to prevent exhaustion of the growth medium. 11β-hydroxysteroid dehydrogenases assays were carried out using 100mg. homogenate equivalents (p. 27 ). Incubations with 1ml of the growth medium in the 11ml enzyme assay volume were also carried out to find out the presence or not of 11β-hydroxysteroid dehydrogenase in the serum of the growth medium.

## RESULTS

No 11β-hydroxysteroid dehydrogenase activity was observed with cells cultured from submandibular salivary gland. Neither 7 day nor 21 day explants nor the growth medium exhibited any 11β-HSD activity. Therefore the possibility that 11β-HSD is present in the serum of the growth medium can be discounted.

In the cell culture fibroblasts appeared first after 2 days followed by the deposition of epithelial cells on the surface of the glass. The epithelial cells were cuboidal or rounder in shape than the fibroblasts.

The explants showed fast degeneration and a 7 day old explant culture showed up spots of secretory deposit (Fig. 39). The appearance of tubules occurs within 7 days as shown in Fig. 40. These tubules can be maintained in culture for several weeks and even grow during this period. The culture of ductal tissue occurs preferentially to acinar tissue (Fig. 41) due to the rapid degeneration of acinar tissue. However, a 26 day old explant culture showed collagen and enucleated eosinophilic material (probably from ductal cell material). This material showed no haemotoxylin stain or histochemical enzyme activity (succinic dehydrogenase or 11β-HSD).

Figure 39. (x90) 7 days old explant culture of male rat submandibular gland showing spots of secretory deposit (S).

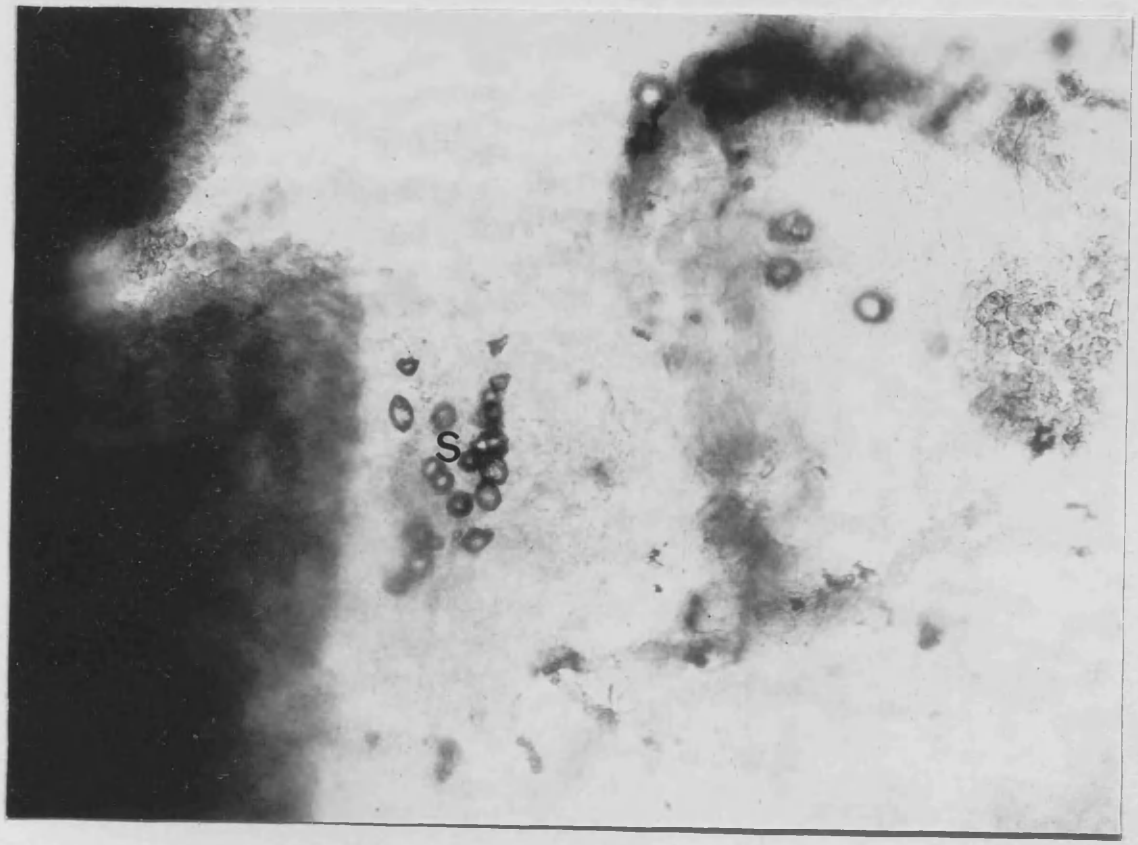

S

Figure 40. (x225) 7 days old explant culture of male rat submandibular gland showing growth of 'tubules' from the explant.

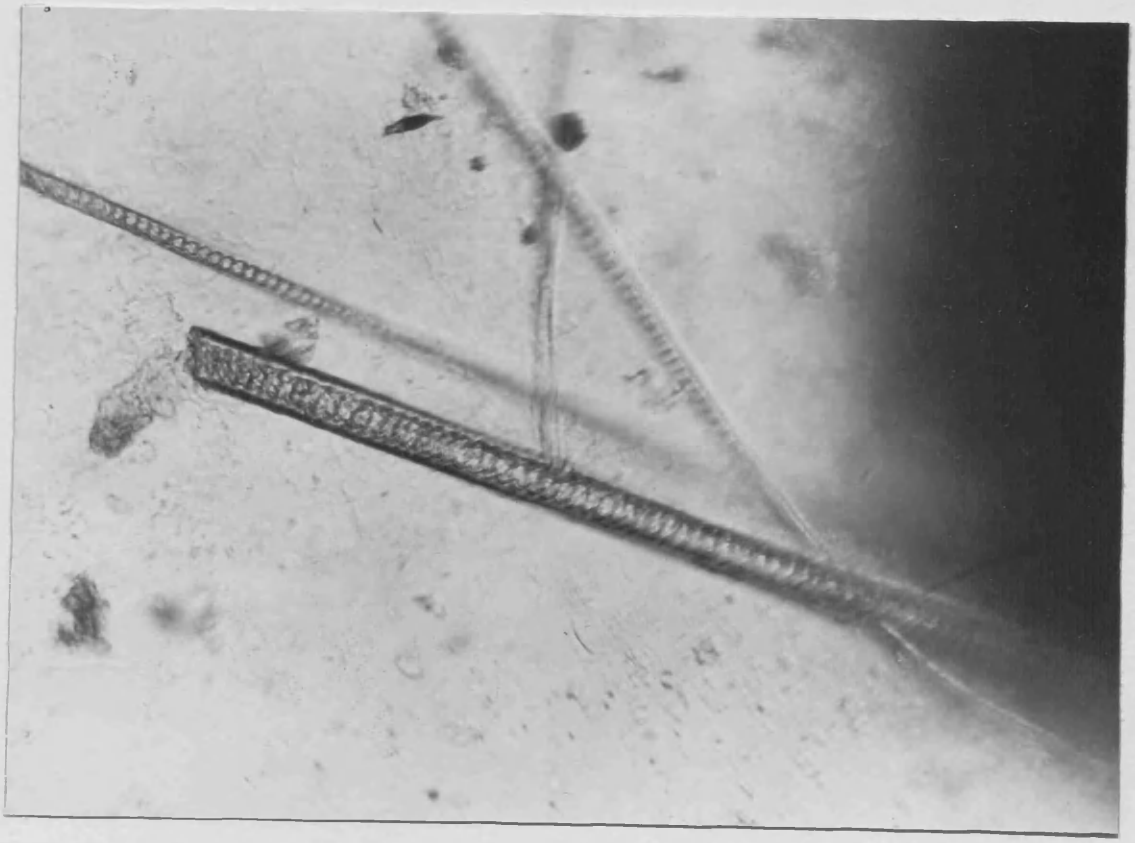
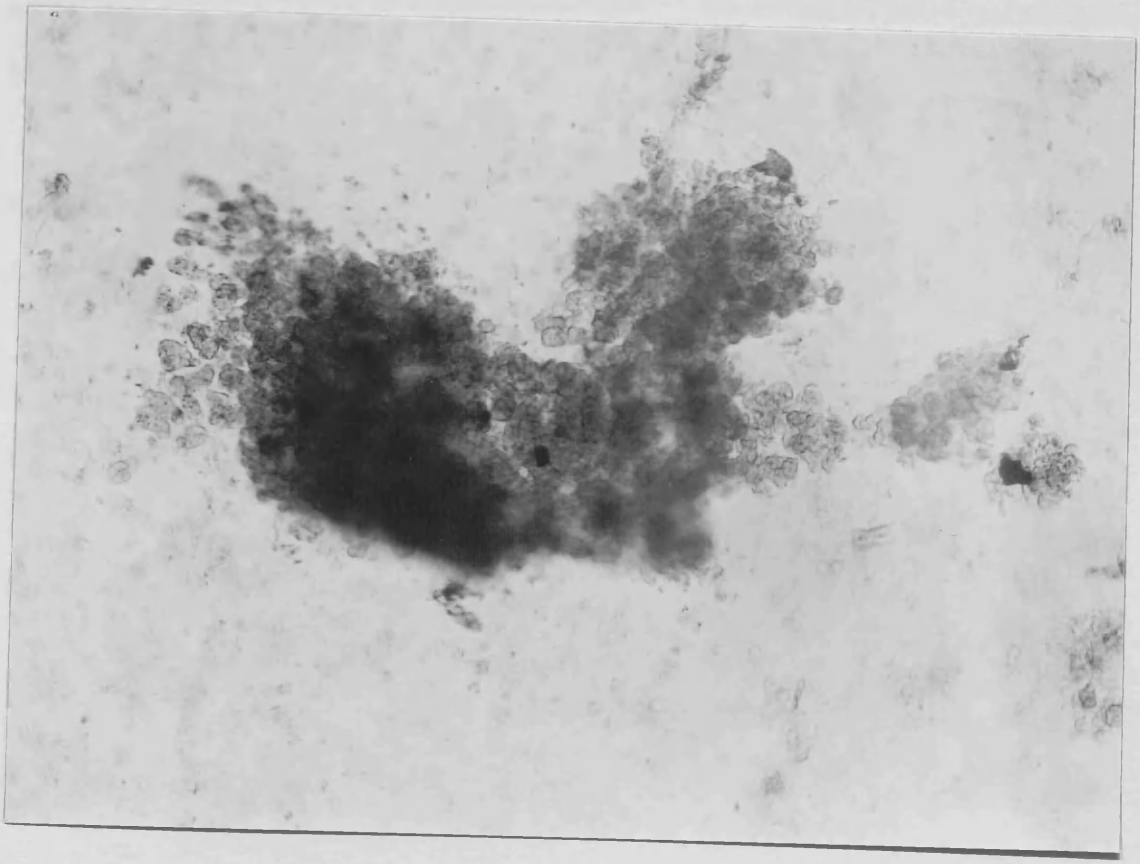


Figure 41. (x90) Explant culture of male rat submandibular gland showing predominance of ductal tissue.



## DISCUSSION

11β-HSD activity has been reported in connective tissue in fibroblast cultures *in vitro* (Berliner & Dougherty, 1961). Their report indicates that cortisol and corticosterone are concentrated by the entire reticuloendothelial and connective tissue systems. Portions of these hormones undergo metabolism as unconjugated products in these tissues. There is also considerable exchange with interstitial fluids, and Samuels (1966) has suggested that, with cortisol, extravascular and intracellular binding may play an important role. Osinski (1960) also found the placental 11β-HSD strongly bound to connective tissue.

Fibroblastic synthesis results in collagen production, and the effect of collagen is relevant to epithelial morphogenesis (Kallman & Grobstein, 1965, 1966). In the present study, fibroblasts were the first cell type to be formed followed later by the appearance of epithelial cells. No 11β-HSD activity was seen and it can only be concluded that submandibular gland fibroblasts or epithelial cells do not contain the induction processes for 11β-HSD formation. However, trypsin-collagenase was used in the cell isolation and this probably has a detrimental effect on the steroid enzyme. The same conclusion can be formed for the explant culture in that the induction processes for 11β-HSD formation are absent. Several cultures did not survive for 2 days but degenerated to loosely packed and shrunken cultures, and along with the development of the secretory deposit produces evidence that the cultures suffer anoxic stress. This confirms the work of Tapp (1967) who tried to reduce anoxia in organ cultures of submandibular gland. Anoxia is one of the factors which contributes to the development of watery vacuolation when small pieces of the gland are immersed in a physiological medium *in vitro* (Tapp & Trowell, 1967). Ligation of the submandibular gland *in vivo* (Standish & Shafer, 1957; Tamarin, 1971) showed that the glands atrophy rapidly. The acinar cells appeared to atrophy rapidly whereas the duct cells survived. From these findings one

can confirm the preferred culture of ducts and tubules by the submandibular gland.

There are two main theories as to the reason why the cultures of submandibular gland did not produce 11β-HSD.

1. In the cultures there is anoxic stress which allows fluid to enter the cells, some of which may be killed, and, when the oxygen becomes available, the fluid is secreted causing decrease in cell size and an accumulation of enzymes, possibly lytic enzymes, in the culture medium. These changes could be detrimental to the induction of 11β-HSD.

2. The disadvantage of cell and tissue culture techniques to their employment is the uncertainty that attends the application to the intact animal or fresh homogenate of results obtained with cultures. There are artificial conditions for the maintenance of viability and the removal of cultures from this influence of contiguous and differentiated cells may themselves have so altered the homeostasis of the cells in the gland as to make comparison with the in vivo cell incorrect. The tissues and cell culture are grown in conditions that mimick adrenalectomy, thydroidectomy, castration, etc., and although it seems certain that the presence of hormones is not essential for cell survival it is likely that they are required for the full development and function of differentiated cells (Paul, 1970). Probably the induction of 11β-HSD requires some extraneous cofactors to those produced in the cultures.

## SUMMARY

1. No 11β-HSD activity was observed in cell cultures of rat submandibular salivary gland.

2. No 11β-HSD activity was observed in explant cultures of rat submandibular salivary gland or in serum used in the growth medium.

3. Explant degeneration was followed by the selective growth of submandibular gland tubules.

4. In the discussion the results are correlated with the reports of other workers and two theories are proposed for the absence of 11β-HSD in the cell and tissue cultures.

## GENERAL DISCUSSION

## METABOLISM OF 11β-HYDROXYSTEROID DEHYDROGENASE IN SALIVARY GLAND

1. In relation to water and electrolyte metabolism.

2. In relation to secretion and interconversion of 11β-hydroxy and 11-oxo groups.

3. In relation to general hormone effects.

## GENERAL DISCUSSION

### 1. Water and Electrolyte Metabolism.

The adrenocortical hormones are in the widest sense concerned with maintenance of homeostasis. They enable the organism to react to internal and external stress by sensitising it and calling forth appropriate defence reactions, although they are not specific for these reactions.

The corticosteroids show marked differences in the nature and intensity of their activity. The distinction between mineralocorticoids and glucocorticoids is an unfortunate one since the physiological action of cortisol, the most important 'glucocorticoid', depends much less on its effect on carbohydrate and nitrogen metabolism than on its effect on mineral and water metabolism. This latter action of cortisol is seen particularly in stress, when the most important requirements are maintainance of the circulation, fluid balance and renal function. All corticosteroids cause retention of sodium and an increase in the excretion of potassium and water but cortisol has an additional effect on water metabolism to that of aldosterone and deoxycorticosterone. This substance regulates the distribution of water and salts in the body independently of the renal excretion of sodium. The decrease in water diuresis seen in adrenocortical insufficiency can be reversed by administration of cortisol but not of aldosterone. Cortisol appears to be necessary for maintenance of a tubular function adequate for normal water diuresis but on the other hand does not inhibit the activity of the antidiuretic hormone of the posterior pituitary.

Salivary ducts were proposed by the early histologists (Merkel, 1883; Werther, 1886) on morphological grounds not to be merely passive conduits between the glandular tissue and the oral cavity but were instead responsible in some manner for the modification of acinar secretion. The support of this suggestion of active ductal tissues was later upheld by the description of a rich vascular network surrounding the ducts (Kowalevsky, 1885; Zimmermann,1898; Flint, 1901; Spanner 1937).

The ultrastructure of the salivary duct epithelium has indicated, by the presence of microvilli, secretory antecedents and complex basal infoldings, that the striated ducts are the principal site of the electrolyte and water transfer in the salivary duct system (Scott & Pease, 1959; Leeson & Jacoby, 1959; Parks, 1961; Tandler, 1963), although Tamarin (1964) indicates that the excretory duct ultrastructure is also compatible with ion transfer. Parks (1961) has observed a similarity of salivary gland ultrastructure to that of the renal collecting tubules. The duct epithelium has a high metabolic activity as shown histochemically by the high level of enzymes involved in general metabolism (Hill & Bourne, 1954; Greenspan et al., 1964; Ferguson, 1967) and it has been suggested by Ferguson et al. (1970) that the utilisation of cortisol in striated and excretory ducts is indicative of 11β-hydroxysteroid mediated control of the composition of saliva by these components of the duct system.

A number of investigators have established that the secretion of saliva consists of two phases: a primary acinar secretion and a secondary modification by the duct system which involves water and electrolyte transfer (Burgen, 1956; Logothetopoulos & Myant, 1956; Burgen & Seeman, 1957, 1958; Langley & Brown, 1960; Henriques, 1961; Schneyer & Schneyer, 1961). The electrolyte content of saliva and urine is influenced by several corticosteroids although mineralocorticoids have been found to exert a more profound effect than glucocorticoids (Grad, 1952; White et al., 1955; Martin, 1958; Bates, 1958; Mills, 1966). The salivary concentrations of sodium and potassium in man vary in a circadian subsystem, the $Na^+$:$K^+$ ratio being highest just prior to wakening and lowest about mid-day (Grad, 1951; De Traverse & Coquelet, 1952). The relationship noted between plasma ACTH (adrenocorticotrophic hormone) and the salivary $Na^+$:$K^+$ ratio means that corticosteroids other than aldosterone influence this ratio (Dreizen et al., 1952; Grad, 1952). Imrie et al. (1963) discussed the relationship between corticosteroids, other than aldosterone, and urinary secretion of

electrolytes.

There may well be some relationship between the circadian subsystem of $Na^+$:$K^+$ and the plasma cortisol level which also exists in a circadian subsystem (Peterson, 1957; Doe et al., 1960; Mills, 1966). Bates (1958), however, could not demonstrate any such connection in human parotid saliva, whereas Katz & Shannon (1969a) have shown that parotid fluid corticosteroids appear to be a good measure of plasma cortisol. They also demonstrated that, when salivary flow is stimulated, the concentration of cortisol plus cortisone appears to vary inversely with flow rate resulting in an approximately constant excretion rate. The 11β-hydroxyl group in the steroid molecule is unnecessary for a mineralocorticoid effect, but it does exert a considerable influence on glucocorticoid function. With the localisation of 11β-HSD in the salivary gland ducts and other epithelial sites of water and electrolyte transport it would seem reasonable to propose that the ducts may be the site of a glucocorticoid mediated salivary modification (Ferguson, 1966).

2. Secretion and Interconversion of 11β-Hydroxy and 11-Oxo Groups.

It has been known from circumstantial evidence for a long time that cortisol and cortisone interconvert rapidly in the human body. It was generally held that the interconversion occurred in the liver (Rosenfeld, et al., 1967). 11β-HSD has been shown to have a higher activity in the livers of many mammalian species while lower activities appear to be present in preparations of other tissues (Hurlock & Talalay, 1959; Koerner & Hellman, 1964). However, although the liver operates extremely effectively in the reductive direction, as far as 11β-HSD is concerned, the problem to account for is that approximately two-thirds of the total major $C_{21}O_5$ metabolites of cortisol, or cortisone, in human urine are in the 11-oxo form. This leads to the theory that the extrahepatic oxidation of cortisol to cortisone is responsible for the major part of the 11-oxo metabolites in human urine. The suggestion of Bush (1969) is that the relatively low

activity of 11β-HSD in peripheral tissues would be made up by their large mass and the extremely low concentration of cortisone in the circulating blood would be explained by the very large, possibly 100% hepatic clearance for cortisone. Bailey & West (1969) have noted that the initial cortisol-cortisone interconversion must mainly occur in the general body tissues and the speed of interconversion precludes the liver from being the main site of interconversion.

The theory proposed by Bailey & West (1969) is that the tissues, under basal conditions, are receiving from the adrenals approximately equal concentrations of cortisol and cortisone. Within the body tissues as a whole the cortisol and cortisone are interconverted and metabolised which would allow one type of cell to have a raised effective concentration of cortisol by making the reaction cortisol-cortisone proceed predominantly to the left and another type of cell to have a low effective concentration by converting cortisol to cortisone. The submandibular duct cells seem to have the latter ability. Bush (1969) postulated the idea of two cell types in the liver, assigning the reductive function to the Kuppfer cells lining the hepatic sinusoids and the oxidative function to the microsomes of the parenchymatous cells. Bush (1956) drew attention to the fundamental problem of whether the catalytic actions of adrenal steroids involved a cyclic reversible reaction with the hormone or not, and emphasised the possible role of the 11β-HSD if the former possibility could be substantiated. Among the large number of pairs of related 11β-hydroxy and 11-oxosteroids, the oxo form is systemically active because it is readily reduced to the 11β-hydroxyl by the hepatic 11β-HSD. Robel (1971) postulated that in hydroxysteroid dehydrogenase action, the metabolites which are inactive by themselves could regulate the amount of active hormone available to receptors and/or favour its renewal. In the submandibular salivary gland the active steroid is that bearing the 11β-hydroxyl group rather than the 11-oxo group. Body weight tends to decrease in rats treated by cortisone

while nitrogen excretion increases. The most marked catabolic effect is observed in lymphoid tissues and muscle but the liver is an exception to this inhibitory reaction and in animals treated by glucocorticoid liver weight and protein synthesis increases. In the submandibular gland of the rat, however, cortisone was found to have no catabolic effect (Zelles et al., 1971) whereas on parotid gland there was an intense catabolic effect. Organ weight, activity of various dehydrogenases and adenine nucleotide contents of submandibular gland were not influenced by cortisone treatment. However, Takeuchi et al. (1971) reported that the administration of aspirin and cortisone caused significant decreases in most of the components of protein-bound carbohydrates of dog submaxillary saliva.

Katz & Shannon (1964) have shown that dog parotid tissue converts cortisol to cortisone showing the presence of 11β-HSD in the parotid gland and the findings of the present study show 11β-HSD in submandibular gland. Shannon, Katz and coworkers (1959, 1967, 1969a, 1969b) have investigated in great detail the presence of free 17-hydroxycorticosteroids (17-OHCS) in saliva. The presence of 17-OHCS in plasma is a sensitive index of the functional status of the human adrenal cortex. Human parotid saliva contains a measurable amount of 17-OHCS and parallels have been demonstrated between serum and parotid fluid 17-OHCS levels in both health and disease. The rapid transfer of cortisol from the bloodstream to parotid fluid suggests that parotid fluid steroid measurements possess potential in monitoring adrenocortical responses of even a fleeting nature. No glucuronide-conjugated steroids, the form used for renal excretion, were present in submaxillary fluid suggesting that their secretion is not an excretory process. The water solubility of glucuronide conjugates is relatively greater than is that of their parent steroids. Such a characteristic makes the conjugates more readily available for renal excretion. Thus the steroids, mainly cortisol and cortisone, would tend not to subsume an excretory function, but rather to participate in a process analogous to the

enterohepatic circulation. Submaxillary fluid lacks significant corticosteroid binding protein activity and therefore free steroid is available to the cells of the submaxillary gland.

3. General Actions of Steroid Hormones.

In the last fifteen years or more there have been various approaches to the action of steroid hormones. Biochemists and endocrinologists have focussed their attention on the control of cellular functions such as regulation of enzyme activity by steroid hormone ever since the discovery that enzymes are key regulators of metabolic processes. There have already been extensive reviews on actions of steroid hormones reported previously (Bush, 1962; Williams-Ashman, 1965; Grant, 1969; Torda, 1971; Lang, 1971; Robel, 1971).

Following a stimulating paper by Talalay & Williams-Ashman (1958), suggesting that oestrogens act as co-enzymes for the soluble pyridine nucleotide transhydrogenase in human placenta, several workers have tried to show that enzymes catalysing the conversion of steroid hormones might be involved in the action of such hormones. However, data were accumulated which seemed incompatible with the general thesis advanced. Hurlock & Talalay (1959) were not able to show transhydrogenation with hepatic 11β-HSD (rat) although the enzyme, like most other HSD's, can use either NAD or NADP as co-enzyme. The 11β-HSD in rat submandibular gland can also use either NAD or NADP with cortisol as substrate but the inability of cortisone to use $NADPH_2$ or $NADH_2$ seems to discount any possibility of transhydrogenation being related to 11β-HSD action in salivary gland.

Yielding & Tomkins (1960) observed that oestrogens can inhibit crystalline glutamate dehydrogenase by disaggregating the enzyme molecule from a polymeric into a monomeric form. This effect gives rise to the concept that hormones may influence enzyme activities through bringing about changes in the physical properties of enzyme molecules. The concentrations of steroids used in their experiments were far above the physiological levels

and no discernible physiological significance can be shown. Grant (1969) has suggested that these studies must be regarded as offering models of how steroids might interact with proteins rather than as throwing light on physiological processes.

Cyclic AMP (adenosine monophosphate) has been considered as a mediator of the hormone effect since Robison et al. (1971) showed the primary effect of hormone action is the activation of adenyl cyclase which leads to cyclic AMP as a secondary messenger and this brings about further secondary events. Torda (1971) has stated that the steroid hormones require a two-step mechanism (cellular and nuclear) for full expression of the message they carry. Steroid hormones pass the cell membranes in a carrier bound form. The carrier is either a non-specific translocator, or a specific receptor. Thereafter, the hormones are stored in cytosol pools. In order to realise their functions they must be remobilised and enter the nucleus in a carrier-bound form. A non-specific carrier may act by preparing an allosteric binding site of a hormone-specific receptor to bind the incoming steroid hormone. When the specific receptor serves as carrier, the steroid hormone is either released to form a geneomic coupling on the chromatin, or the receptor-hormone-complex becomes attached to the chromatin.

There is considerable evidence that corticosteroids stimulate protein and nucleic acid synthesis in liver while the opposite is the case in lymphoid tissue. Knox (1951) showed that cortisol induces the liver enzyme, tryptophan pyrrolase. The use of protein and RNA synthesis inhibitors gave the indirect proof of the induction of new enzyme by cortisol. A great deal of work has been concerned with the theory that the steroid hormones achieve their effect by influencing genetic expression. Lang (1971) has reported that cortisol has a primary action on the nucleus and RNA polymerase activity is stimulated which gives rise to an increase in RNA synthesis. A part of the newly synthesised RNA can be regarded as an RNA which carries the information for the synthesis of specific

proteins and enzymes.

It is proposed that the 11β-HSD in different cellular particles might be associated with separate functions. In the mitochondria and membranes the action may be with membrane permeability and in the nucleus, as in rat submandibular gland, it may be to do with genetic expression.

SUMMARY

## SUMMARY

The enzyme responsible for catalysing the oxidation-reduction of the 11-oxygen functions of steroids is 11β-hydroxysteroid dehydrogenase (11β-HSD). Previous physiological and histochemical studies have shown that the enzyme occurs in salivary gland, as demonstrated by the conversion of cortisol to cortisone in significant quantities. The aims of this thesis were to report more fully on the investigation of 11β-HSD in rat submandibular gland.

A reverse isotope dilution technique using cortisol-4-$^{14}C$ as substrate was used to assay the enzyme in homogenate, spectrophotometric and fluorometric assays being unsuitable. Cortisol was converted to cortisone, as identified by formation of cortisone derivatives and recrystallisation to constant specific activity.

A variety of assay parameters were investigated to find the optimal conditions of enzyme activity. Nicotinamide adenine dinucleotide was the preferred cofactor to nicotinamide adenine dinucleotide phosphate with the optimal pH conditions for the cofactor-linked enzyme being pH8.1-8.9 and pH7.6 respectively. Both cofactors were in saturating amounts at 1.25mM in the assay volume of 11ml. A phosphate buffer showed no side effects whereas borate and tris buffer showed chemical reactions and therefore phosphate buffer was used to control pH. The optimal temperature for the NAD- and NADP-linked 11β-hydroxysteroid dehydrogenase was 37°C for incubations of 2 mins. and 7 mins. respectively. Km and Vmax values for the NAD-linked 11β-hydroxysteroid dehydrogenase were 0.48μM and 1.27 n moles/min., the NADP-linked 11β-hydroxysteroid dehydrogenase values being 0.95μM and 0.37n moles/min. The substrate concentration chosen for enzyme assay was approximately 2xKm of the NAD-linked enzyme. Verification of enzyme assay for 100mg gland homogenates was shown by increasingly proportional enzyme activity with enzyme (gland) concentration around this value.

Reversibility of the reaction using cortisone-4-$^{14}$C as substrate could not be shown although reduced cofactors and a variety of pH values and incubation periods were used. One of the effects of 11β-HSD in salivary gland is, therefore, to convert physiologically active steroids into inactive steroids by 11β-hydroxyl group transfer to 11-oxo group steroids.

Corticosterone, the naturally secreted adrenocorticoid in the rat, was metabolised by the submandibular gland 11β-HSD.

Rat kidney was also used to investigate 11β-hydroxysteroid dehydrogenase activity. The conversion of cortisol to cortisone was confirmed but using cortisone as substrate it was converted to a product other than cortisol thus indicating the difference between kidney and salivary gland metabolism of cortisone.

The intracellular localisation of 11β-hydroxysteroid dehydrogenase was studied by incubating subcellular particles of the rat submandibular salivary gland for the NAD-linked 11β-hydroxysteroid dehydrogenase. The subcellular particles were isolated from homogenate by differential centrifugation in sucrose. The main enzyme activity was found in the crude nuclear fraction and this led to the investigation of what part of the nuclear fraction was responsible for the enzyme activity. Nuclei were isolated using 1.8M Sucrose and these were found to retain the major enzyme activity. Attempts to solubilise the nuclear enzyme using detergent and hypotonic media were unsuccessful. It is of interest to see that the enzyme is nuclear bound as the liver enzyme has been located in the microsomal (105,000g/hr.) fraction. The enzyme in the salivary gland may influence nucleic acid metabolism. However, further work will have to be undertaken to investigate this possibility.

It was recognised that the rat submandibular gland was a tissue of differentiated cell types. In the study of 11β-hydroxysteroid dehydrogenase it was considered important to investigate the activity of the steroid

enzyme in separated tubules using the enzyme, collagenase, to break up the gland. However, little activity was recovered in the tubules probably because the collagenase used included a number of protease and peptidase impurities.

The histochemical localisation of the enzyme was in the duct cells. It was not located in any single component of the salivary duct system but was evident in striated, lobular and interlobular excretory ducts. Acinar tissue did not exhibit any activity. Enzymes involved in central metabolic pathways were present principally in the ducts whereas alkaline phosphatase was present in the acinar tissue and not in the duct system. Acid phosphatase appeared in equal amounts in both the acinar and ductal tissue. The presence of these enzymes gives further proof of the concept of a highly active metabolic duct epithelium.

The culture of salivary gland ducts was investigated but no 11β-hydroxysteroid dehydrogenase activity was present in cultures which were 7 days and 3 weeks old. The enzyme may require the addition of various cofactors and hormones which are lacking in the tissue culture.

# NOMENCLATURE

Steroid Nomenclature: Rules for Nomenclature of Steroids from Biochem. J. (1969) 113, 5-28; (1972) 127, 613-617.

| Trivial Name | Proper Name |
|---|---|
| Adrenosterone | 4-Androstene-3, 11, 17-trione |
| Aetiocholanolone | 3α-Hydroxy-5β-androstan-17-one |
| Aldosterone | 18, 11-Hemiacetal of 11β, 21-dihydroxy-3, 20-dioxo-4-pregnen-18-al |
| Androsterone | 3α-Hydroxy-5α-androstan-17-one |
| Cholesterol | 5-Cholesten-3β-ol |
| Corticosterone | 11β, 21-Dihydroxy-4-pregnene-3, 20-dione |
| Cortisol | 11β, 17, 21-Trihydroxy-4-pregnene-3, 20-dione |
| Cortisone | 17, 21-Dihydroxy-4-pregnene-3, 11, 20-trione |
| Cortisone Acetate | Cortisone 21-acetate |
| 11-Dehydrocorticosterone | 21-Hydroxy-4-pregnene-3, 11, 20-trione |
| Deoxycorticosterone | 21-Hydroxy-4-pregnene-3, 20-dione |
| 5α-Dihydrocortisol | 11β, 17, 21-Trihydroxy-5α-pregnane-3, 20-dione |
| 20-Dihydrocortisone | 17, 20, 21-Trihydroxy-4-pregnene-3, 11-dione |
| 11β-Hydroxyandrostenedione | 11β-Hydroxy-4-androstene-3, 17-dione |
| 17α-Oestradiol | 1, 3, 5(10)-Estratriene-3, 17α-diol |
| Oestrone | 3-Hydroxy-1, 3, 5(10)-estratrien-17-one |
| Prednisolone | 11β, 17, 21-Trihydroxy-1, 4-pregnadiene-3, 20-dione |
| Pregnenolone | 3β-Hydroxy-5-pregnen-20-one |
| Progesterone | 4-Pregnene-3, 20-dione |
| Testosterone | 17β-Hydroxy-4-androsten-3-one |

Enzyme Nomenclature (1965) Recommendations 1964 of the International Union of Biochemistry, Elsevier, Amsterdam, London, New York.

| Trivial Name | Systematic Name | Number |
|---|---|---|
| Alcohol dehydrogenase | Alcohol: NAD oxidoreductase | EC. 1.1.1.1. |
| α-Glycerophosphate dehydrogenase | L-Glycerol-3-phosphate: NAD oxidoreductase | EC. 1.1.1.8. |
| Lactic dehydrogenase | L-Lactate: NAD oxidoreductase | EC. 1.1.1.27. |
| β-Hydroxybutyrate dehydrogenase | D-3-Hydroxybutyrate: NAD oxidoreductase | EC. 1.1.1.30. |
| Malic dehydrogenase | L-Malate: NAD oxidoreductase | EC. 1.1.1.37. |
| Glucose-6-phosphate dehydrogenase | D-Glucose-6-phosphate: NADP oxidoreductase | EC. 1.1.1.49. |
| 3α-Hydroxysteroid dehydrogenase | 3α-Hydroxysteroid: NAD(P) oxidoreductase | EC. 1.1.1.50. |
| 3 (or 17) β-Hydroxysteroid dehydrogenase | 3 (or 17) β-Hydroxysteroid: NAD(P) oxidoreductase | EC. 1.1.1.51. |
| Oestradiol 17β-hydroxysteroid dehydrogenase | Oestradiol: NAD 17β-oxidoreductase | EC. 1.1.1.62. |
| Testosterone 17β-hydroxysteroid dehydrogenase | 17β-Hydroxysteroid: NAD 17β-oxidoreductase | EC. 1.1.1.63. |
| Testosterone 17β-hydroxysteroid dehydrogenase (NADP) | 17β-Hydroxysteroid: NADP 17β-oxidoreductase | EC. 1.1.1.64. |
| Succinic dehydrogenase | Succinate: (acceptor) oxidoreductase | EC. 1.3.99.1. |
| Glutamate dehydrogenase (NAD(P)) | L-Glutamate: NAD(P) oxidoreductase (deaminating) | EC. 1.4.1.3. |
| Transhydrogenase | Reduced-NADP: NAD oxidoreductase | EC. 1.6.1.1. |
| Peroxidase | Donor: hydrogen-peroxide oxidoreductase | EC. 1.11.1.7. |
| Tryptophan pyrrolase | L-Tryptophan: oxygen oxidoreductase | EC. 1.13.1.12. |
| 17α-Hydroxylase | Steroid, reduced-NADP: oxygen oxidoreductase (17α-hydroxylating) | EC. 1.14.1.7. |
| N-acetylglucosaminyltransferase | UDP-2-acetamido-2-deoxy-D-glucose: chitin acetylaminodeoxyglucosyltransferase | EC. 2.4.1.16. |

| Trivial Name | Systematic Name | Number |
|---|---|---|
| RNA polymerase | Nucleosidetriphosphate: RNA nucleotidyltransferase | EC. 2.7.7.6. |
| Ali-esterase | Carboxylic-ester hydrolase | EC. 3.1.1.1. |
| Lipase | Glycerol-ester hydrolase | EC. 3.1.1.3. |
| Acetylcholinesterase | Acetylcholine hydrolase | EC. 3.1.1.7. |
| Alkaline phosphatase | Orthophosphoric monoester phosphohydrolase | EC. 3.1.3.1. |
| Acid phosphatase | Orthophosphoric monoester phosphohydrolase | EC. 3.1.3.2. |
| 5'-Nucleotidase | 5'-Ribonucleotide phosphohydrolase | EC. 3.1.3.5. |
| Glucose-6-phosphatase | D-Glucose-6-phosphate phosphohydrolase | EC. 3.1.3.9. |
| α-Amylase | α-1, 4-Glucan-4-glucano-hydrolase | EC. 3.2.1.1. |
| β-Amylase | α-1, 4-Glucan maltohydrolase | EC. 3.2.1.2. |
| Lysozyme | Mucopeptide N-acetyl-muramylhydrolase | EC. 3.2.1.17. |
| β-Galactosidase | β-D-Galactoside galactohydrolase | EC. 3.2.1.23. |
| β-Glucuronidase | β-D-Glucuronide glucuronohydrolase | EC. 3.2.1.31. |
| Amino peptidase | Amino-acyl-oligopeptide hydrolase | EC. 3.4.1.2. |
| Trypsin | - | EC. 3.4.4.4. |
| Collagenase | - | EC. 3.4.4.19. |
| ATP ase | ATP phosphohydrolase | EC. 3.6.1.3. |
| Aldolase | Ketose-1-phosphate aldehyde-lyase | EC. 4.1.2.7. |
| Carbonic anhydrase | Carbonate hydro-lyase | EC. 4.2.1.1. |
| Steroid Δ-isomerase | 3-oxosteroid $\Delta^4$-$\Delta^5$ isomerase | EC. 5.3.3.1. |

Other Nomenclature

Where abbreviations occur in the text of the thesis, the proper names have been inserted alongside the first quotation to them. Those not inserted in the text of the thesis are:

| Trivial Name | Proper Name |
|---|---|
| Naphthol AS acetate | 2-Acetoxy-3-naphthoic acid anilide |
| Naphthol AS-BI | 6-Bromo-2-hydroxy-3-naphthoyl-O-anisidine |
| Naphthol AS-MX | 3-Hydroxy-2-naphtho-2, 4-xylidide |
| OV-17 | Phenyl methylsilicone, 50% phenyl |
| Tris hydrochloride | Tris (hydroxymethyl) aminomethane hydrochloride (Tris; THAM; 2-Amino-2-(hydroxymethyl)-1, 3-propanediol) |

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