THE UNIVERSITY OF MANITOBA

ARYLSULFATASE ACTIVITY IN SECRETORY TISSUES WITH SPECIAL REFERENCE TO RAT SUBMANDIBULAR GLAND

A Comparative Study

by

Roger Vernon Watson, B.Sc.

A Thesis Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements for the Degree
of Master of Science

Department of Oral Biology
Winnipeg, Manitoba

October, 1976

"ARYLSULFATASE ACTIVITY IN SECRETORY TISSUES WITH SPECIAL REFERENCE TO RAT SUBMANDIBULAR GLAND"

by

ROGER VERNON WATSON

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

dedicated
to my parents
who taught me
to have
a dream

ACKNOWLEDGEMENTS

I am deeply indebted to the following people, each of whom has helped to make this thesis possible. I would like to thank -

E. THACKERAY "THACK" PRITCHARD,
my mentor, whose creative intelligence and considered compassion helped me acquire direction and who, while doing so, became something more than an advisor.

HARVEY KNULL,
the kind of person who gives intelligence a good name, for his terrific sense of humour.

DAN LANDMAN,
whose rapid-fire ideas have been a source of information and bemusement, for sharing his ideas.

JOANNE CUSHNIE,
whose calmness has been reassuring, for giving technical assistance with care.

ANNE DAGG,
who conscientiously typed and retyped this manuscript throughout its formative stages, for her stoic lucubrating.

This investigation was supported by the Medical Research Council of Canada (grant MT 3536) and the author expresses his appreciation of this support.

CONTENTS

Page

LIST OF FIGURES

Figure | Page

LIST OF TABLES

ASA ..... arylsulfatase A

ASB ..... arylsulfatase B

ASC ..... arylsulfatase C

ATP ..... adenosine triphosphate

BSA ..... bovine serum albumin

DEAE ..... diethylamine ethyl

E_{515} ..... molar extinction coefficient

MES ..... 2-(N-morpholine) ethanesulfonic acid H_2O

MLD ..... metachromatic leucodystrophy

NC ..... nitrocatechol

NCS ..... nitrocatechol sulfate

OD ..... optical density

Pi ..... organic phosphate

PPi ..... pyrophosphate

SLG ..... sublingual salivary gland

SMG ..... submandibular salivary gland

TCA ..... trichloroacetic acid

TES ..... N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid

TRIS ..... tris (hydroxymethyl) aminomethane

I. PURPOSE OF THE STUDY

The objectives of this study were three-fold: (1) to study the properties of arylsulfatase in secreting tissues, (2) to investigate the nature of arylsulfatase of rat tissue under various physiological conditions and (3) to seek methods for the purification of arylsulfatase of rat submandibular gland.

Two new findings have enhanced interest in arylsulfatases. The known specificity of the enzyme has been expanded to include ascorbic 2-sulfate as a substrate. Secondly, metachromatic leukodystrophy, a disease wherein arylsulfatase A is lacking, has been partially corrected by enzyme replacement therapy.

II REVIEW OF THE LITERATURE

A. History of Arylsulfatases

There are many enzymes which hydrolyze sulfate esters of which the following are typical: arylsulfatases, steroid sulfatases, glycosulfatases, choline sulfatases, alkylsulfatases, myrosulfatases, etc. (122).

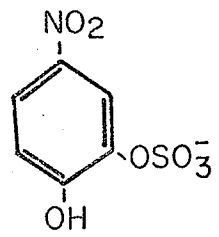
Arylsulfatases (arylsulfate sulfhydrolases, E.C. 3.1.6.1), are enzymes that hydrolytically cleave sulfate from organic sulfate esters, and appear to be ubiguitous in nature yet their specific functions in mammalian systems are unknown. They were first described by Bauman in 1876 (13). The earlier literature has been adequately reviewed by Framageot (43-47) and Dodgson and Spencer (27). Many of the most recent studies originating from the laboratories of Roy (114), Dodgson (26) and Jatzkewitz (70) will be reviewed herein.

A simple, convenient spectrophotometric assay for arylsulfatase has been developed (111) based on the colorimetric determination of nitrocatechol released from p-nitrocatechol sulfate (2-hydroxy-5-nitrophenyl sulfate, Fig. 1). This ester still remains the substrate of choice although others have been used, eg., p-nitrophenyl sulfate, p-acetyl phenylsulfate, 4-methylumbelliferyl sulfate (134) and L - ascorbic acid 2 - sulfate (123). See Fig. 1.

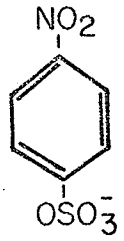
Arylsulfatases have been arbitrarily divided into two classes or types depending upon whether or not their activity is inhibited by inorganic sulfate. Type I arylsulfatases are not, while Type II is, inhibited by inorganic sulfate. Both types catalyze hydrolysis as follows:

$$R\text{-}O\text{-}\ SO_3^- + HOH \rightarrow R\text{-}OH + SO_4^{2-} + H^+ \quad (131)$$

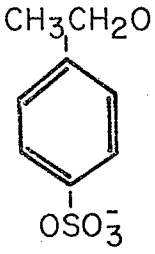
where R could be nitrocatechol , 4 - nitrophenyl -, p - acetophenyl -, phenyl -, etc. Fig. 1). This reaction appears to be irreversible



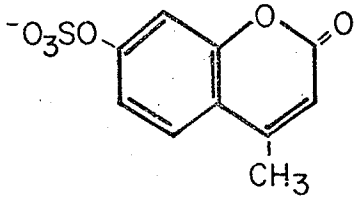
(1) 4 – nitrocatechol sulfate



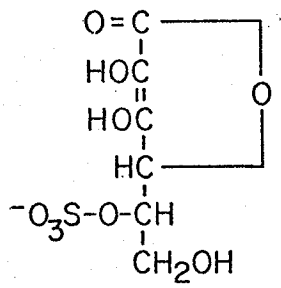
(2) p-nitrophenyl sulfate



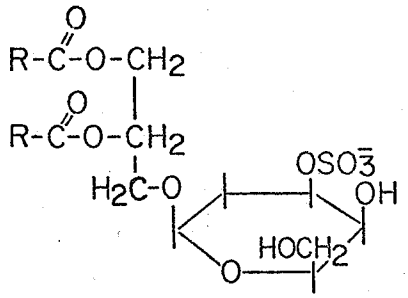
(3) p-acetophenyl sulfate



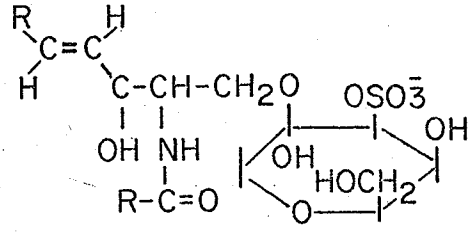
(4) 4-methylumbelliferyl sulfate



(5) L-ascorbic acid-2-sulfate



(6) monogalactosyl diglyceride sulfate



(7) cerebroside sulfate

Figure 1. Substrates used for assay of arylsulfatase activity.

with no metal ion requirement.

Arylsulfatases are widespread in their occurrence in both the animal and plant kingdoms. Dodgson et al (30) studied their relative distribution in human tissue (Table 1). The major arylsulfatases thus far discovered in mammalian tissues have been named A and B (both Type II) and C (Type I) (Table 2). The former are apparently lysosomal enzymes whilst the latter is firmly bound to the microsomal membranes (122) (Table 3).

A comparative study (119) on the livers of rat, mouse, guinea pig, rabbit, ox, hen, frog and stickleback showed a similar subcellular distribution of all three arylsulfatases. No sex-dependent differences in the level of the enzymes were observed.

The A and B forms of arylsulfatase have been identified in urine (21,27,5,12) but not in white blood cells (98,130). The latter investigations detected a higher level of total arylsulfatase activity in granulocytes when compared to antibody-producing lymphocytes. This was expected because of the high level of lysosomes in phagocytic granulocytes.

Arylsulfatase A has been found in human blood platelets (101,113), cultured skin fibroblasts (102-4,133) and cells cultured from human amniotic fluid (74).

Arylsulfatase A has been isolated and purified from ox brain and liver (16,95), rabbit sperm acrosomes (144), boar testes (143), chicken brain (37) and human brain, liver, placenta and urine (61,50,20.27,134). A 21,000 fold purification has been achieved by following various purification procedures including centrifugation, ion exchangers, molecular sieves, freeze drying, dialysis (Fig. 2), ammonium sulfate fractionation and acetone precipitation (50,61,30). Unconventional procedures included

TABLE 1

Activity of Type I (Insoluble) and Type II (Soluble) Arylsulfatases in Human Acetone-Dried Tissues.

Tissue	Activity per g whole acetone-dried tissue (µg "phenol" liberated)	Percentage of activity of whole tissue "insoluble" fraction	"soluble" fraction
Liver	13,260	26	103
Pancreas	8,000	11	101
Kidney	22,750	13	108
Lung	4,780	16	123
Brain	2,900	19	120
Heart	2,100	22	123
Large Intestine	390	15	98
Small Intestine	4,200	28	116
Spleen	3,900	Fractionation not practicable	

(Dodgson et al. Biochem. J. 62, 500-507, 1956)

TABLE 2.

The General Properties of the Arylsulfatases of Ox Liver

	Sulfatase		
	TYPE II		TYPE I
	A	B	C
Substrate: nitrocatechol sulphate			
optimum pH	5.0	5.6	7.5
optimum substrate conc. (mM)	3.0	15	20
K_m (mM)	0.8	1.8	8.0
Relative activity in:			
0.025 M-SO_4^{2-}	0.29	0.63	.
0.1 M-Cl^-	1.21	0.78	.
0.025 M-$H_2PO_4^{1-}$	0.01	0.01	.
Substrate: p-nitrophenyl sulphate			
optimum pH	5.4	5.7	8.0
optimum substrate conc. (mM)	80	50	7.5
K_m (mM)	23	4.4	2.0
Relative activity in:			
0.025 M-SO_4^{2-}	0.16	0.32	1.00
0.1 M-Cl^-	0.94	5.0	1.00
0.025 M-$H_2PO_4^{1-}$	0.03	0.01	.
V_{NCS}/V_{NPS}	3.0	55 (0.6*)	0.5

* Value for NPS determined in 0.1 M-KCl.

(Roy, A.B. and Trudinger, P. The biochemistry of inorganic compounds of sulfur. p. 135, 1970.)

TABLE 3.

Intracellular Distribution of Arylsulfatase in Rat Liver.

	Sulfatase Activity Range %	Mean %	Recovery of 36 µg added nitrocatechol %
Nuclei	9-20	15	98
Mitochondria	43-62	50	98
Microsomes	20-23	22	99
Soluble fraction	11-21	14	99

(Roy, A.B., Biochim. Biophys. Acta 14, 149, 1954)

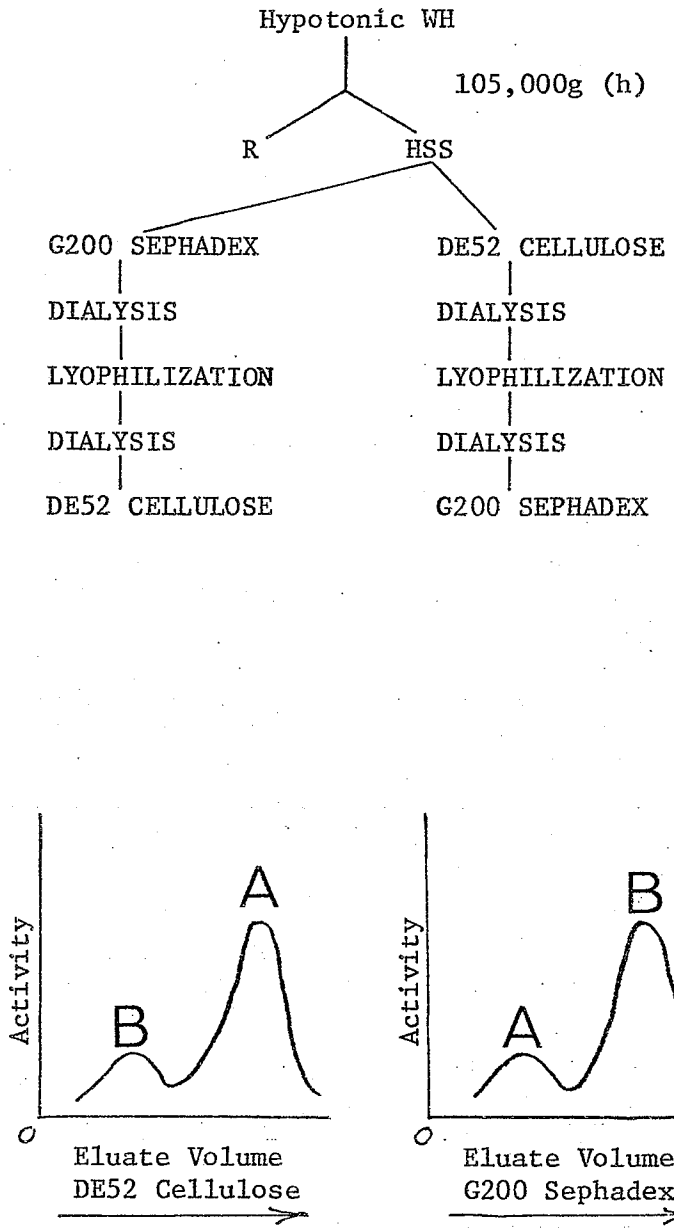


Figure 2 Flow diagram illustrating some typical techniques for separation of arylsulfatase A and B.

Taken from Harinath,B. and Robins,E. J.Neurochem. 18,245-257, 1971, Farooqui,A.A. and Bachhawat,B.K. Biochem.J. 126, 1025-1033,1973, and Stevens,R.L. et al. J.Biol. Chem. 250,2495-2501, 1975.

affinity chromatography and utilization of the polymerization of arylsulfatase A in order to effect purification (20,95).

The optimum pH for activity ranged from 4.6 - 5.5 and varied slightly with the origin of the arylsulfatase and the substrate utilized (37,61,115,133).

The activities of arylsulfatases A and B are related to their origin. Although the ratio varies, arylsulfatase B in rat tissues is present at much higher levels than arylsulfatase A (141), the opposite of findings for human tissues. Pathological changes in one enzyme with concurrent normal levels of the other implies a certain independence, at least in the human (14).

The assigned role of lysosomal arylsulfatase A, in human CNS, is the cleavage of the sulfate ester from sulfatide with the resultant degradation of this compound. No physiological function has yet been attributed to arylsulfatase B (90).

A mathematical correlation between the reaction velocity and the incubation time was recently derived from work on arylsulfatase A of human kidney (135). This allowed a study of inhibiting anions and gave rise to an apparent Michalelis constant and a rate of inactivation for arylsulfatase A. The proposed mechanism is shown in Fig. 3 (see following page).

It has been noted that during incubations the enzyme is slowly modified until it becomes inactive (10,18,97,135). These investigators proposed that a second site became exposed during the reaction and that if substrate, products or anions became bound to it, the enzyme would be inactivated.

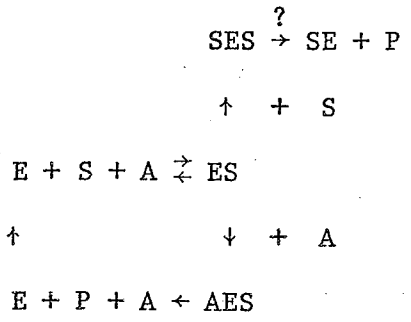


Figure 3. Reaction of the enzyme (E) with either the substrate (S) or the anion (A) may cause exposure of a second active site. Further binding gives rise to the inactive SES or the active AES complex with the latter giving rise to product (P).

Investigations of arylsulfatase A in subphylum invertebrata have led to the postulate that the enzyme may function in an endocrine regulatory mechanism involving steroid hormones (145). It also seems to be involved with the hydrolysis of some types of sulfate groups present in the diet (22) which is supported by the observation that the level of the enzyme changes in proportion to the amount of sulfate ingested (23,26,62,81).

B. Characteristics of "Purified Arylsulfatases"

Studies of arylsulfatase A, purified 7000-fold from ox liver (95), indicate that the enzyme exists as a monomer (105,000 daltons) at 0.10 ionic strength and pH 7.5, but as a tetramer (411,000 daltons) at pH 5.0. It was concluded, from the smaller negative charge on the monomer, that the polymerization at pH 5.0 was due to attractive hydrophobic intermolecular bonds. The monomer was shown to contain four subunits of 24,000 daltons.

Further investigations indicated that a tyrosyl residue was essential for arylsulfatase A activity (72). It was suggested that this tyrosyl residue resides at the active center of the enzyme and

that the sulfation of this residue correlates with the desulfation of the substrate. It was postulated that an adjacent histidyl residue aided in this desulfation.

A 21,000-fold purification of arylsulfatase A from ox brain has been reported (18). Arylsulfatase B has been isolated from ox brain (16,17) and ox liver (3,96). The presence, in ox liver, of two subunits of arylsulfatase B (B-alpha and B-beta) were shown. Their size of 25,000 daltons is similar to the arylsulfatase A subunit's size of 24,000 daltons, and they also undergo polymerization under appropriate conditions. It was concluded, based on different isoelectric points and other properties, that no direct relationship existed between the subunits of arylsulfatase A and arylsulfatase B. However, an interconversion between lysosomal hexosaminidase A and hexosaminidase B after neuraminidase action has been noted. A similar interconversion was postulated for arylsulfatases and other hydrolases (52) but is presently unsubstantiated.

A study of ox liver arylsulfatase A confirmed the glycoprotein nature of the enzyme (53). Referring to the monomeric form (107,000 daltons) it was noted that there were 8 molecules of galactose, 14 of mannose, 18 of glucosamine and 8 of sialic acid. Neuraminidase treatment identified sialic acid as the terminal sugar. Desialyated arylsulfatase A retained the properties of the native enzyme and remained distinct from arylsulfatase B with respect to polymerization, sedimentation coefficient, Km, ability to hydrolyze cerebroside sulfate and inactivation of the enzyme during hydrolysis.

C. Possible Physiological Roles of Arylsulfatases

Cerebroside sulfatase has been purified 6000-fold from pig

kidney (90). Electrophoresis divided the fraction into two peaks: one with arylsulfatase B activity but no arylsulfatase A nor cerebroside sulfatase activity; the second with both arylsulfatase A and cerebroside sulfatase activity.

A heat-stable activator for cerebroside sulfatase activity was recently purified from human liver and was shown to be stable at 100°C for 20 minutes with no loss of activity towards the enzyme (38).

The enzymic degradation of many other glycosphingolipids has been shown to require heat-stable activator proteins. One of these has been shown to be a glycoprotein (63,64,83,84).

A sulfoglycerogalactolipid, isolated from rat testes, was similar to cerebroside sulfate in its predilection for human arylsulfatase A as compared to B. It was postulated as another physiological substrate for the enzyme (40).

A role for lysosomal arylsulfatase in fertilization was proposed from the hypothesis that sperm acrosomes are actually specialized lysosomes (4,128,144).

Cerebroside sulfatase acts optimally at pH 4.5 although its activity decreases with increasing buffer concentration (135). The Km for cerebroside sulfate is 2.9×10^{-4} mole per liter (90).

Cerebroside sulfatase has varied specificity towards different substrates. Work done on sulfatides (Fig. 1) has shown the importance of the sulfate group being located at the C-3 of the galactose moiety to facilitate optimum enzyme activity (91). The enzyme was approximately thirty times more active towards the synthetic p-nitrocatechol sulfate than towards the natural sulfatide. Cerebroside

sulfatase was inactive towards steroid sulfate and chondroitin sulfate.

In metachromatic leukodystrophy, not only were cerebroside sulfatase, psychosine sulfatase and sulfoglycerogalactolipid sulfatase deficient, but also arylsulfatase was absent (35,40).

That cerebroside sulfatase is possibly arylsulfatase A is further corroborated in data showing that various inhibitors have the same effects on both preparations (90).

D. Pathological Conditions

Arylsulfatases have been implicated in several disease states - metachromatic leukodystrophy (MLD), hemorrhagic shock, maroteaux-lamy syndrome (MLS) and certain carcinomas of the breast and colon.

a) Metachromatic Leukodystrophy (MLD)

The genetic disorder metachromatic leukodystrophy (MLD) results in improper expression of arylsulfatase A gene. MLD is transmitted as an autosomal recessive trait and can be detected in cultured skin fibroblasts from parents of a child with juvenile MLD (82). Parental consanquinity is exhibited.

The observation of decreased brain levels of arylsulfatase in patients with MLD stimulated research on characterization of the enzymes involved (7). Both arylsulfatase A and B were shown to be predominately lysosomal and, in MLD, the level of A was seen to be decreased to almost undetectable levels (6,8) while the other lysosomal enzymes levels were unchanged.

Therapeutic treatment of MLD by enzyme replacement has recently been investigated (139,104). Cultured human fibroblasts, derived from patients with late infantile MLD, were found to have inclusion granules as a result of an accummulation of sulfatides.

After addition of arylsulfatase A the granules were cleared and the MLD fibroblasts became indistinguishable from controls. The ultimate goal is to extrapolate from temporary replacement in cultures to enzyme replacement in patients with MLD.

A study of arylsulfatase A levels of activity in leukocytes of two families with a history of late adult MLD revealed that this enzyme could be used to detect a) recessive carriers, or, b) victims with the deficiency (100).

Gargoylism (Hurler's syndrome) is clinically manifested by enlargement of the head and coarsening of the facial features followed by mental deterioration, deafness and corneal clouding. This autosomal recessive disease has a deficiency of α-L-iduronidase resulting in excessive levels of urinary dermatan sulfate and heparin sulfate. A study indicated that arylsulfatase A and other lysosomal enzymes existed at normal levels except for a marked increase in arylsulfatase B activity (8). However, this enzyme has yet to be implicated with Hurler's syndrome.

b) Hemorrhagic Shock

Labilization of lysosomes due to hypotension during hemorrhagic shock results in an increase of most lysosomal enzyme activities in the plasma (67). However, arylsulfatase A shows a decrease in activity. Two hypothesis have been put forward; 1) decreased blood flow to organs hinders the release of the enzyme and 2) inhibitors may be released into the plasma which reduce arylsulfatase activity.

c) Maroteaux - Lamy Syndrome (MLS)

Also known as polydystrophic dwarfism, Maroteaux - Lamy Syndrome (MLS) has a prognosis of marked retardation of growth.

Biochemically there is an elevated level of dermatan sulfate and a lowered level of arylsulfatase B in the urine (14). Arylsulfatase B was shown to be present at very low or undetectable levels in various tissues from patients with MLS (14) and this deficiency appears to give rise to the basic metabolic defect. A study of cultured skin fibroblasts, peripheral leukocytes and long-term lymphoid cell line cultures showed normal levels of nine other lysosomal enzymes, including arylsulfatase A. It was proposed that normally, arylsulfatase B acts on dermatan sulfate to produce N - acetylgalactosamine and therefore, in MLS, dermatan sulfate accumulates in the tissues and is present in urinary secretion.

d) Carcinoma

It has been reported, based on histochemical evidence, that arylsulfatases seemed to have no lysosomal localization in carcinomas of the human breast and colon (78). The precipitates of arylsulfatase activity were localized in the neighbourhood of the cell membranes. The histochemical procedure does not distinguish between arylsulfatase A, B and C and, when coupled with a dearth of literature on arylsulfatase in human tissues, makes judgement of the conclusions difficult.

E. Proposed Functions

That arylsulfatases are involved in the metabolism of many sulfate esters is well known. However, the unique physiological roles are much less well established and a number of postulates have been put forward.

Investigations of MLD have shown that a decrease in arylsulfatase A is followed by an increase in the amount of sulfatide. The

obvious deduction is that arylsulfatase A is involved in the turnover of sulfatides. The similarity and proposed convertibility of arylsulfatase A and cerebroside sulfatase has been discussed in Section II, C.

Studies in mammals have suggested that arylsulfatase acts to alter the level in the blood of two hormones - triiodothyronine (112) and serotonin (21). It was proposed that the enzyme acted at the level of conversion between the transport and storage forms of the hormones. Unfortunately, this work has not yet been substantiated.

Investigations with micro-organisms have indicated that arylsulfatases are involved in the production of some sulfur containing amino acids (109,59,60). It was assumed that the sulfatase acts in micro-organisms to provide sulfate for growth. The level of arylsulfatase is regulated by derepression with substrates and negative feedback with products (68). Methionine, when used as the sole source of sulfur, induces sulfatase synthesis, whereas sulfate, thiosulfate, sulfite and cysteine all repress synthesis. Cystine is believed to be the main repressor but the method by which it acts has yet to be fully elucidated. Methionine, and possibly tyramine, cause induction and derepression of sulfatase synthesis in Aerobacter aerogenes (55-58).

Some investigators have tried to find a link between arylsulfatases and the active transport of sulfate in some bacteria (126), fungi (142) and rat kidney cortex (140) without success.

Sulfatides have been hypothesized to act as a carrier or receptor for sodium ion transport (75). The salt (nasal) gland of eider duck and herring gull has two markedly different characteristics -

1) a predilection for sodium ion transport by $Na^+ - K^+$ - dependent adenosine-triphosphatase activity and
2) a high level of sulfatides. The ratio between these is similar among tissues with high sodium ion transport capacity.

Hence, a role for arylsulfatase may be to modify the sulfatide in a sulfatide - $Na^+ - K^+$ pump arrangement.

Other Proposed Substrates

L-ascorbic acid 2-sulfate (Fig. 1) has been shown to be a substrate for arylsulfatases of human and ox (41,123) and may be indicative of a much wider range of specificity than had previously been suspected. The enzyme may act to transfer sulfate from L-ascorbic acid 2-sulfate to cholesterol either by a direct oxidative transfer or by simple hydrolysis and subsequent attachment. However, when compared to controls, no difference was detected in the level of ascorbate-2-sulfate in urine from MLD patients (41).

Human arylsulfatase A was shown to hydrolyze a glycolipid from rat tissues i.e. the sulfoglycerogalactolipid [1-0-alkyl-2-0-acyl-3-0 (β-D-galactopyranoside-3^1-sulfate) - glycerol (40)] (Fig. 1). Competitive inhibition by cerebroside sulfate along with identical pH optimum, Km and other parameters suggests that both sulfatides are hydrolyzed by arylsulfatase A and may help to explain the presence of the enzyme in tissues where little cerebroside sulfate but substantial sulfoglycero-galactolipid is present. The assumed presence of the latter sulfolipid as a membrane component in spermatocytes (40) suggests a role for arylsulfatase A in reproduction. The presence of the enzyme in rabbit sperm acrosomes (144) led to the postulation of a role in fertilization at the level of ovum production but may also be related to enzymatic

action from inside the acrosome to facilitate release of other hydrolytic enzymes for action within the ovum.

Recent evidence indicates that, in rat liver, arylsulfatase and oestrogen sulfatase are the same enzyme. In support of this conclusion are data indicating that the levels of the two enzymes parallel each other during post-natal development, their activities correlate each other in several different tissues, the subcellular profiles are identical and various kinetic experiments did not serve to differentiate the two activities (32). The enzyme may be involved in regulation of free oestrogen concentrations and may be related to the suggestion of a role for oestrogens in the binding of ribosomes to the endoplasmic reticulum of rat livers (92).

Steroid sulfatases of human brain have been postulated to act in the hypothalamic-hypophyseal regulation of steroids through their action on biologically active sulfated steroids (99). Here the similarity to arylsulfatase C was not substantiated since less specific enzyme assays were performed.

In molluscs, steroid sulfatases are excreted from the hepato-pancreas (digestive gland) into the intestinal tract suggesting a role in digestion (15). The enzymes may act to regulate the level of biologically active sulfated steroids in a manner similar to its proposed role in mammalian tissues.

III EXPERIMENTAL METHODS

A. MATERIALS

DE-52, (diethylaminoethyl) - cellulose, was obtained from Whatman Biochemicals Ltd. (England), Sephadex G-200 and Dextran Blue 2000 from Pharmacia Canada Ltd. and p-nitrocatechol sulfate from Sigma Chemical Co. (U.S.A.).

All other chemicals and reagents were of the highest quality obtainable.

B. TISSUE PREPARATION

(i) Whole Homogenates

Male rats of the Long-Evans strain were used throughout this study. The ages are specified in the legends to tables and figures.

Rats were lightly anaesthetized and decapitated. The required tissues were quickly removed and placed in cold 10 mM-TRIS buffer pH 7.4. The glands were then cleaned, weighed, minced with a razor blade and homogenized with a Polytron PT-10 homogenizer. The homogenate was filtered through fine mesh bolting cloth and then adjusted to 10% w/v with 10 mM-TRIS buffer pH 7.4 and will be referred to as the whole homogenate (WH).

(ii) Soluble Supernatant

The WH was centrifuged at 105,000 g (average) for 1 hour in an International centrifuge Model B-60 (Rotor Type A-321). The resulting high speed supernatant (HSS) was either utilized immediately or frozen and stored at -10°. There was no detectable loss of activity after storage periods of several months. Special treatments of the high speed supernatant will be dealt with in Section IV.

C) COLUMN CHROMATOGRAPHY

(i) Sephadex G-200

Sephadex G-200 effectively separates globular proteins in the range of 20,000-400,000 daltons. Since the molecular weights of all reported arylsulfatases are in the range of 60,000-400,000, this resin was used in our attempts to purify the enzyme(s) from rat submandibular gland (SMG) HSS (129).

The Sephadex G-200 was hydrated, with 0.1 M sodium acetate buffer pH 5.0, at 100° for 5 hours. After decantation of fines the gel was cooled to 4° and the slurry was poured into an 80 x 2.5 cm Pharmacia column. Acetate buffer, pH 5.0, was utilized in order to effect aggregation of arylsulfatase A should it be present (141). The column was pre-washed with this buffer at 4° for 24 hours before use.

Dextran Blue 2000 was used to determine the elution volume and the proper packing of the column. The operating pressure never exceeded 100 mm (129).

After introduction of the tissue sample, (usually HSS containing 7.6 mg protein), approximately 50 fractions of 8 ml were collected, with the aid of an LKB UltroRac 7000 fraction collector, and each fraction was assayed for arylsulfatase activity and protein content. Appropriate samples were pooled, concentrated and used for further study.

(ii) DEAE-Cellulose

DE 52 anion exchange-cellulose was used to attempt a separation of arylsulfatase A from arylsulfatase B of SMG-HSS, as has been successfully done on other species and tissues (16,37,50,80,94,134,143,144).

The cellulose was prepared in 10 mM-TRIS buffer pH 7.4 and after decanting the fines a 24 x 1.5 cm Pharmacia column was filled with the slurry. The column was washed for 24 hours with 10 mM-TRIS pH 7.4 before application of HSS, containing 7.6 mg protein, which had been concentrated 10X in an Amicon (Minicon) B15 concentration. After application of the sample, the column was washed with 100 ml of buffer. A linear gradient of NaCl (0 to 300 mM) in 10 mM-TRIS, pH 7.4, was applied. Fractions were collected, and assayed for arylsulfatase activity and protein levels. Appropriate fractions were pooled, dialyzed against 10 mM-TRIS to remove salt and then frozen and stored at -10^{o}.

D) ACRYLAMIDE GEL ELECTROPHORESIS

A Bio-Phore matched gel electrophoresis system was used with a Model 150A cell (Bio Rad Laboratories, (Canada) Ltd.). Pre-caste, 4 and 7.5% 10 mm gels were infused with the appropriate buffer: 0.188M Tris-glycine, pH 8.9, or 0.188M Acetate-Glycine, pH 3.6, for base and acid systems respectively. Gels were run at the lowest reasonable voltage (< 80V) to limit heat production, and cold (10^{o}) water was continuously circulated about the cell. Acceptable separation of protein was achieved within 2 hours.

It was found that these pre-caste gels could be examined for arylesterase activity, prior to fixation and staining, with little subsequent change in the protein band patterns. Activity was located in the gels by allowing gels to soak for 1 hour in 5 mM-nitrocatechol sulfate buffered with 0.5M acetate, pH 5.0 followed by several water washes and finally an immersion in 0.5N-NaOH. The intense red color

of nitrocatechol appeared within a few minutes. These gels could then be fixed and stained for protein. Protein bands were fixed by immersion in isopropanol-acetic acid-water (40:10:50 v/v) for 5 hours.

Protein staining was effected with 1% Amido Black in 7% acetic acid (1 hour at 20°). Coomassie Blue, whilst reportedly more sensitive, failed to detect any protein bands that were not stained by Amido Black, and, furthermore, produced a much more colored background to the gel than did Amido Black. Destaining was achieved with 7% aqueous acetic acid for 24-36 hours. Densitometric scanning of the gels was done with a Joyce Loebl Chromoscan, Mark II.

Gels were stored in 7% acetic acid in specially-made glass tubes.

E) ANALYTICAL PROCEDURES

(i) Protein

Protein content was determined by the method of Lowry et al. (86). Protein standards were prepared with bovine serum albumin.

(ii) Arylsulfatase Assay

As brilliantly described by Dodgson and his co-workers (65,141) the estimation of arylsulfatase A in the presence of B is difficult whereas estimation of total (A + B) activity is rather simple. The results with most tissue preparations are reported as "arylsulfatase activity" which means total (A + B) activity. The procedure is essentially that of Dodgson (29).

Duplicate or triplicate tissue preparations, usually at 2 or 3 different dilutions, were incubated at 37° for various times (in 10 mM - nitrocatechol sulfate in a total of 1 ml 0.5M acetate buffer,

pH 5). The effects of varying pH, buffer strength, adding inhibitors, and other modifications will be reported in the Experimental Results.

Reactions were stopped by addition of 0.8 ml 1N - NaOH, and after thorough mixing, 4 ml of water. The color was stable for several hours. The absorbance was determined at 515 nm in a 1 cm cell (Unicam SP-600 with automatic siphon).

The enzyme activity is reported as nmoles nitrocatechol released/hour/mg protein by reference to a standard curve (which was checked with each analysis). (See Fig. 4)

In certain instances the procedure of Baum et al (12), originally developed for arylsulfatase A in human tissues, was employed by the present investigators. This is noted appropriately in legends and figures. This procedure is similar to that noted above except 0.5M-NaCl and 0.5 mM pyrophosphate are present in the incubation medium to inhibit arylsulfatase B.

Arylsulfatase C was determined as described by Milsom, Rose and Dodgson (1972) (92) using potassium p-acetylphenyl sulfate as substrate and measuring absorbance changes at 323 nm (Unicam SP-700).

Note For brevity, throughout the following text the incubation medium for assay of total arylsulfatase activity will be referred to as Medium A and that of Baum et al. as Medium B.

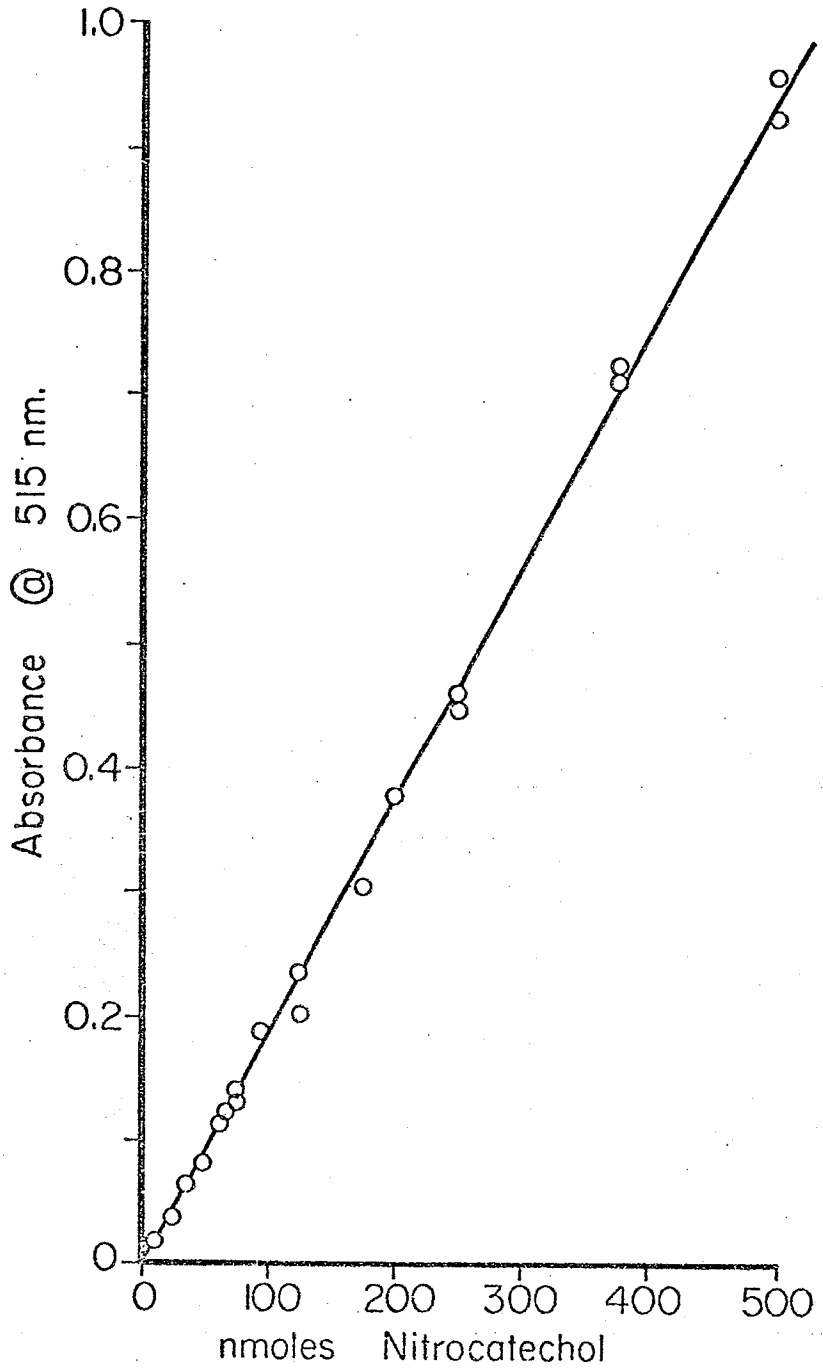


FIGURE 4. CALIBRATION CURVE FOR ARYLSULFATASE ASSAY

SECTION IV

EXPERIMENTAL RESULTS

Initially this study was intended to effect the isolation and purification of an arylsulfatase from submandibular gland (SMG) that would, hopefully, have "sulfolipid sulfatase" activity. This would help to explain the high turnover rate of SMG sulfolipids recently reported by this laboratory (107). Although this aim has not been fully achieved, the search has produced new information about arylsulfatases in salivary glands and other tissues of the rat under a variety of conditions.

A. REACTION CHARACTERISTICS

1. Period of Incubation
2. Concentration of Tissue
3. Substrate Concentration
4. Influence of Temperature
5. Stability of Enzyme Activity
6. Incubation Conditions
   - (i) buffers
   - (ii) pH
   - (iii) anions and cations

Dodgson and others (11 , 28 ,-30) have evaluated arylsulfatase assays and have suggested that results have been improperly interpreted by many investigators. There are definite differences in the types of arylsulfatases in various species which have introduced difficulties in the interpretation of experimental results except in experiments where the investigator was fully aware of the limitations of a particular assay. The assay procedure developed by Baum, Dodgson and Spencer (10,11,12) for human tissues, where A > B, which has been widely used without modification, cannot be directly adapted to most animal tissues where the B form of the enzyme may predominate.

The use of the Baum procedure (Medium B in text) would produce not unreasonable results when examining conditions within a given tissue, or, perhaps, even within a single species. However, interspecies comparisons would not be valid nor would the values for total enzymic activity.

The difference between total arylsulfatase activity, as measured in Medium A, and that measured by the procedure of Baum (Medium B) is strikingly demonstrated by the results shown in Figure 5. It is seen that the latter procedure measures only about one third obtained with Medium A. It can be seen that NaCl and pyrophosphate markedly inhibit toal aryl-sulfatase activity (See Figure 6.). At the concentration normally used in Medium B (arrow on Figure 6) the inhibition is approximately 60 to 70 percent. All rat tissues tested gave very similar results.

In ignorance, Medium B was used in some initial studies during the present investigation. However, in almost all instances the results have been checked using Medium A and the interpretation of results of comparative studies were unchanged.

(1) PERIOD OF INCUBATION It is obvious from Figure 5 that the reaction was reasonably linear under both assay conditions for almost 2 hours. The usual incubation period was 1 hour.

(2) CONCENTRATION OF TISSUE The effect of increasing tissue levels on the enzymic activity is illustrated in Figure 7. The lack of an exact relationship (i.e. relative increase in enzymic activity with increasing tissue concentration) was not unexpected as the level of substrate used in most assays was not saturating (See below). The use of sub-optimum levels of substrate in arylsulfatase assays is common - mainly because of the expense of the substrate. Generally the level of tissue protein was kept relatively low so that the optimum possible rate could be obtained (cf. Table 4).

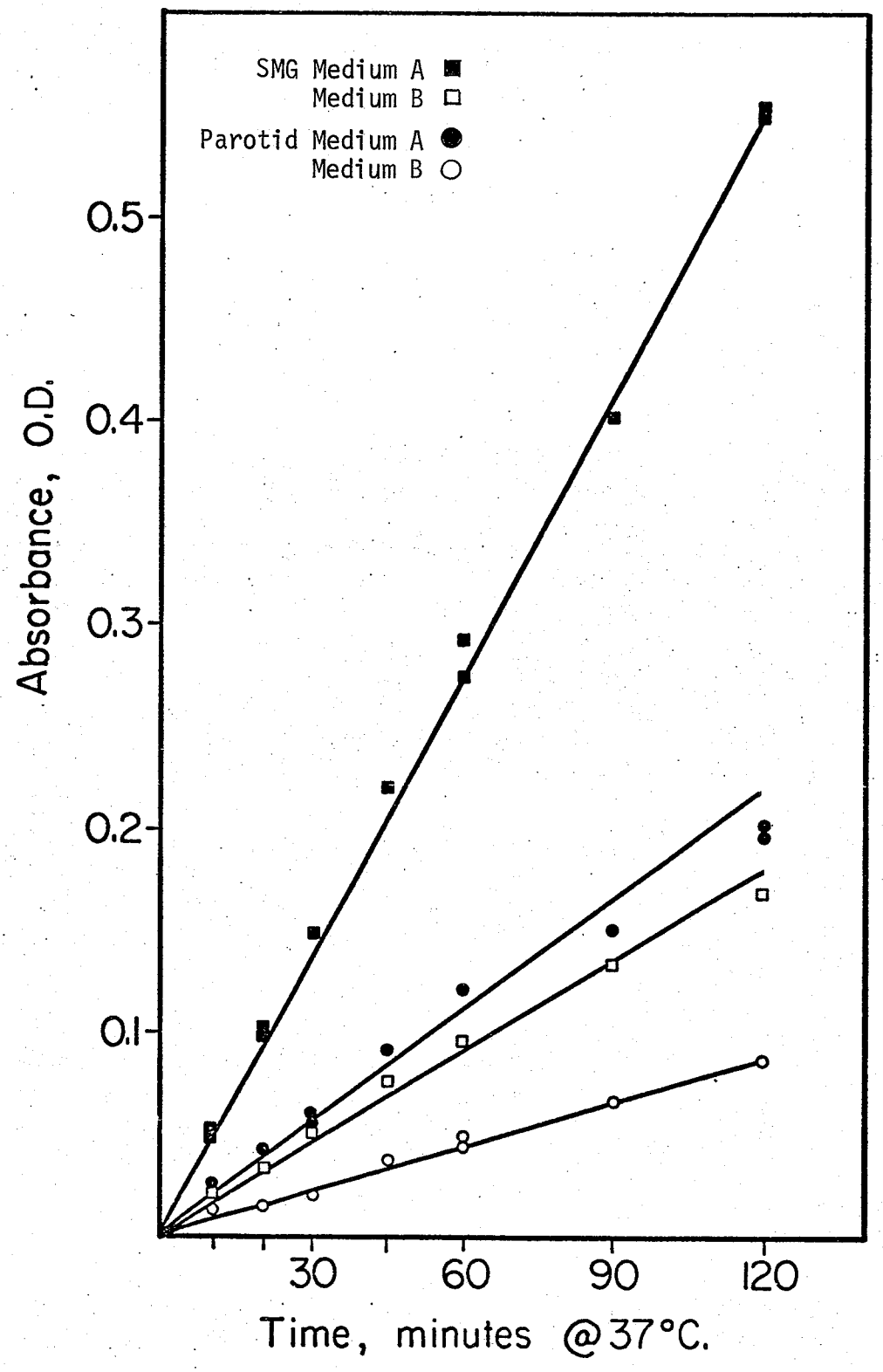


FIGURE 5. EFFECT OF INCUBATION TIME ON ARYLSULFATASE ACTIVITY IN RAT SUBMANDIBULAR AND PAROTID GLAND HOMOGENATES

Portions of tissue homogenates, containing equivalent protein levels (200 ug), were incubated at 37° for the indicated periods of time in Medium A and Medium B.

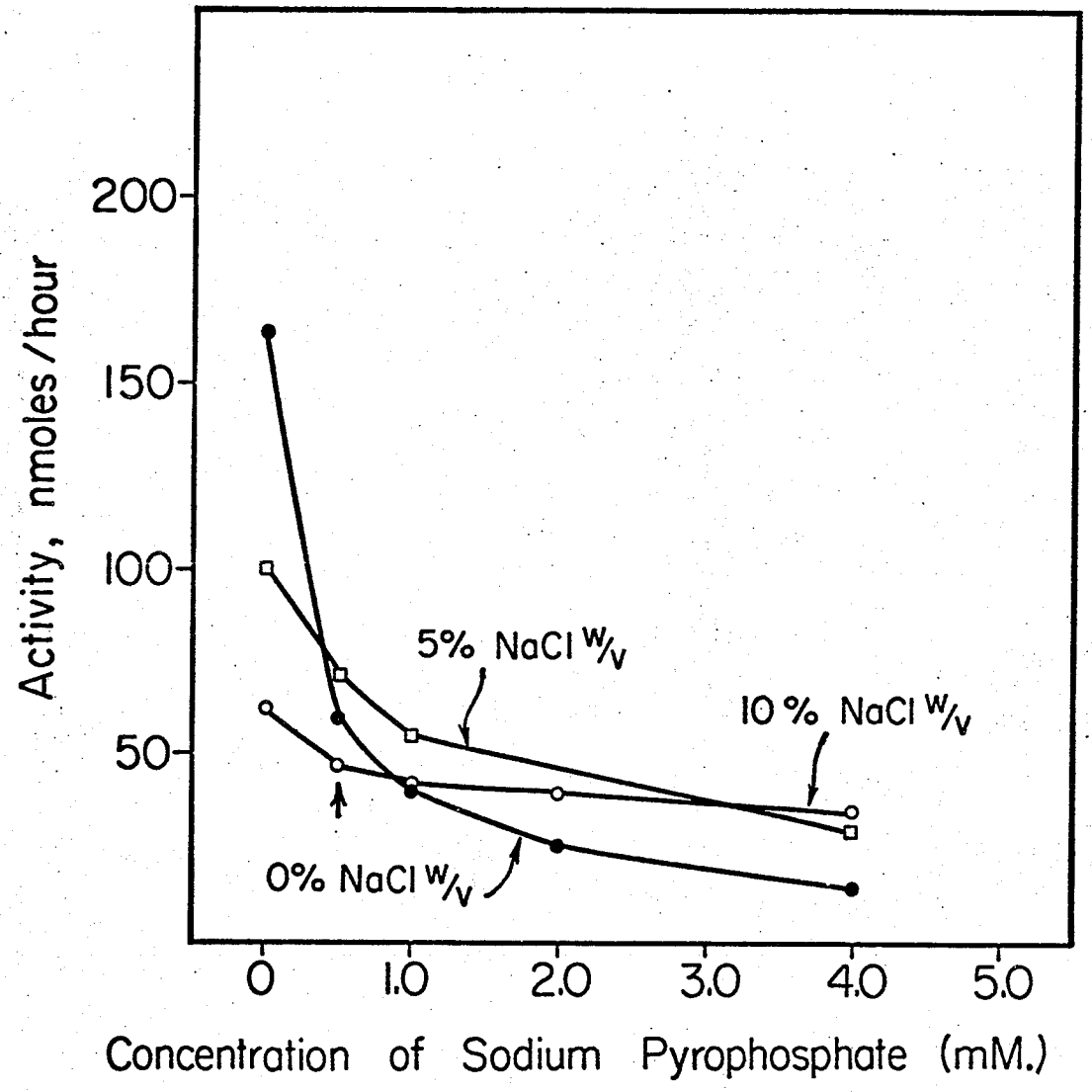


FIGURE 6. INFLUENCE OF SODIUM PYROPHOSPHATE AND SODIUM CHLORIDE ON ARYLSULFATASE ACTIVITY IN SMG

SMG whole homogenate (204 ug protein / test) was incubated at 37° for 1 hour in Medium A with the additions illustrated. Arrow indicates the concentration of pyrophosphate present in Medium B at 10 % NaCl.

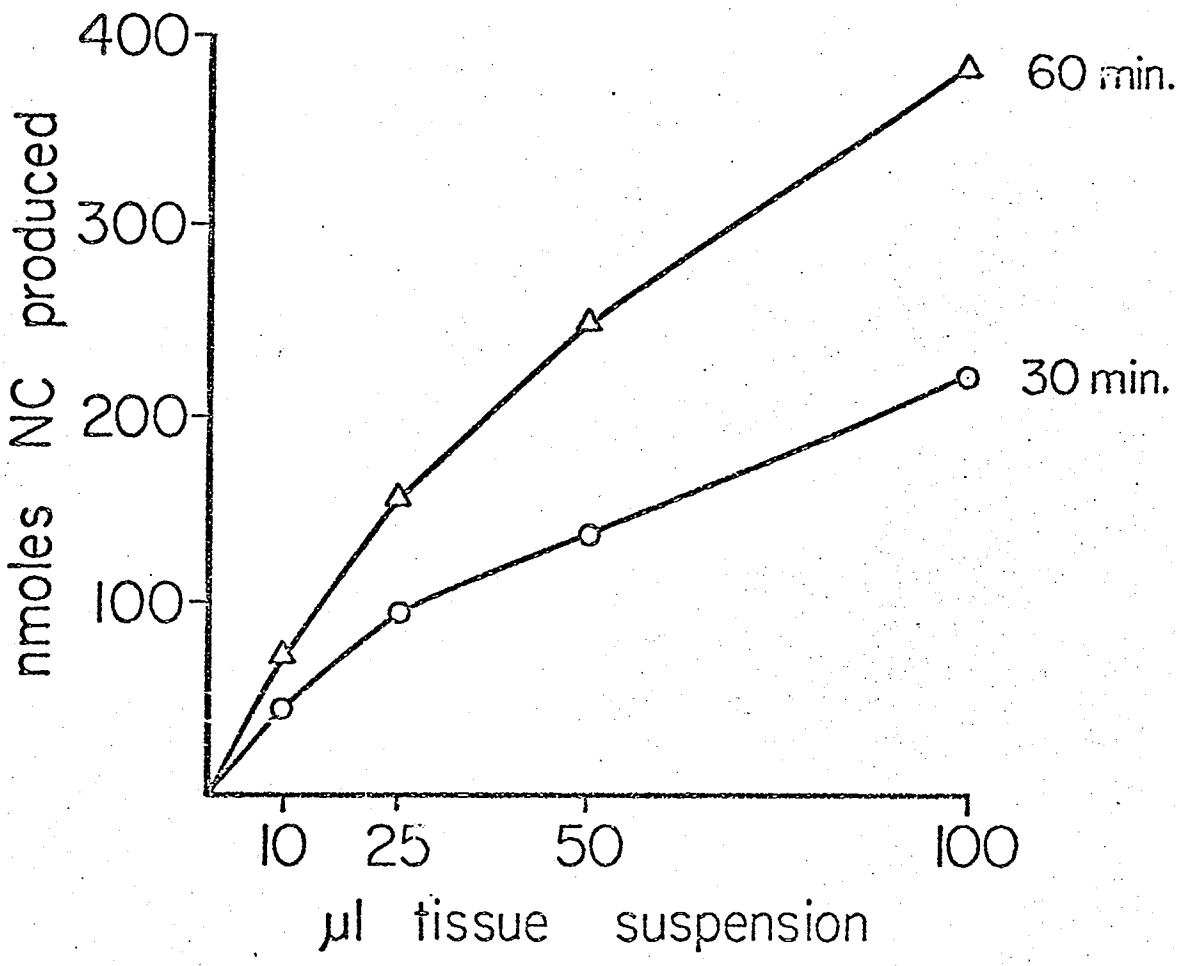


FIGURE 7. INFLUENCE OF TISSUE CONCENTRATION ON TOTAL ARYLSULFATASE ACTIVITY OF RAT SMG

Suitable portions of rat SMG HSS were incubated at 37° in Medium A for 30 min. and 1 h.

TABLE 4.

INFLUENCE OF TISSUE CONCENTRATION ON TOTAL ARYLSULFATASE

	nmoles NC released / mg protein / hour		
Incubation time @ 37°	30	60	90
192 ug tissue protein	78	141	208
398 ug tissue protein	63	126	183

SMG HSS incubated in Medium A for 1 hour at 37°.

TABLE 5.

SOME CHARACTERISTICS OF HSS OBTAINED FROM RAT KIDNEY, PAROTID AND SUBMANDIBULAR GLANDS

	nmoles NC released / mg protein / hour			
	Original homogenate	Frozen, kept at -10° for 1 month	Heated 100° 3 min.	Lyophilized, stored -10°, reconstituted
Submandibular gland	309	315	0	590
Parotid gland	164	170	0	311
Kidney	2045	2040	0	3613

Incubation was in Medium A for 1 hour at 37°.

(3) SUBSTRATE CONCENTRATION The effect of increasing the substrate (nitrocatechol sulfate) on the enzymic activity is shown in Figure 8. The apparent Km for this reaction was about 3.1 mM. The usual concentration of substrate present in the assay mixtures was 10 mM although in more recent work the value has been increased to 20 mM. Both these values are less than the ideal level (about 30 mM) but the present systems have produced useful and reproducible results.

(4) INFLUENCE OF TEMPERATURE Suitable portions of submandibular and kidney homogenates were incubated at various temperatures for 1 hour in Medium A containing 10 mM nitrocatechol sulfate. The reactions were terminated by the addition of NaOH directly into the incubation mixture. The results are presented in Figure 9. As expected, enzymic activity increased with increasing temperature. The somewhat indifferent increases noted at lower temperatures are most likely explained by the fact that arylsulfatase A, which is present in the mixture to the extent of about 20 - 30 percent, exhibits higher activity at low temperature than it does at high temperature (141).

Arrhenius plots of the data of Figure 9 are shown in Figure 10. These plots exhibit a discontinuity in the region of 14 to 16°C, a phenomena that is commonly observed with enzymes in tight association with lipid (33, 76).

(5) STABILITY OF ENZYME ACTIVITY The unusually high stability of crude arylsulfatase preparations has been reported by several investigators (30,61,143). After homogenization in 10 mM-Tris pH 7.4, SMG high speed supernatant (Figure 11) and whole homogenate (Figure 12) were held at the temperatures shown for periods of up to 25 hours, then portions were removed and assayed for arylsulfatase activity in Medium A (Figure 11) and Medium B (Figure 12). It can be seen that the activity is remarkably stable for 24 hours at temperatures up to 40°. Treatment at 100° for 3 min. completely abolished all activity (Table 5).

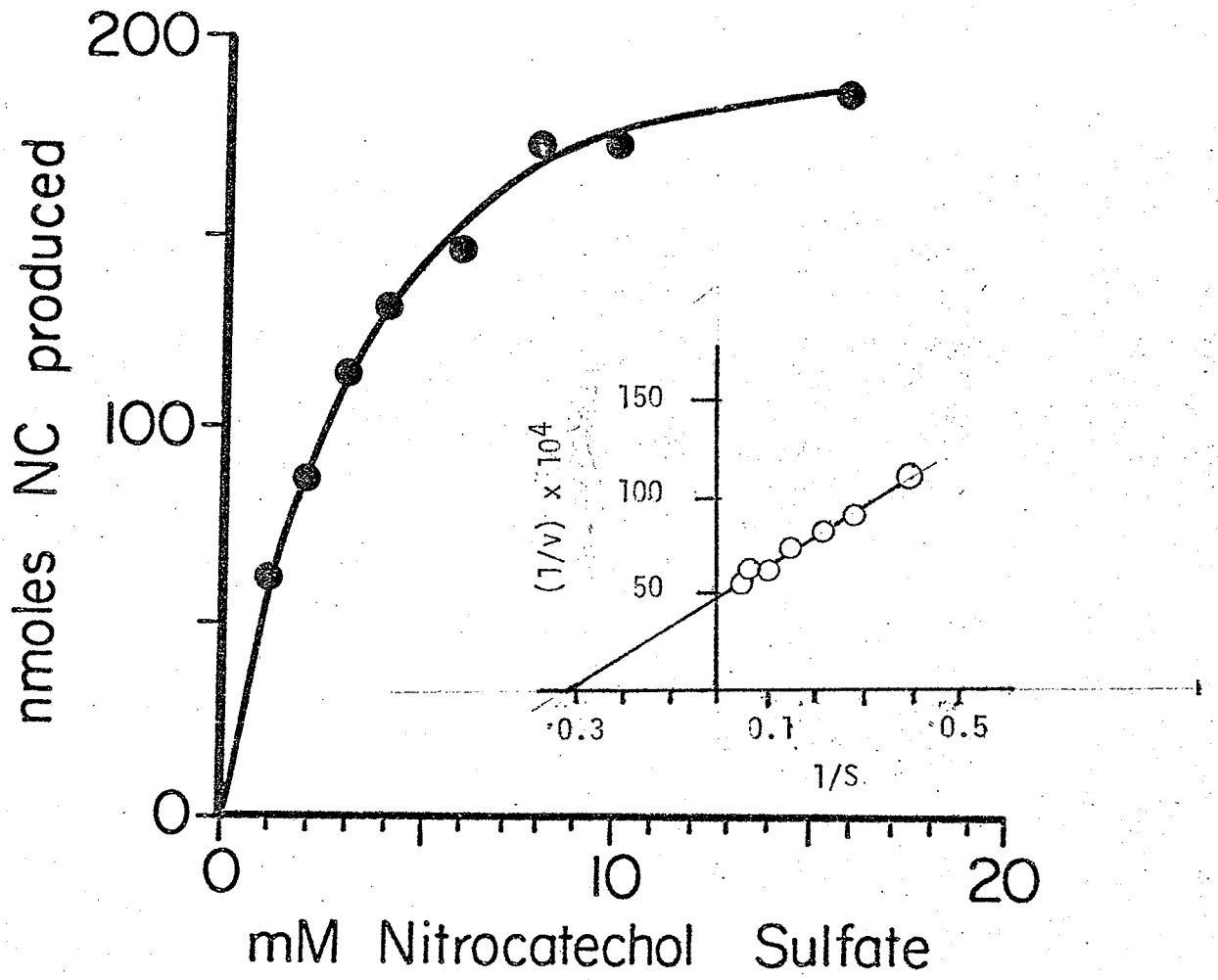


FIGURE 8. EFFECT OF NITROCATECHOL SULFATE CONCENTRATION ON THE ACTIVITY OF SMG ARYLSULFATASE

Equal portions of SMG HSS (304 ug protein / test) were incubated at 37° for 1 hour in Medium A which contained the concentrations of nitrocatechol sulfate shown.

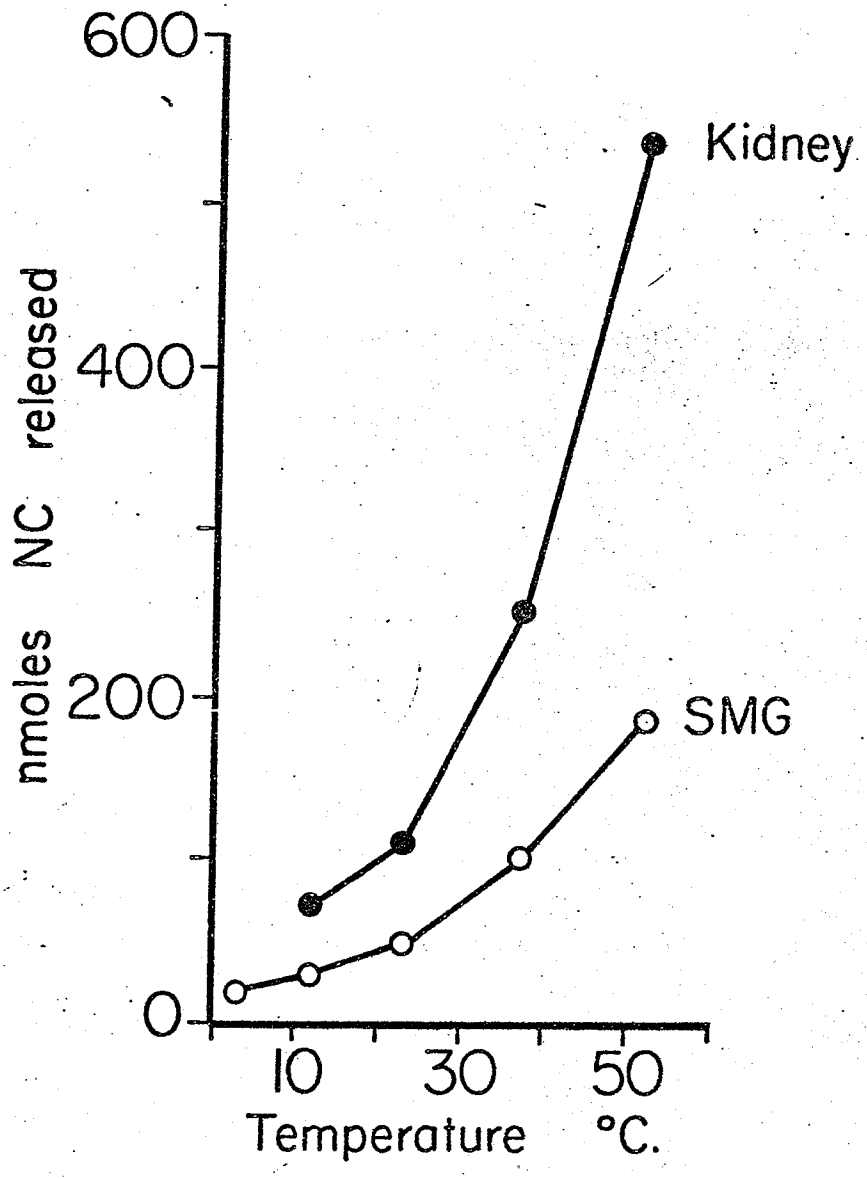


FIGURE 9. INFLUENCE OF INCUBATION TEMPERATURE ON NITROCATECHOL SULFATE HYDROLYSIS

Portions of rat kidney and SMG homogenates were incubated in Medium A at the indicated temperatures for 30 minutes. Reactions were quickly terminated by the addition of NaOH.

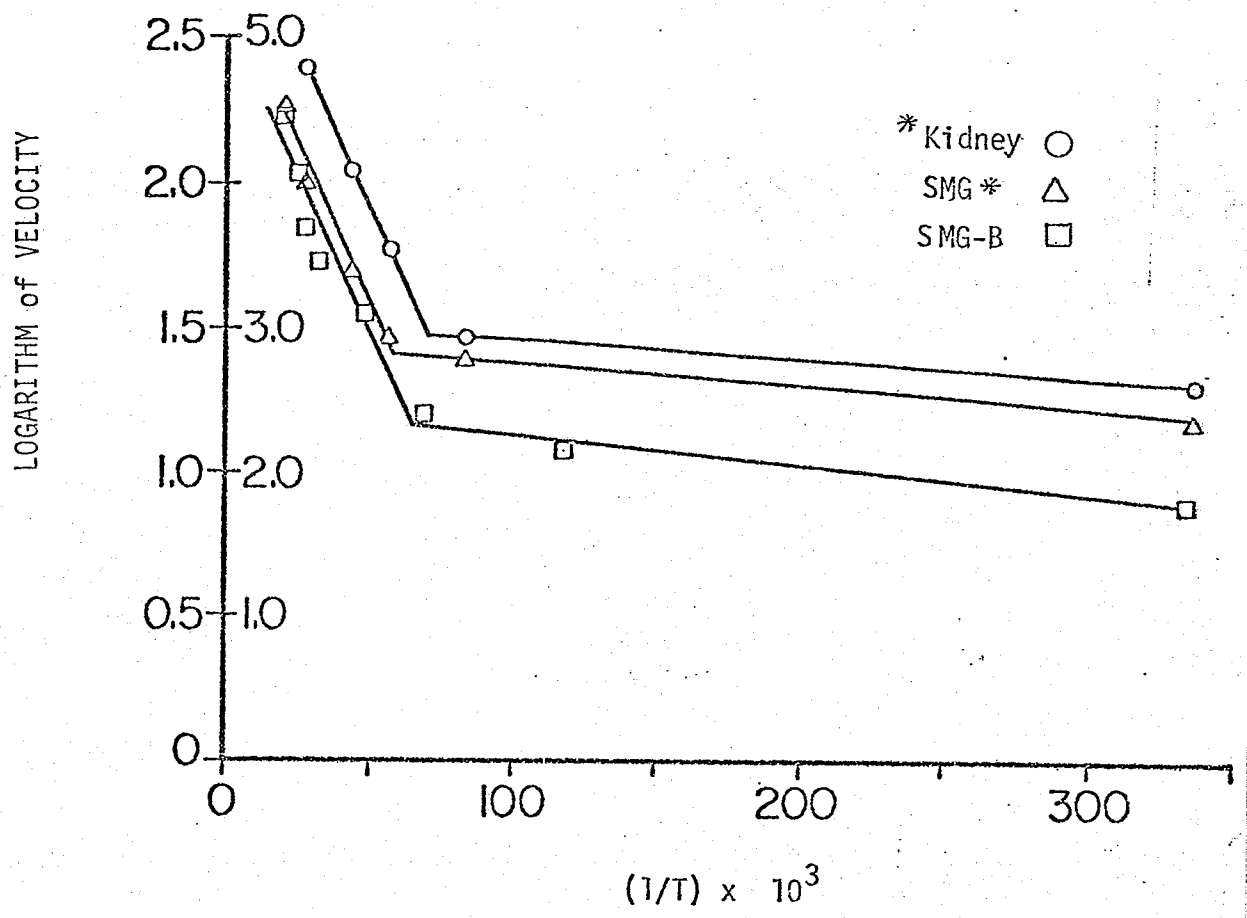


FIGURE 10. ARRHENIUS PLOT OF RAT SMG AND KIDNEY ARYLSULFATASE PREPARATIONS

* Data taken from Figure 9. SMG-B was material obtained from the active peak after Sephadex G-200 gel chromatography.

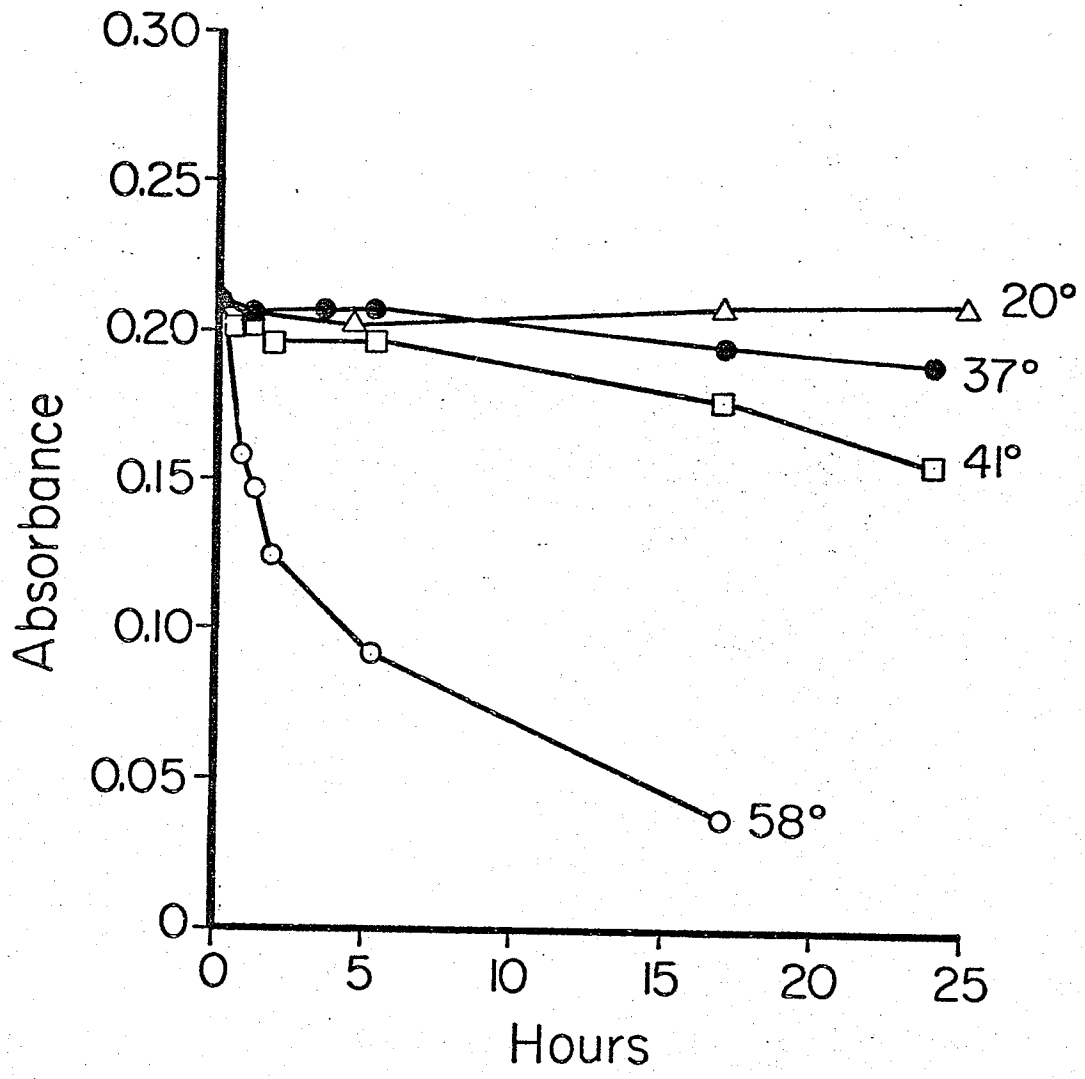


FIGURE 11. STABILITY OF SMG ARYLSULFATASES WHEN KEPT AT ELEVATED TEMPERATURES

SMG HSS in 10 mM Tris buffer, pH 7.4 was kept at the indicated temperature. At the time intervals shown, 0.2 ml portions were withdrawn and their arylsulfatase activity measured in 0.8 ml of Medium A (1 hour at 37°).

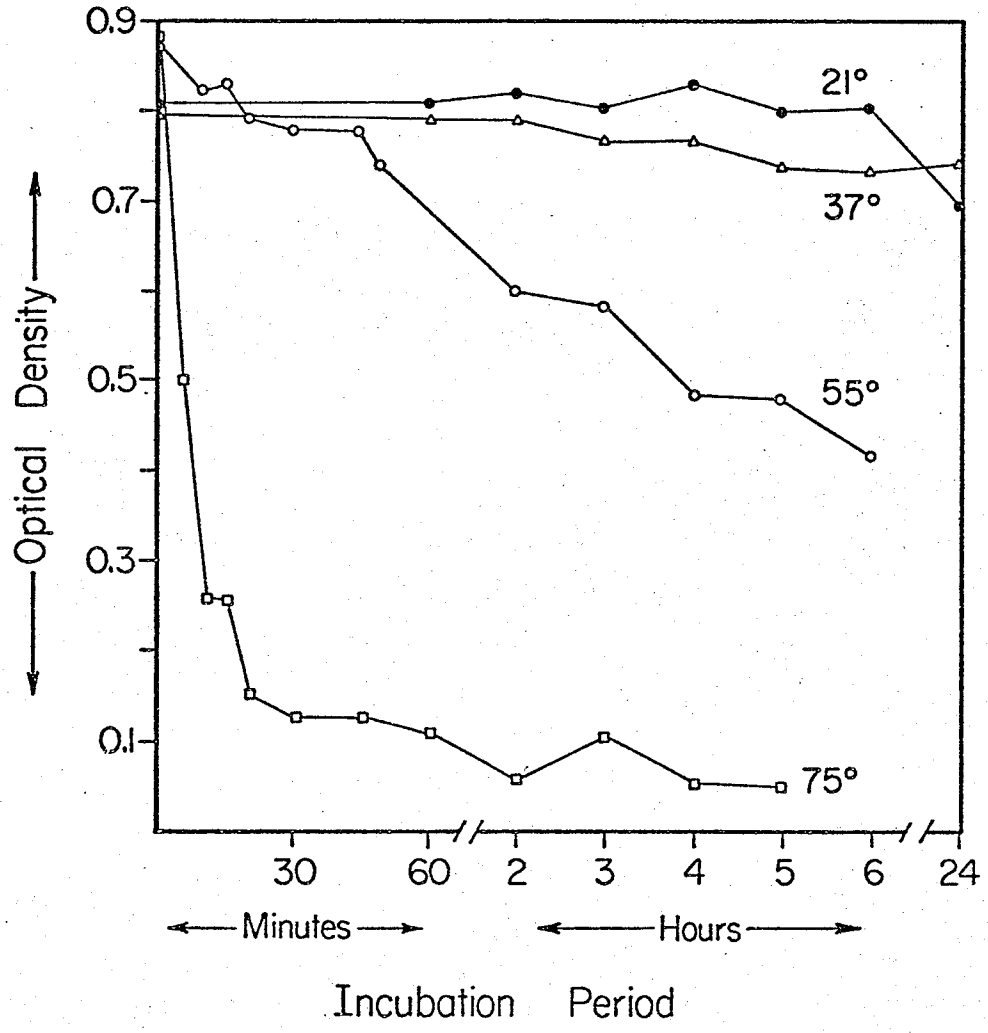


FIGURE 12. STABILITY OF SMG ARYLSULFATASE ACTIVITY WHEN KEPT AT ELEVATED TEMPERATURES

SMG whole homogenate in 10 mM Tris buffer, pH 7.4 was kept at the indicated temperature. At the time intervals indicated, 0.2 ml portions were withdrawn and their arylsulfatase activity measured in Medium B (1 hour at 37°).

The enzyme activity in whole homogenates and HSS was not inactivated by freezing and storage at -10^{o} nor by lyophilization, storage and reconstitution (Table 5). Subsequently, it was also found that extraction of HSS preparations at -40^{o} with acetone or acetone-ether did not decrease the level of activity, but actually increased it.

(6) INCUBATION CONDITIONS The influence of buffer type and concentration, pH and various ions on arylsulfatase activity will be described in this section.

(i) Buffers The effect of increasing buffer concentration at constant pH was examined with three buffers effective in the pH range where arylsulfatases reportedly exhibit their maximum activity. They were (effective pH range in brackets): acetate (3.7 - 5.6), citrate (3.0 - 6.2) and MES (5.0 - 6.2). All were adjusted to pH 5.5. Figure 13 shows that acetate buffer exhibited maximum effectiveness between 0.3 - 0.5 M while increasing concentrations of the other two tended to markedly suppress arylsulfatase activity. Acetate buffer was used throughout this study mainly because it was the buffer used by other arylsulfatase investigators and it is relatively inexpensive. Other studies, suggest that arylsulfatase is more active in MES buffer than in acetate and is effective at 1/10 the acetate concentration which is a great advantage in purification procedures wherein high salt concentrations are undesirable.

(ii) pH Acetate buffers (0.5 M) of various pH values were prepared by mixing 0.5 M solutions of acetic acid and sodium acetate. Since nitrocatechol sulfate can exert considerable buffering capacity in the pH range studied (114), the pH was always measured after the addition of NCS. The results, reported in Figures 14 and 15, indicated that the optimum pH in Medium B was 5.7 to 6.0 for SMG whole homogenate (Figure 14). The optimum in Medium A was quite similar (Figure 15). Purified (Sephadex G-200, see later) arylsulfatase B from SMG shows a lower pH optimum than the crude preparations. These results with SMG preparations agree well with values reported for arylsulfatases from a variety of tissues taken from

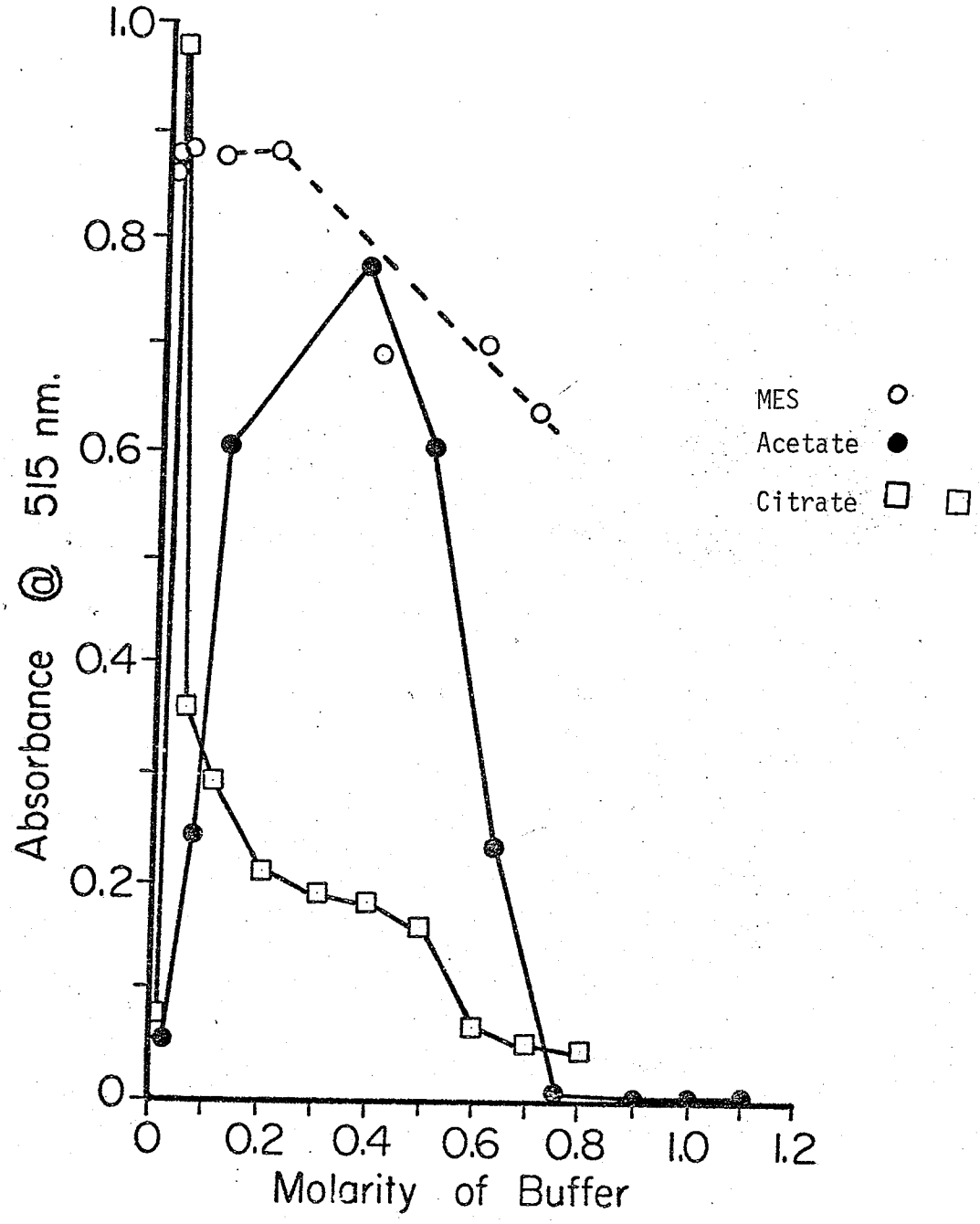


FIGURE 13. EFFECT OF BUFFER CONCENTRATION ON ARYLSULFATASE ACTIVITY OF SMG HOMOGENATES

SMG whole homogenate preparations were incubated for 1 hour at 37° at various concentrations of the three buffers all of which contained 10 mM nitrocatechol sulfate and were at pH 5.5.

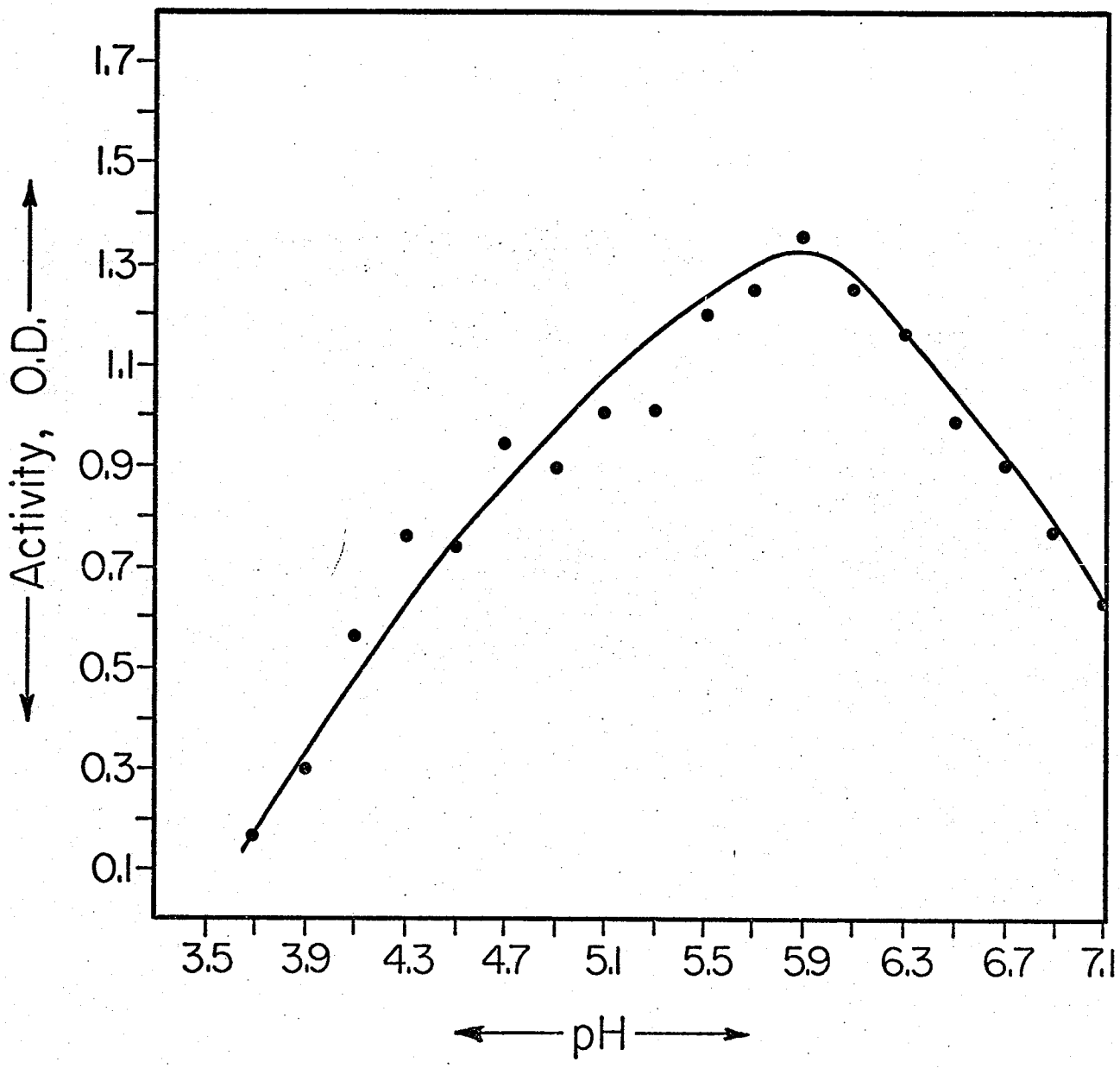


FIGURE 14. EFFECT OF pH ON SMG ARYLSULFATASE

SMG whole homogenate was incubated for 1 hour at 37° in 0.5 M-acetate buffer (adjusted as shown) containing 10 mM nitrocatachol sulfate, 0.5 mM sodium pyrophosphate and 10 % w/v NaCl (Medium B).

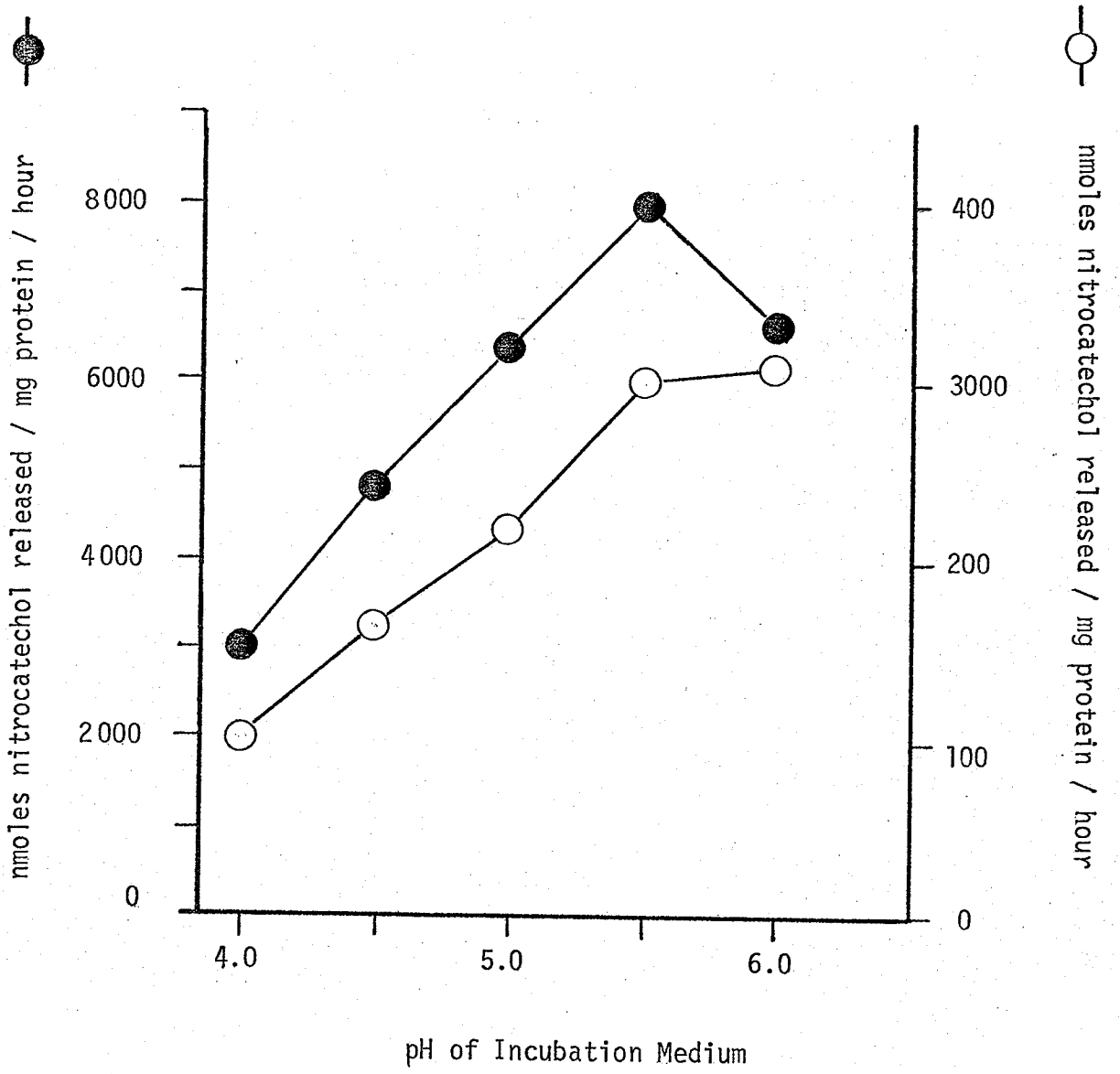


FIGURE 15. EFFECT OF pH CHANGE ON SMG ARYLSULFATASE ACTIVITY

Incubation for 1 hour at 37° in 0.5 M acetic acid-acetate buffer (adjusted to the pH shown) containing 10 mM nitrocatechol sulfate. SMG whole homogenate (—○—) and Sephadex-column purified arylsulfatase B from SMG-HSS (—●—).

different animal species (119).

(iii) Anions and Cations The influence of several electrolytes on SMG arylsulfatase was measured by the addition of various concentrations of electrolyte to Medium A. Cu^{2+}, SO_4^{2-}, SO_3^{2-} and F were observed to strongly inhibit the reaction (Table 6) as did "Topical Fluoride" (a mixture of KF and H_3PO_4). Very similar findings have been reported for arylsulfatase from ox (117) and rat (119) livers. The above observations stress the need to carefully select the ionic composition of any solution used in purification or assay of arylsulfatases.

Inorganic phosphate has long been known to depress both arylsulfatase A and B action(117). The activity in both rat kidney (Figure 16) and rat SMG (Figure 17) HSS preparations was reduced rapidly by low concentrations of inorganic phosphate and this inhibition appeared to be competitive. Although hydroxyapatite column chromatography appeared to effectively separate arylsulfatases, exhaustive dialysis was required to remove inhibiting phosphate ions from the eluted fractions. This led to considerable loss of activity and discontinued use of the column.

SUMMARY

1. Arylsulfatase of whole homogenate and high speed supernatant is extremely stable at temperatures of from 10^{o} to 55^{o}.
2. Arylsulfatase activity increased with temperature up to 50^{o}.
3. The apparent Km for the overall reaction was 3.1 mM NCS.
4. Arylsulfatase activity appeared to be maximum at pH 5.5-6.0.
5. The reaction was markedly affected by the type and concentration of buffers.
6. Removal of lipid from semipurified enzyme did not greatly affect the enzymic activity.
7. Enzymic activity was strongly inhibited by various anions, including inorganic phosphate, pyrophosphate and nitrite as well as by the salts of fluoride and sulfate.

TABLE 6.

Effect of Anions and Cations on Arylsulfatase Activity

	Percent of Control (=100)				
Concentration (mM)	1	5	10	20	45
$CaCl_2$	95	90	88	86	79
$SnCl_2$	95	86	72	43	27
$Cu(C_2H_3O_2)_2$	21	18	18	11	7
*"Topical Fluoride"	14	12	10	7	6
NaCl	100	95	91	87	77
NaCN	100	100	-	.	99
Na_2SO_4	59	46	36	28	21
Na_2SO_3	12	9	9	9	9
$NaNO_3$	98	88	56	33	23
NaF	32	23	19	9	0
KF	33	25	17	4	0

Portions of SMG whole homogenate were incubated in Medium A for 1 hour at 37^{o} with the additions shown.

*Topical fluoride contains a mixture of NaF and H_3PO_4.

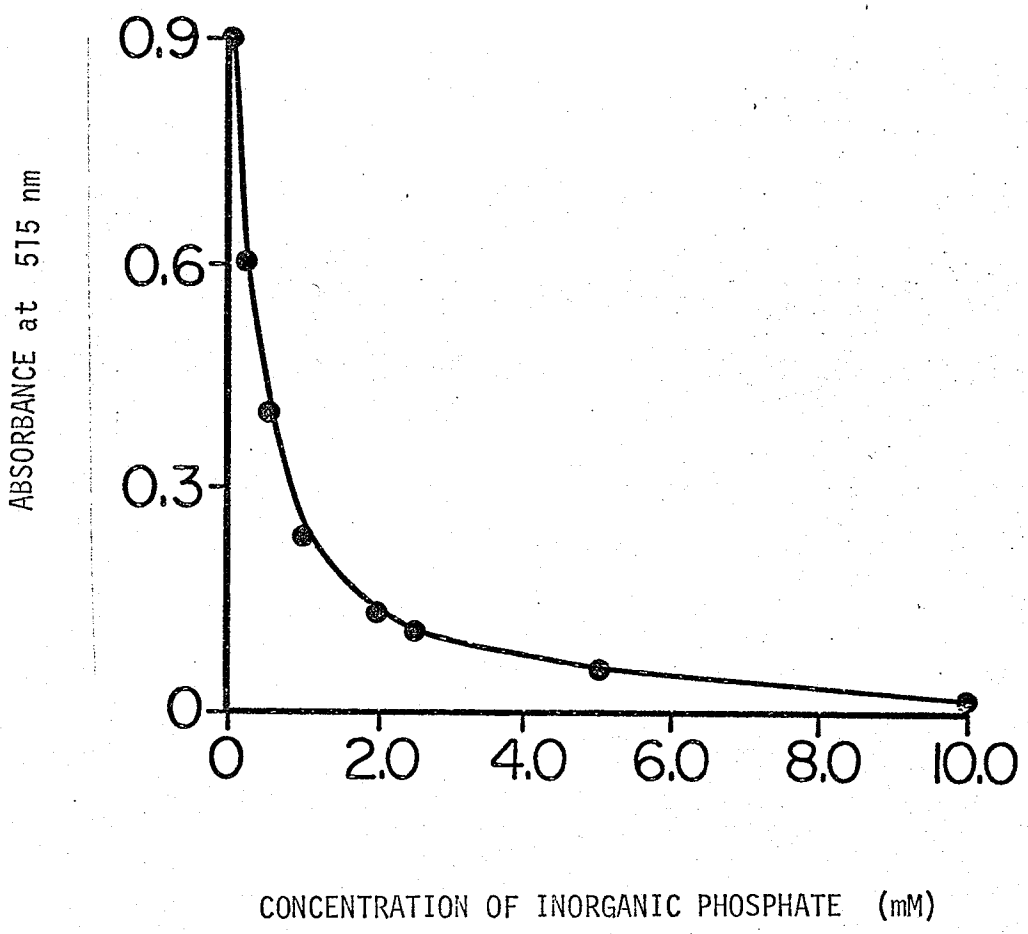


FIGURE 16. EFFECT OF INORGANIC PHOSPHATE ON THE ACTIVITY OF RAT KIDNEY HSS ARYLSULFATASE

Portions of rat kidney HSS were incubated at 37^{0} for 1 hour in Medium A to which the amount of inorganic phosphate shown was added.

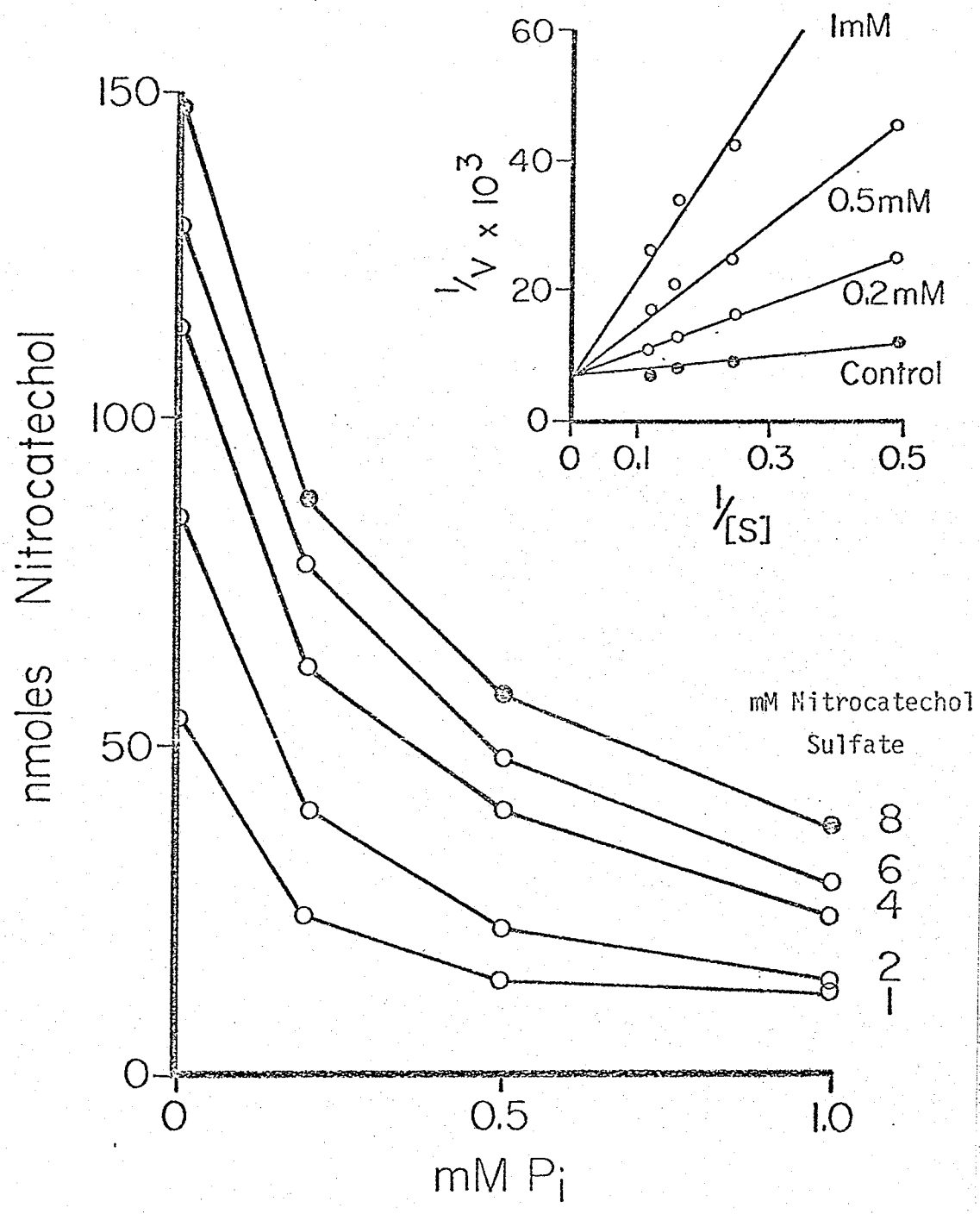


FIGURE 17. EFFECT OF INORGANIC PHOSPHATE ON SMG ARYLSULFATASE

Aliquots of SMG HSS were incubated for 1 hour at 37° in 0.5 M-acetate buffer, pH 5.5 which contained the concentrations of nitrocatachol sulfate and inorganic phosphate shown. In the inset 1/(S) refers to nitrocatechol sulfate and numbers on slopes (1 mM, 0.5 mM, etc) to inorganic phosphate concentrations.

B. ARYLSULFATASE ACTIVITY IN RAT TISSUES

This section is composed of five subsections entitled-

(1) A Comparison of Arylsulfatase Activity Among Several Rat Tissues

(2) Changes During Early Post-Natal Growth

(3) Influence of Fasting on Enzymic Activity

(4) Enzyme Changes in Diabetes

(5) Apparent Arylsulfatase Activity in Saliva and Other Fluids

While there are obvious interrelationships among some of the above, each sub-section has been treated as an entity having some explanation of methodology, a discussion of the experimental results and a concise summary.

(1) A Comparison of Arylsulfatase Activity Among Rat Tissues.

The results of a survey for total arylsulfatase activity are presented in Table 7. The usual procedure for (A + B), as noted in Methods, was modified essentially as described by Worwood, Dodgson, Hook and Rose (141). These authors claim that their method can provide a rough estimate of the relative amounts of the A and B forms of arylsulfatase. The first two columns (Medium A, pH 6.0) represent total (A + B) activity while column 3 (Medium A + NaCl, pH 5.0), according to the above authors, would represent the maximum possible level of A which would appear to be less than 20% of the total. These workers (141) claimed that a preponderance of the arylsulfatase activity in rat tissue is of the B form (91% testes to 97% in kidney). Therefore, the results with various tissues reported herein reflect primarily arylsulfatase B. There is some evidence, however, that this may not be true for all rat tissues as will be discussed in Section IV, C.

Among rat salivary glands, the sublingual had the highest activity (nmoles NC released/mg protein/h). However, if one considers the relative weights of all these glands, the SMG has much more activity per gland than either of the others. The ratio of weights is approximately SMG:SL:P=8/1/4.

It is apparent from the present study that arylsulfatases A and B are ostensibly ubiquitous.

TABLE 7 A

ARYLSULFATASE ACTIVITY OF SOME RAT TISSUES

Tissue	nmoles NC released / mg protein / hour
Submandibular gland	362
Kidney	1744
Spleen	1131
Lung	1182
Brain, white matter	486
Brain, grey matter	250
Large intestine	385
Pancreas	316
Adipose tissue	486
Heart	137
Stomach	110
Small intestine	171
Prostate gland	141

Suitable portions of whole homogenate (10 % w/v) obtained from various tissues of one rat were incubated for 1 hour at at 37° in Medium B.

TABLE 7.

ARYLSULFATASE ACTIVITY OF RAT TISSUES

	Arylsulfatase A + B*			Arylsulfatase C
Incubation time (minutes)	30	90	60	60
Incubation Temperature	37_1	37_1	15_2	37_3
Submandibular	194	514	100	26
Parotid	119	292	75	38
Sublingual	398	1180	218	-
Kidney	1457	2616	604	45
Liver	550	1575	250	154
Testes	345	647	155	29
Spleen	540	1859	281	34

Whole homogenates of each tissue (10 mM Tris buffer, pH 7.4) adjusted to approximately equal protein levels / test, were incubated under conditions noted.

1. Medium A, pH 6.0
2. Medium A, pH 5.0 + 50 mM NaCl
3. Assayed by the method of Milsom, Rose & Dodgson (1972) Biochem. J. 128, 331 - 336. The medium contained 0.1 M phosphate buffer, pH 8.0 and 40 mM potassium p-acetylphenyl sulfate. Absorbance read at 323 nm. Units: nmoles p-hydroxyacetophenone /mg protein.

* Units: nmoles nitrocatechol released / mg protein / time shown

Table 7 also shows the relative activity of arylsulfatase C, considered to be predominately a microsomal enzyme (118), and at least in liver, identical to estrogen sulfatase (32). Liver is the major source of this enzyme (92) and male rat livers apparently contain more activity than do female rat livers although in most other tissues the levels were found to be identical (92).

(2) Changes During Early Post-Natal Growth

It has been observed that there are some enzymic changes with age in many animal tissues (104,105,107, 20A) This is particularly evident in the CNS (107). Eto and his associates (34,20A) reported that rat brain arylsulfatase B activity increases during early developement to a maximum around the 20'th day post-partum and then declines rapidly. The activity in liver, however, has been shown to steadily decline from birth (34, 20A). The results noted in Figure 18 indicate that SMG arylsulfatase activity tends to decrease during early growth when expressed as activity per unit protein. When the activity is expressed per whole gland (Figure 18, inset) there appears to be an overall increase in the amount of enzymic activity.

(3) Influence of Fasting on Enzymic Activity

Arylsulfatase activity, of tissues obtained from fasted rats, tended to show a slight increase (Table 8). Several factors may affect this activity. Enzymes committed to the maintenance of the blood sugar level are known to have altered activities during periods of stress, i.e. with fasting the increase in enzymes involved with gluconeogenesis is paralleled by a decrease in those of glycolysis (65). Perhaps the apparent increase in arylsulfatase activity is similar to the response of gluconeogenic enzymes.

Lysosomal digestion increases with cell death and arylsulfatase activity may be a part of this occurrence (59,60).

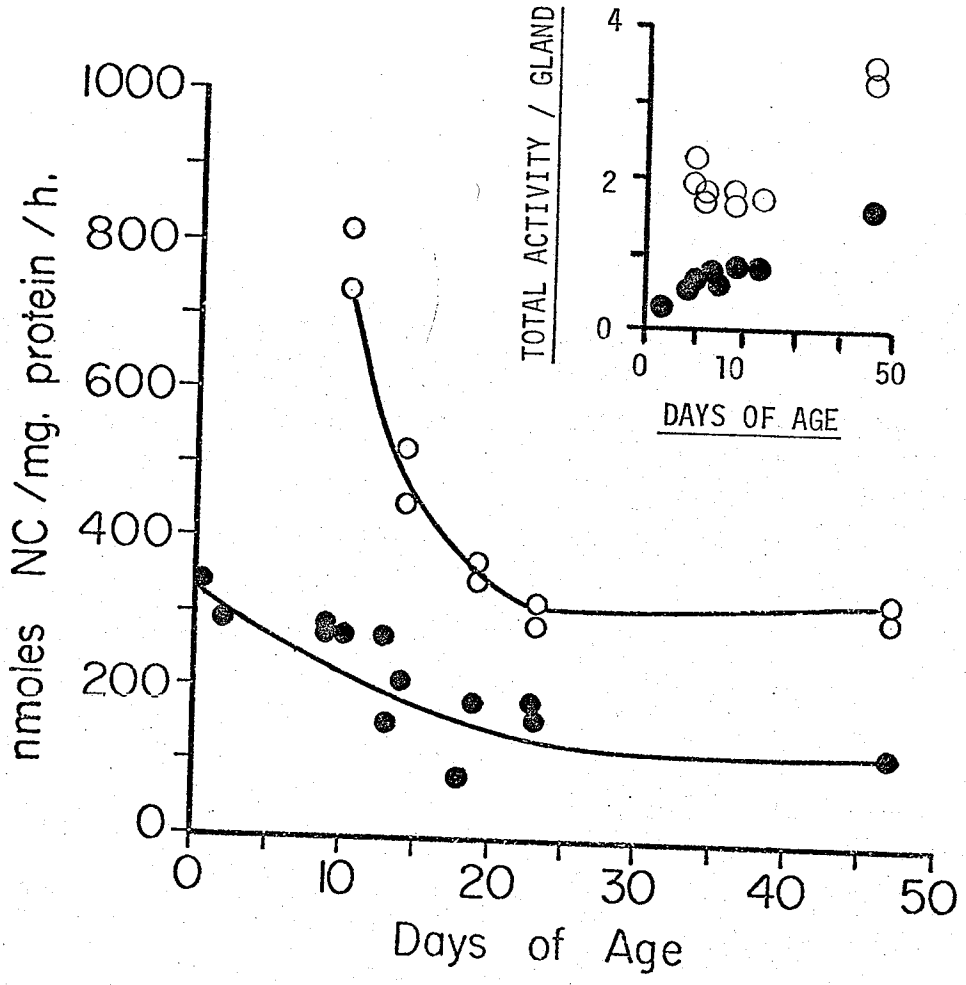


FIGURE 18. CHANGES IN TOTAL ARYLSULFATASE ACTIVITY OF RAT SMG DURING EARLY POSTNATAL GROWTH

SMG whole homogenates were prepared from rats of various ages and suitable portions incubated in Medium A (○) or Medium B (●) for 1 hour at 37°.

Inset The same data as in large figure except the ordinate values have been multiplied by total protein / gland. This figure then represents the variation in total aryl-sulfatase activity of a whole SMG with growth.

TABLE 8.

ARYLSULFATASE ACTIVITY IN TISSUES FROM FASTED MALE RATS

Tissue	nmoles NC / mg protein / h		$\frac{\text{Fasted}}{\text{Fed}} \times 100$
	Fed	Fasted	
Submandibular Gland	367	252	72
Parotid Gland	167	212	126
Sublingual Gland	581	470	81
Kidney	1881	2328	123
Liver	824	920	111
Testes	518	561	105
Spleen	820	1146	138

Five percent w/v homogenates were prepared from tissues of rats fasted for three days, but allowed water ad libitum, and littermate controls. Equivalent portions (in protein) of homogenate were incubated in Medium A for 1 hour at 37°.

TABLE 8A.

CHANGES IN ORGAN AND ANIMAL WEIGHTS WITH FASTING

	Control weight	Fasted in grams
Body Weight	256(3)	183(3)
Salivary Glands-		
Submandibular	128	103
Parotid	126	93
Sublingual	34	24
Kidney	1053	729
Liver	939	780
Testes	1099	1246
Spleen	1096	611

(4) Enzyme Changes in Diabetes

Tissues of diabetic rats, measured in vitro, showed little change in arylsulfatase activity (Figure 19). Four tissues (SMG, parotid, testes, liver) tended to show a slight increase while kidney and spleen indicated a slight decrease in activity. There was, however, a very significant elevation of serum arylsulfatase activity (Table 9)

Insulin effects an increased uptake of glucose into cells and a deficiency of the hormone is accompanied by hyperglycemia, glycosuria, increased glycogenolysis and fatty acid breakdown, and by decreased protein synthesis(132). The decrease in the uptake of glucose leads to increased lysosomal fragility and may enhance release of the lysosomal contents (19,125) which would include arylsulfatase A and B. This may explain the apparent increase in serum activity with diabetes. The production of keto acids from fatty acid catabolism may decrease pH and cause the increase in activity as discussed in Section IV B (3).

Fushimi and Tasui (48,49) have examined the levels of three lysosomal enzymes - β-N-acetylglucosaminidase, β-glucuronidase, and β-galactosidase - in streptozotocin - diabetic rats. They noted decreased activity in kidney and an elevated level in serum of all four enzymes' activity. Insulin administration appeared to restore the levels to normal. As yet these authors have no explanation for these enzymic changes.

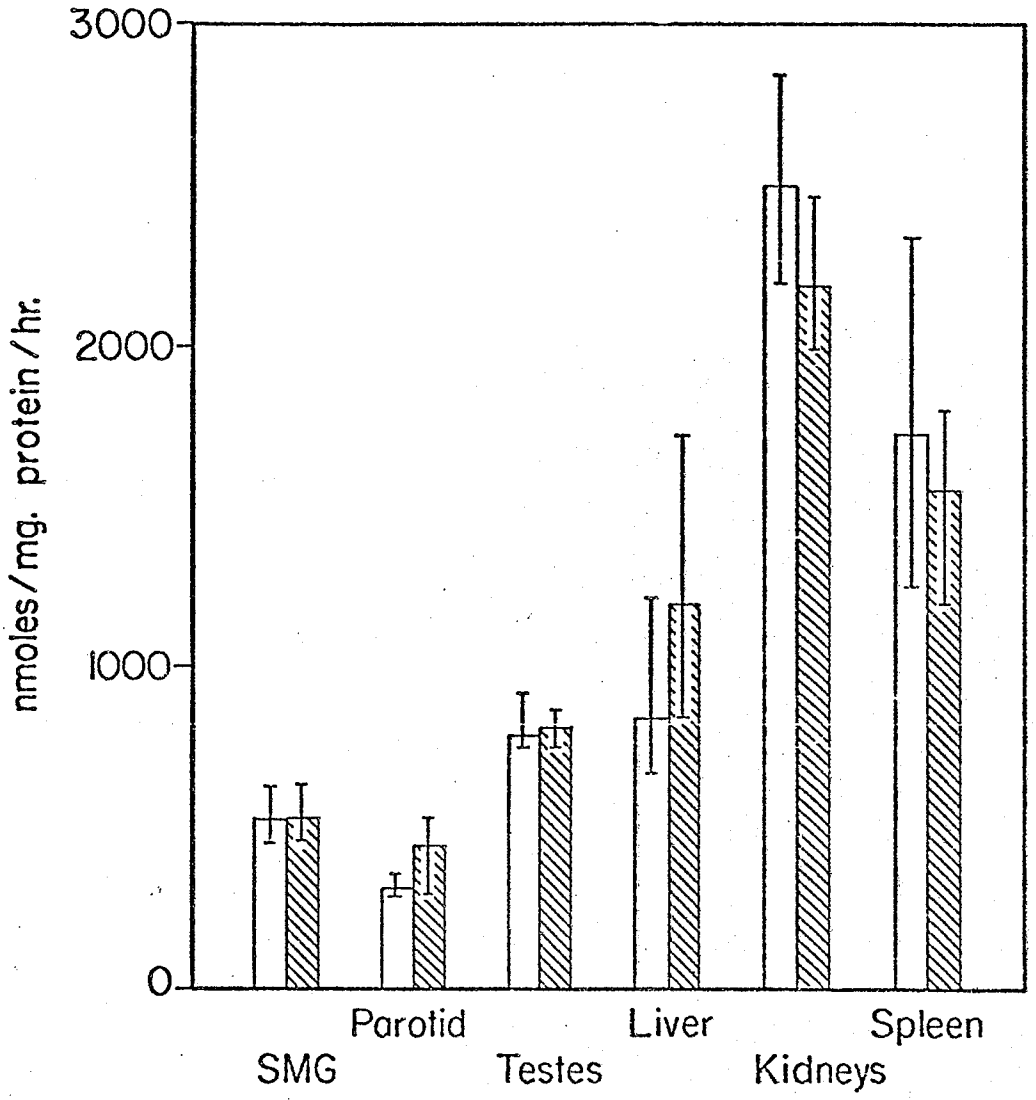


FIGURE 19 INFLUENCE OF DIABETES ON APPARENT ARYLSULFATASE ACTIVITY IN SOME RAT TISSUES

Whole homogenates were prepared from the tissues noted above and suitable aliquots were incubated in Medium A for 1 hour at 37°. The bars on each histogram indicate the limits of the values obtained from 4 rats. No significant differences between control and diabetic tissues were observed.

TABLE 9.

COMPARISON OF SERUM ARYLSULFATASE ACTIVITY BETWEEN NORMAL AND DIABETIC RATS

Type	No. rats	nmoles NC released / ml / hour	
Control	6	2358 ± 36	
Diabetic	12	5363 ± 449	$P < 0.01$
Control	5	1980 ± 128	
Diabetic	6	4115 ± 520	$P < 0.02$
Control	12	2800 ± 119	
Diabetic + insulin	4	2988 ± 198	No difference.

(5) Apparent Arylsulfatase in Saliva and Other Secretions.

The examination of arylsulfatase activity in saliva was an obvious extension of the preceding studies. Several reports (71,110,87) in the literature describe sulfatase activity in human and animal whole mixed saliva ("spit") but none to the investigator's knowledge on pure saliva collected directly from the salivary ducts before it has mixed with the bacteria, cells, food debris, etc. in the oral cavity. The present section will deal with whole and pure saliva.

Methods (a) Rat Saliva. Rat submandibular and parotid saliva were collected from male Sprague-Dawley rats at four months of age. The rats were anaesthetized by intraperitoneal injection of Nembutal (50 mg/Kg body weight), then tracheotomized to facilitate breathing and SMG and parotid ducts were canulated with PE 10 polyethylene tubing. Pilocarpine (80 mg/Kg body weight) was then injected subcutaneously to stimulate secretion and saliva was collected in sterile, cooled tubes. The method was developed in this department by Abe and Dawes(1)

(b) Human Saliva. An individually fitted molded plastic cover and a modified Lashley cannula were used to collect submandibular and parotid salivas, respectively, from human subjects. Sour lemon drop candies were used for gustatory stimulation and subjects adjusted the secretion rate to 1 ml/min by movement of the lemon drop. Sublingual saliva was not collected from humans for technical reasons.

Whole mixed saliva was obtained by means of paraffin wax stimulation. Subjects expectorated into test tubes inserted in an ice bath. The expectorant was separated into supernatant and residual fractions by centrifugation at 1000 g for 15 minutes. The residue contained all oral bacteria, epithelial cells and debris etc. while the supernatant contained the salivary gland secretions (77,124). This separation via centrifugation may not be absolute as some bacterial products may not be precipitated with the residual material. All

saliva was stored at -10° until required.

(c) Human Tears and Sweat. Onion-stimulated tears from 4 females and sweat from 1 male after heavy exercise were collected by means of Pasteur pipettes.

(d) Snake Venoms. Venoms were collected from four different species of snakes: Crotalus horridus horridus (timber rattlesnake), Crotalus viridus viridus (prairie rattlesnake), Croalus atrox (western diamondback rattlesnake), and Agkistrodon piscivorus piscivorus (eastern cottonmouth).

Snakes were fasted for one week prior to venom collection and 24 hours before "milking" the snakes were placed in a 4° cold room. This latter procedure ensured sedation. The collection device was a rubber sheathed funnel attached to a vacuum flask. The fangs were inserted through the sheath and the poison gland gently squeezed. Venom was centrifuged to remove debris and stored frozen at -10°.

Results and Discussion

Human saliva contains a small but significant amount of arylsulfatase activity (Tables 10 & 11), the level of which fluctuates markedly among individuals. The amount of activity in the supernatant fluid obtained from whole mixed saliva agrees reasonably well with that obtained by cannulation. The contribution to the activity in whole mixed saliva by bacteria is considerable (Residue, Table 10) but not 100% as others have suggested (87). The cannulation studies (Table 11) satisfactorily illustrate a significant contribution of oral arylsulfatase is provided by glandular secretions. These secretions most likely provide for the level of supernatant activity reported in Table 10.

Whole mixed saliva was collected daily from a male individual at the same periods of time for 5 consecutive days. The saliva was centrifuged to remove bacteria and debris and the supernatants were analyzed for arylsulfatase activity (Figure 20). A distinct circadian rhythm was obtained with an apparent acrophase (peak) at, or before, 8 a.m. The activity steadily decreased reaching the nadir about 7 p.m.

TABLE 10.

APPARENT ARYLSULFATASE ACTIVITY IN WHOLE HUMAN SALIVA

Whole Saliva	"Supernatant"	"Residue"
45 - 280	25 - 85	30 - 280

Whole saliva (spit) was collected from four individuals and separated into "supernatant" and "residue" by centrifugation at 1000 x g for 15 minutes. Arylsulfatase was measured in Medium A. The results are expressed as nmoles nitrocatechol released per ml and the range is given above.

TABLE 11.

APPARENT ARYLSULFATASE ACTIVITY IN PURE SALIVA FROM RATS AND HUMANS

	nmoles NC released / mg protein / hour		
	Parotid Gland	Submandibular Gland	Sublingual Gland
Rat	75	21	742
Human	25 - 36	6 - 40	-

Pure saliva was collected from the glands by cannulation as described in the text. Incubation for estimation of arylsulfatase activity was in Medium B.

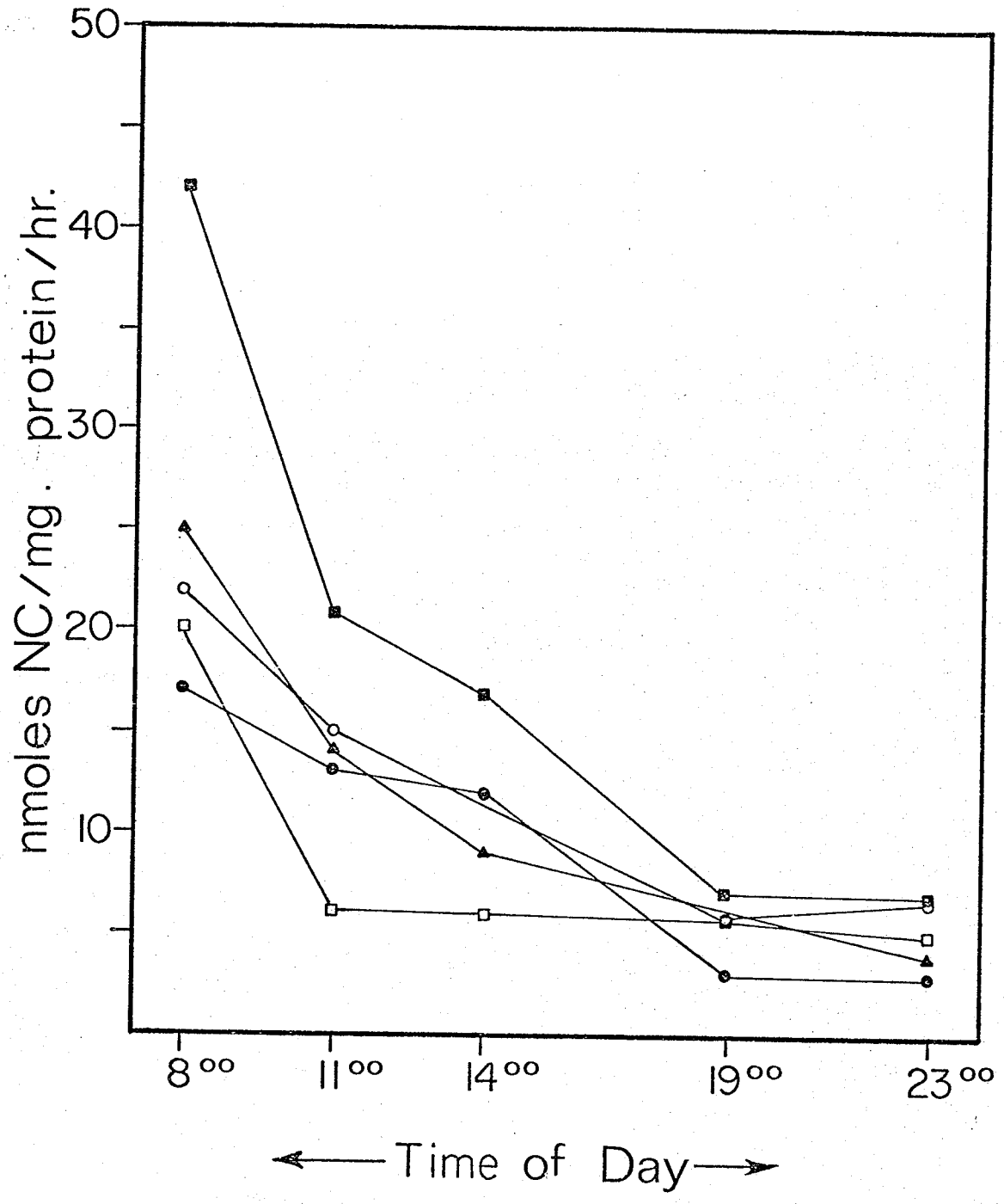


FIGURE 20. CIRCADIAN RHYTHM OF ARYLSULFATASE ACTIVITY OF HUMAN WHOLE SALIVA

Whole mixed saliva was collected at times noted above and aliquots of 2000g (1 hour) supernatant were incubated in Medium A for I hour at 37°. Day 1, ● ; 2, ▲ ; 3, ■ ; 4, ○ ; 5, □ .

Since salivary flow is negligible during sleep (24,126), no collections were made between midnight and 8 a.m. Surprisingly, the total arylsulfatase activity did not parallel the total protein rhythm which has an acrophase in the late afternoon (ca 4 p.m.).

As yet there is no evidence warranting the assignation of an important physiological role to salivary arylsulfatases. The present survey contained only five persons. A much larger sample would be required to find the true physiological range of this activity in the human population. Furthermore, it is possible that salivary arylesterases, known to be present in pure saliva (138) are responsible for the observed arylsulfatase activity.

Table 12 indicates that human tears and sweat show arylsulfatase activity but that the levels in snake venoms are negligible.

SUMMARY

1. The total arylsulfatase activity in rat tissue appears to be highest in kidney, spleen and liver with the latter, due to its large size, having by far the greatest total content of enzymic activity. The specific activity of arylsulfatase in SMG homogenates tended to decrease with growth, as has been reported for liver, but the total activity per gland increased.

2. Diabetes and fasting, which have many similar physiological effects, did not change the specific activity of arylsulfatase in submandibular, parotid, testes, liver, kidney or spleen. However, in diabetic animals, blood serum levels were significantly elevated; an effect which was abolished by insulin injection.

3. Pure saliva, sweat and tears contained significant arylsulfatase activity.

4. Arylsulfatase activity of whole saliva exhibited a circadian rhythm that was not in phase with known rhythms of protein concentration of cannulated saliva.

TABLE 12.

ARYLSULFATASE ACTIVITY OF HUMAN SWEAT & TEARS & SOME SNAKE VENOMS

	nmoles NC released / mg protein / hour
Tears	59
Sweat	474
Crotalus horridus horridus	7
C. viridus viridus	5
C. atrox	7
Agkistrodon piscivorous piscivorous	4

SECTION C

SEPARATION OF ARYLSULFATASES

It has been assumed that most arylsulfatase activity in rat SMG is due to the B type enzyme. This assumption is based on the reports from Dodgson's and Roy's laboratories. Both these workers have shown that the B form predominates in rat adrenal, brain, liver, spleen testis and kidney by a ratio of approximately 10:1. They did not examine salivary glands.

As noted in the Review of the Literature section, there are at least two major differences between the A and B forms of arylsulfatase, to wit, the A type polymerizes at pH 4 - 5 to a much higher molecular weight than the B, which doesn't polymerize (42), and the pI of A is at an acidic pH whilst that of B is at a basic pH. Because of these differences the two forms of the enzyme should be separable by gel filtration in acid media by ion exchange chromatography. Such separations have been effected and reported (2,42,65,141).

Freezing and thawing whole homogenates prepared in hypotonic buffer (10 mM Tris, pH 7.4) solubilized arylsulfatase as effectively as Triton X-100 (Table 13). Thus, the freezing and thawing procedure eliminated any possibility of Triton X-100 interfering with the separation. A HSS of the "freeze-thawed" whole homogenate was prepared by centrifugation at 100,000 g for 1 hour.

Table 14 shows the results of an examination of the HSS from various tissues by means of molecular filters. At pH 7.4, where both A and B enzymes have approximately the same molecular weight, considerable activity was lost when a 125,000 M.W. cut-off filter was used. More activity was retained when a 75,000 M.W. cut-off filter was used. In addition, the total activity increased to twice that in the crude homogenate perhaps because inhibitors of arylsulfatase were excluded through the 75,000 M.W. filter. These results encouraged use of molecular sieving via column chromatographic procedures.

TABLE 13

SOLUBILIZATION OF ARYLSULFATASE ACTIVITY FROM SMG WHOLE HOMOGENATES

Tissue	Percent of Total Activity Solubilized (1)	
	Hypotonic + Freeze-thaw	Triton X-100
SMG	89	88
Parotid gland	84	87
Sublingual gland	75	73
Kidney	89	89
Liver	83	80
Testis	83	83

(1) After treatment the whole homogenate was centrifuged at 100,000 g for 1 hour to obtain the soluble activity (HSS). Results are expressed as (Total Activity in HSS/ Total activity in equivalent amount of original whole homogenate) x 100.

Hypotonic solution was 10 mM Tris buffer, pH 7.4
Triton X-100 was used at a level of 1 % w/v

TABLE 14

SEPARATION USING AMICON MINICON FILTER UNITS

Filter Type and Molecular Weight Retained	nmoles NC formed / mg protein / hour			
	SMG	Parotid	Liver	Kidney
Original samples	886	379	2125	5600
A-75 Filter. M.W. 75,000	1612	689	3371	12188
S-125 Filter M.W. 125,000	0	0	1550	2500

Original material was 5 % w/v HSS in 10 mM Tris buffer, pH 7.4

Gel filtration of SMG-HSS on a small Sephadex G-200 column (0.9 x 30 cm) produced the result shown in Figure 21. Arylsulfatase activity was in the fractions collected soon after the passage of the void volume. There was only one peak of activity. When a larger column (2.6 x 77 cm) was employed, there appeared to be a small peak of enzyme activity, appearing immediately after the void volume, which could correspond to the A form of arylsulfatase. The bulk of the activity appeared somewhat later (Figure 22). The increase in specific activity of both peaks was approximately 6 times that of the original HSS. It is possible that dilution of the enzymes during chromatography leads to inactivation. Unfortunately, both dialysis and lyophilization decrease the apparent enzyme activity (Table 15). Purified arylsulfatase was also more heat labile than arylsulfatase in the original HSS. These results suggest that either a co-factor or a "protective factor" may be lost during the processing of HSS (e.g. dialysis, concentration, etc.) after column chromatography. Protein co-factors have been isolated and shown to enhance the activity of lipid hydrolytic enzymes (38,83).

As expected the DEAE-cellulose column produced the reverse pattern from that of Sephadex G-200 (Figure 23) with the major peak of activity, apparently the B form, emerging first followed by the smaller peak corresponding to the A type. Again the purification factor was about 6 and enzyme instability was observed. Very recently the cold lability of very highly-purified human arylsulfatase B has been reported (2). This phenomenon may be responsible, in part, for the difficulties experienced in the present study with both column chromatography and storage of the resultant samples.

The results of CM-cellulose chromatography of SMG-HSS were very similar to those from DEAE chromatography with only a 5 times increase in the specific activity over the original material. A few trials with an hydroxyapatite column gave good protein separations but the eluting buffer - 50 to 500 mM phosphate - completely inhibited arylsulfatase activity. The necessity of dialyzing all fractions, with the possibility of losses of arylsulfatase activity, was considered too time consuming for the present study.

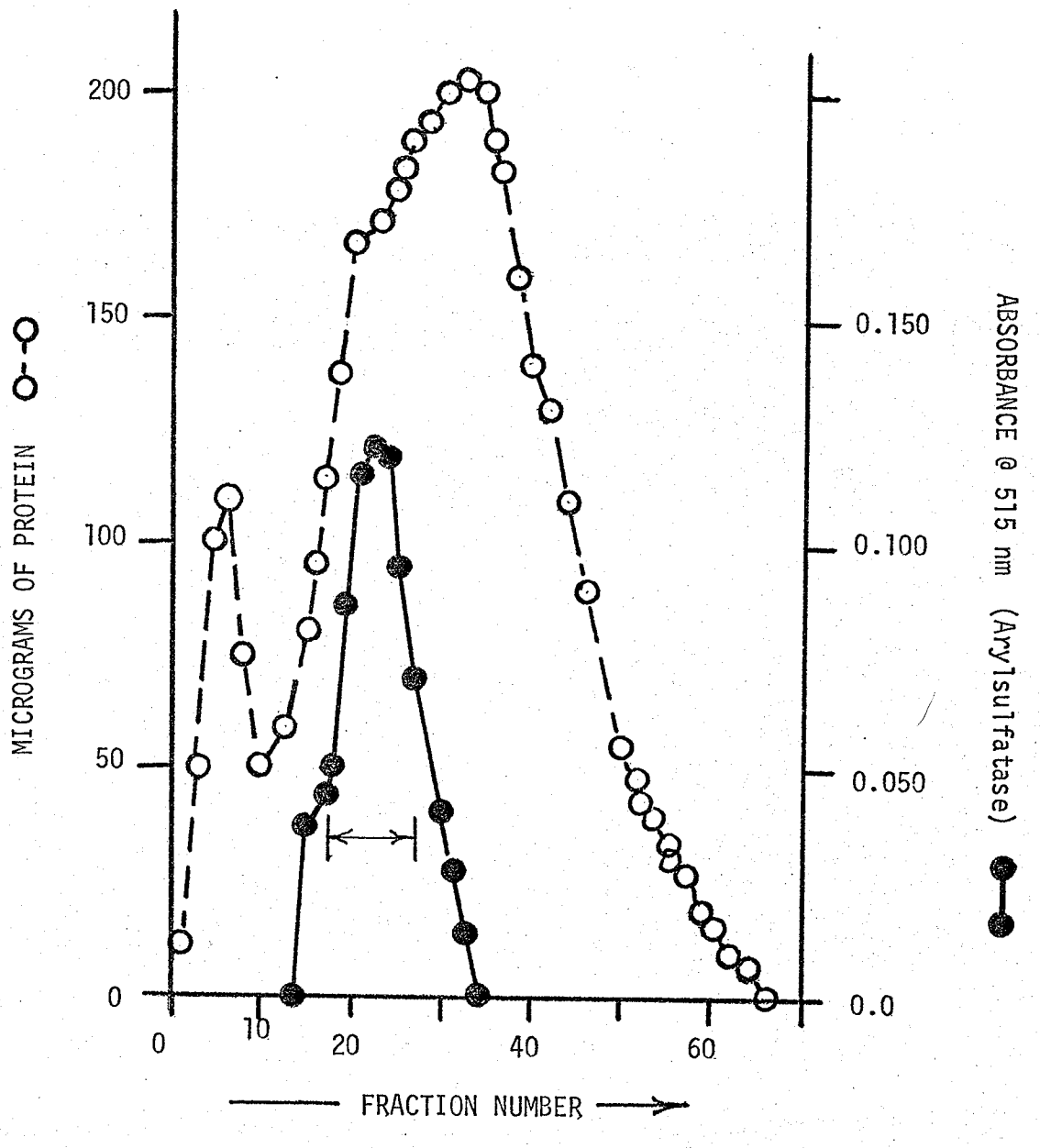


FIGURE 21. SEPHADEX G-200 COLUMN CHROMATOGRAPHY OF SMG-HSS

HSS was eluted from column (0.9 x 30 cm) with 500 mM sodium acetate buffer, pH 5.0.

TABLE 15

STABILITY OF ARYLSULFATASE ACTIVITY DERIVED FROM SEPHADEX G-200 COLUMN CHROMATOGRAPHY

Treatment	Activity (nmoles NC formed / mg protein / hour)
None, original HSS	350
Activity from G-200 peak (HSS)	2000
After dialysis of peak material above	1150
After concentration (Amicon B-15 Filter) and centrifugation of peak material	
Precipitate	350
Soluble supernatant*	2800
*Supernatant heated 1 h @ 41°	2200
*Supernatant heated 1 h @ 51°	1500
*Supernatant lyophilized & then re-suspended	960

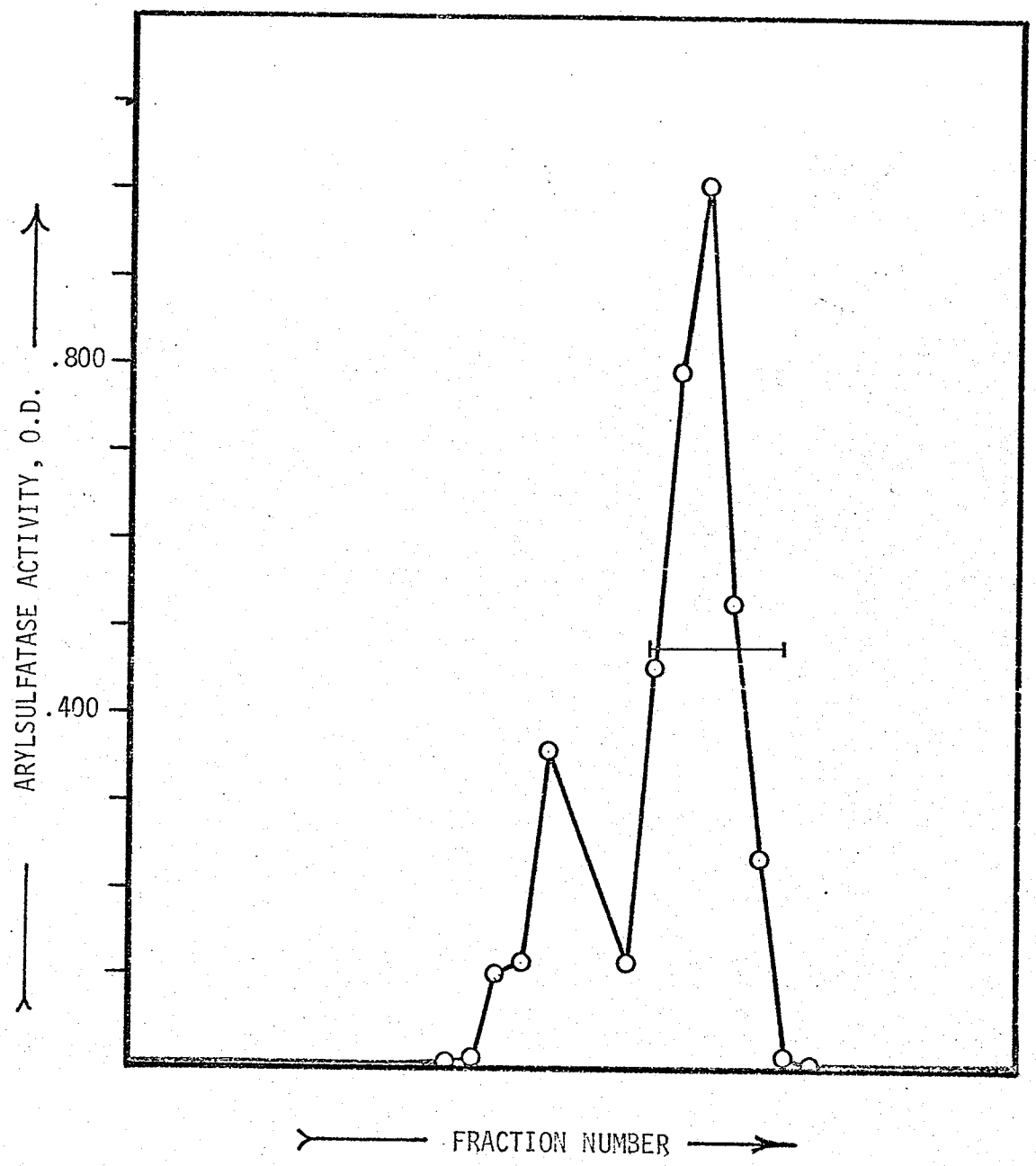


Figure 22. SEPHADEX G-200 COLUMN CHROMATOGRAPHY OF SMG-HSS

HSS was eluted from column (2.6 x 70 cm) with 500 mM sodium acetate buffer, pH 5.0. Approximately 70 fractions of 4 ml. were collected.

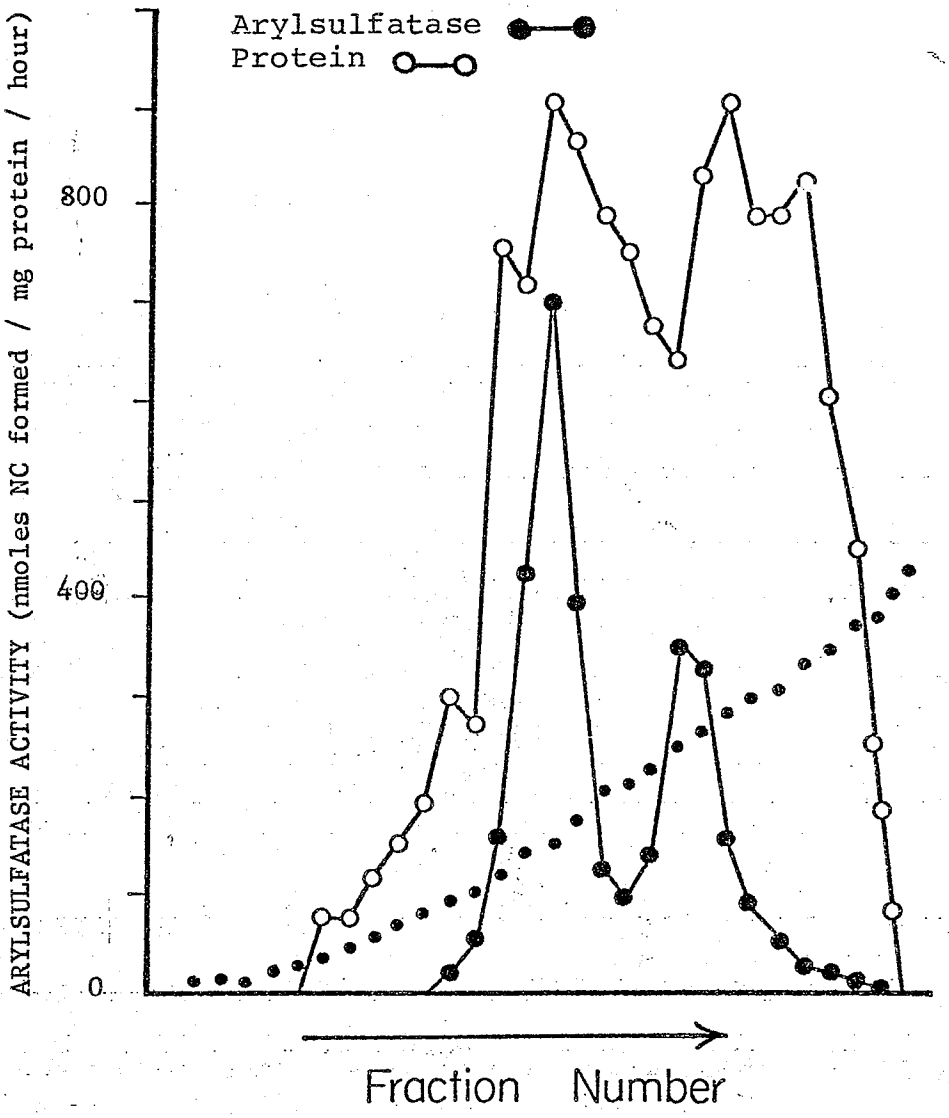


FIGURE 23 DEAE-CELLULOSE COLUMN CHROMATOGRPHY OF SMG-HSS

HSS was eluted from column (2.6 x 77 cm) with increasing concentrations of NaCl (0 to 300 mM) buffered with 10 mM Tris, pH 7.4. Approximately 60 fractions of 6 ml were collected. Change in conductivity is noted by the dotted line.

Acrylamide Gel electrophoretic separation was performed on several arylsulfatase preparations including the active material eluted from both Sephadex and DEAE-cellulose columns. Initial trials with 7.5% gels were somewhat unsatisfactory in the separation of proteins and, as was learned later, arylsulfatase activity remained at, or near, the origin. In 4% gels, run in either acidic or basic buffer systems, the material eluted from Sephadex columns was less contaminated than that from DEAE columns, although both preparations were less complex than the original HSS (Figure 24 & 26).

After several trials, experimental conditions for the location of arylsulfatase activity on acrylamide gels were developed to a reasonable level of accuracy. The exact procedure is described in the Methods section. One obvious drawback to the method, wherein substrate and product are diffusing in and out of the gel simultaneously, is that the precise location of the enzyme can not be made to less than a few mm. Separation of the gel on a gel fractionator might be more satisfactory, although initial attempts with this technique were unsatisfactory due to very high protein values caused, it is thought, by some innate material in the gels and not by protein.

Utilization of the above procedure did result in some interesting observations (Figure 25). Based on the data for pI reported by other workers, it would be logical to assume that the B type arylsulfatase would move much more rapidly than the A form in an acidic buffer system. In all tissues tested, save one, the major band of arylsulfatase activity was associated with a reasonably rapid running moiety (Figure 25). Kidney and brain, which are not shown in this figure, gave similar results. The parotid gland HSS was the exception to the foregoing findings in that its major arylsulfatase activity was associated with the slower running area. The parotid, unlike all other tissues tested, perhaps has a higher level of A type than B. Other unique findings were the presence of three bands of activity in testis and presence of only one area (fast running) in brain myelin (not shown).

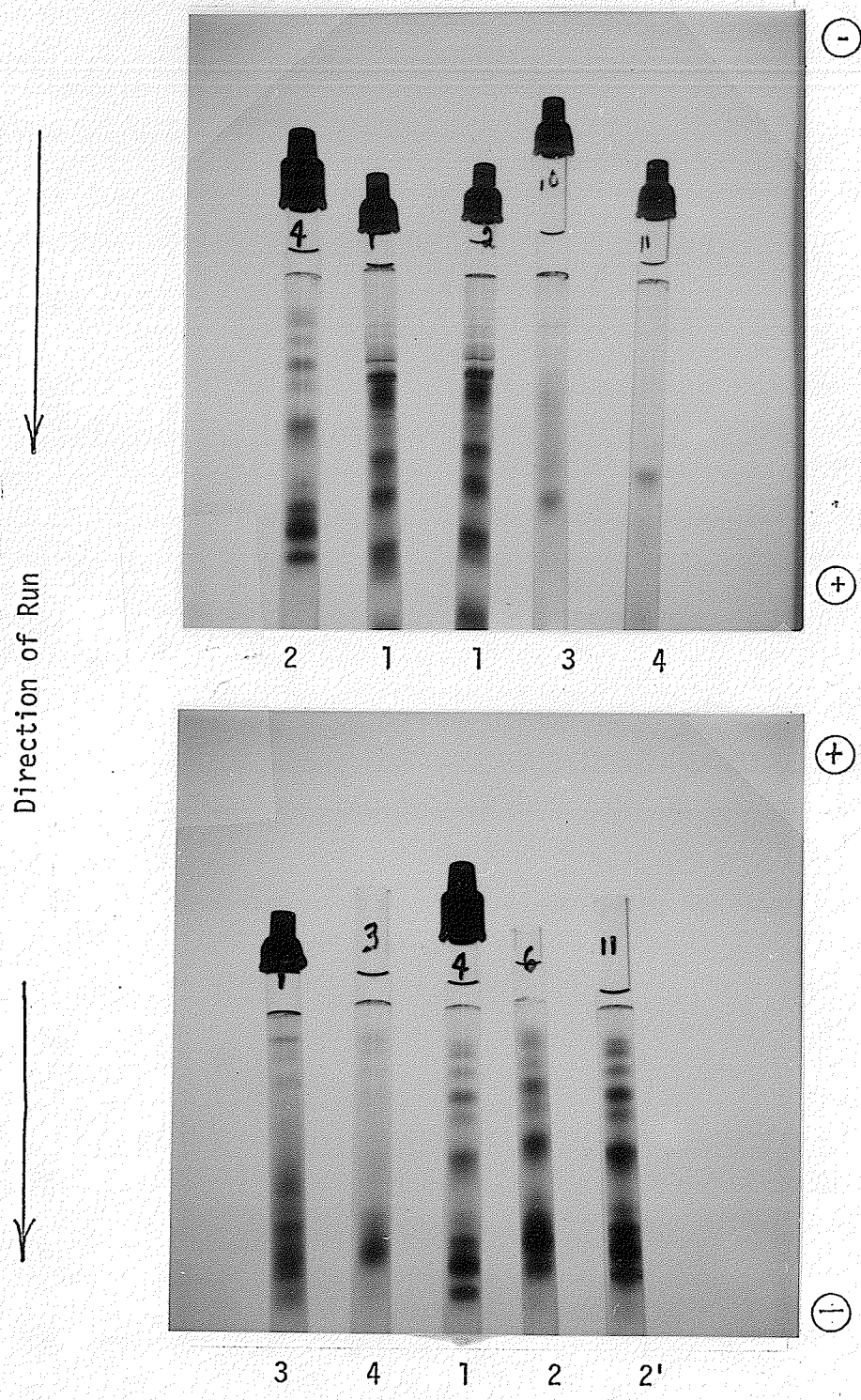


FIGURE 24. ACRYLAMIDE GEL ELECTROPHORESIS OF SMG HSS PREPARATIONS

Numbers refer to (1) HSS, (2) HSS extracted with acetone, (2') HSS extracted with acetone and then ether, (3)DEAE arylsulfatase peak, (4) Sephadex G-200 arylsulfatase peak. Stain: Amido Black (protein).

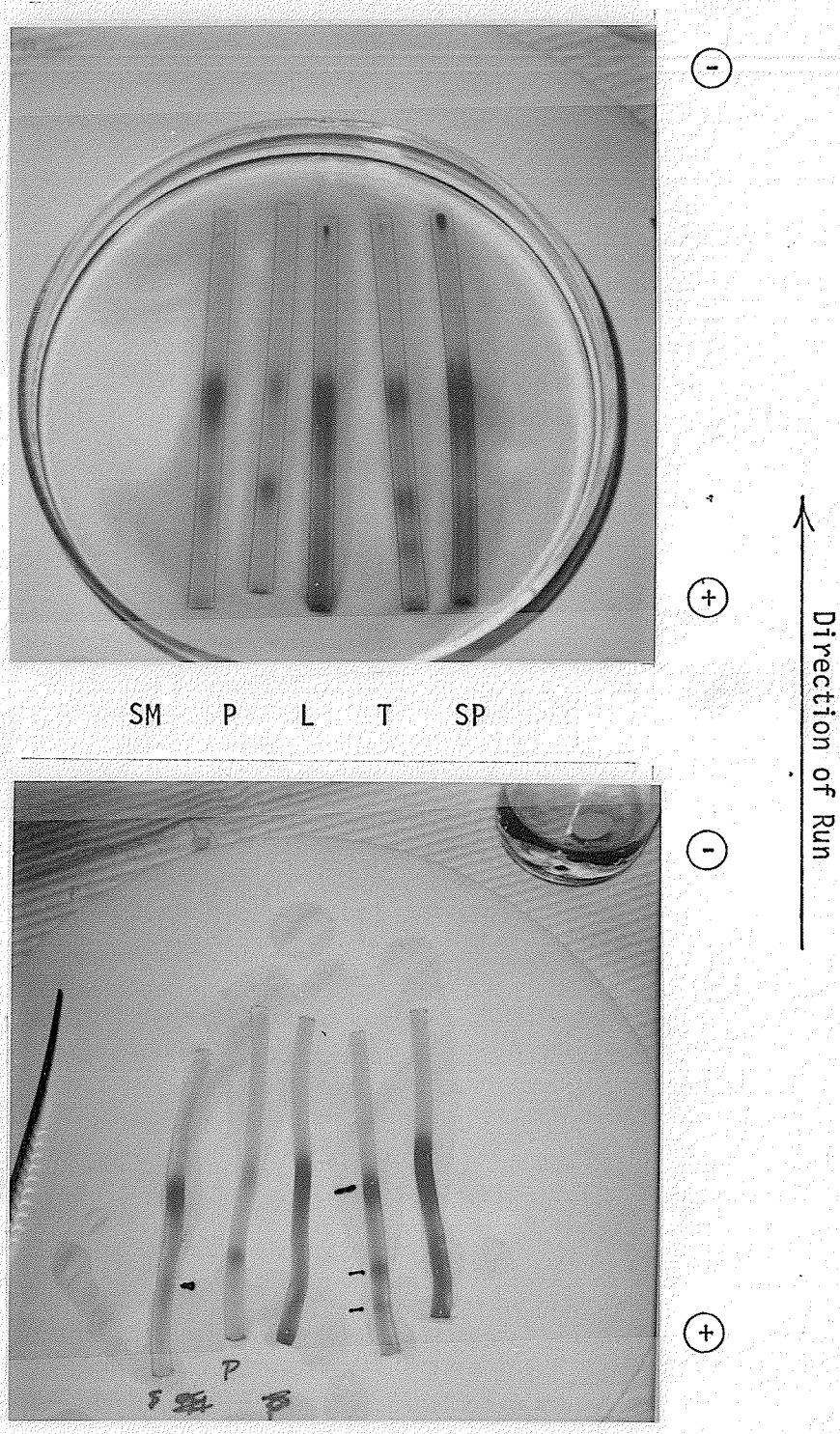
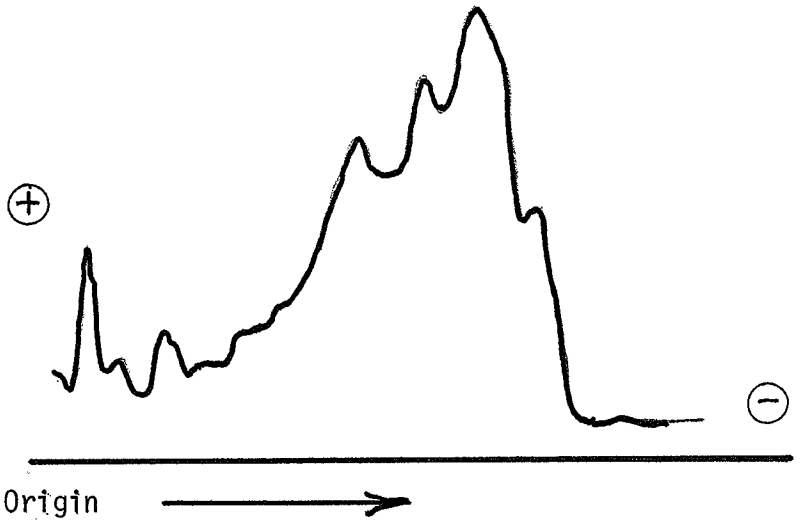


FIGURE 25. ACRYLAMIDE GEL ELECTROPHORESIS OF HSS PREPARATIONS

HSS were run in acidic buffer system and the 4 % gels examined for arylsulfatase activity. Red areas indicate enzymic activity. Letters refer to HSS from SM = submandibular gland, P = parotid gland, L = liver, T = testis and SP = spleen.

(a) Protein from Peak Obtained by DEAE-Cellulose Column Chromatography (Fig. 24 (B), 3.



(b) Protein from Peak Obtained by Sephadex G-200 Column Chromatography (Fig. 24 (B), 4).

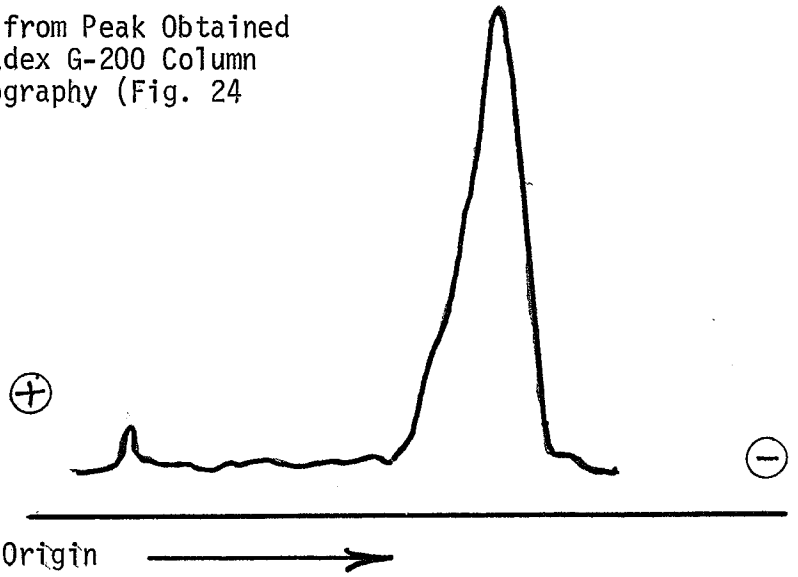


FIGURE 26. PATTERN OF ACRYLAMIDE GEL-SEPARATED PROTEINS

Separation was effected in acidic system using 4 % gels. After fixation, the gels were stained with Amido Black. Scanning was with a Joyce Loebl Chromoscan at 620 nm. The patterns shown are tracings from the original chart recordings.

(c) Protein from HSS (SMG)
after acetone and ethanol
extraction. (Fig. 24 (B) 2')

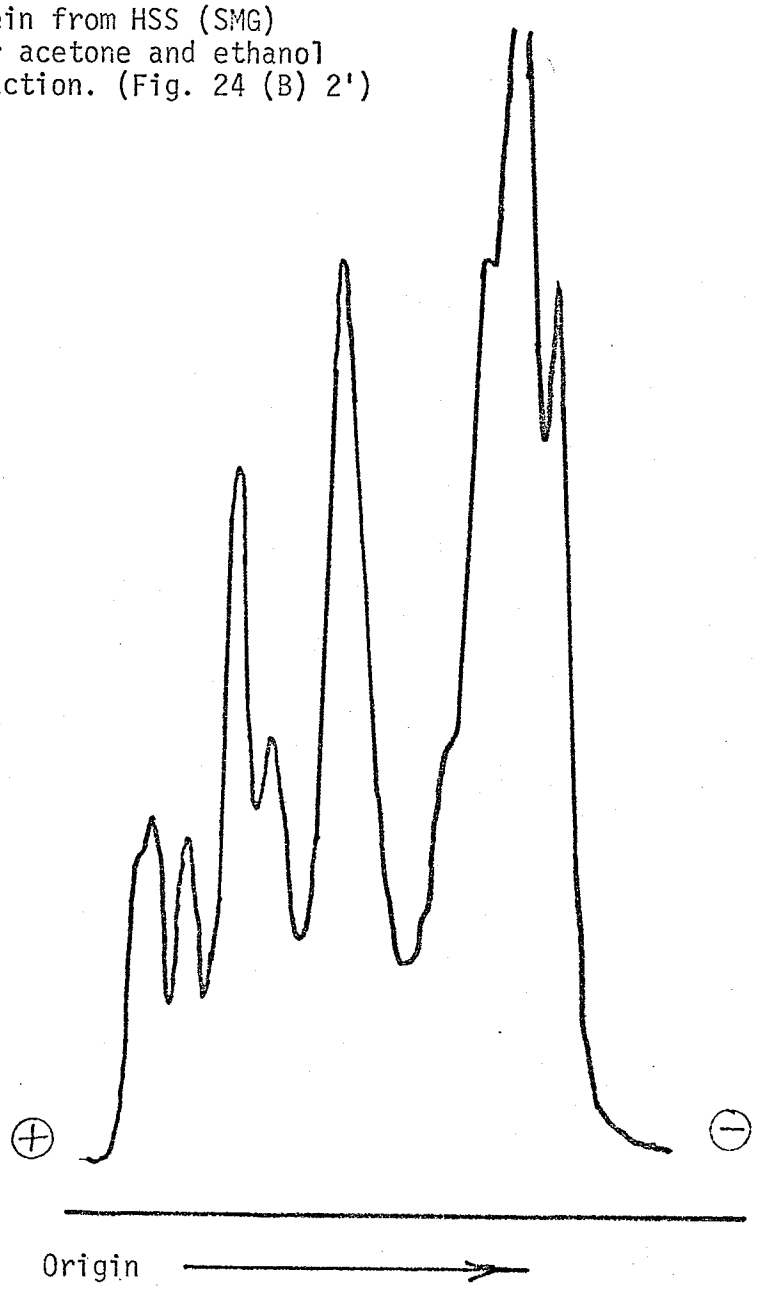


FIGURE 26. (continued)

The foregoing results indicate that preparative acrylamide gel electrophoresis would be an excellent method to isolate pure arylsulfatases after some preliminary "cleaning-up" procedures such as column chromatography. Studies, subsequent to this one, will utilize this technique.

SUMMARY

1. Sephadex G-200 and DEAE-cellulose column chromatography effected a six times purification of arylsulfatase.
2. The partially-purified enzyme preparations were much less stable than the HSS. They lost activity on dialysis and lyophilization while the HSS did not.
3. Acrylamide gel electrophoresis indicated that SMG and parotid gland have different levels of the two major arylsulfatases. SMG appears to have much more "B" type than parotid whereas parotid has more "A".
4. Testis appears to contain at least three forms of arylsulfatase.

REFERENCES

1) Abe, K. and Dawes, C.
Circadian variations in pilocarpine and isoprenaline-induced protein secretion by rat parotid gland.
Archs. oral Biol. 20, 543-546, 1975.

2) Agogbua, S.I. and Wynn, C.H.
Purification and properties of arylsulfatase B of human liver.
Biochem. J. 153, 415-421, 1976.

3) Allen, E. and Roy, A.B.
The sulfatase of ox liver.
XI The isoelectric focusing of a purified preparation of sulfatase B.
Biochim. Biophys. Acta 168, 243-251, 1968.

4) Allison, A.C. and Hartree, E.F.
Lysosomal enzymes in the acrosome and their possible role in fertilization.
J. Reprod. Fertil. 21, 501-515, 1968.

5) Ammon, R. and Ney, K.H.
Arylsulfatase des menschlichen Harns.
Arch. Biochem. Biophys. 69, 178-185, 1957.

6) Austin, J., Armstrong, D. and Shearer, L.
Metachromatic form of diffuse cerebral sclerosis.
Arch. Neurology 13, 593-614, 1965.

7) Austin, J., Balasubramanian, A., Pattabiraman, T., Saraswathi, S., Basu, D. and Bachhawat, B.
A controlled study of the enzymic activities in three human disorders of glycolipid metabolism.
J. Neurochem. 10, 805-816, 1963

8) Austin, J., McAfee, D., Armstrong, D., O'Rourke, M., Shearer, L., and Bachhawat, B.
Abnormal sulfatase activities in two human diseases (metachromatic leukodystrophy and gargoylism).
Biochem. J. 93, 15c-17c, 1964.

9) Balasubramanian, A. and Bachhawat, B.
Purification and properties of an arylsulfatase from human brain.
J. Neurochem. 10, 201-211, 1963

10) Baum, H., Dodgson, K. and Spencer, B.
The anomalous kinetics of arylsulfatase A of human tissue: the anomalies.
Biochem. J. 69, 567-572, 1958.

11) Baum, H. and Dodgson, K.
The anomalous kinetics of arylsulfatase A of human tissue: interpretation of the anomalies.
Biochem. J. 69, 573-582, 1958.

12) Baum, H., Dodgson, K. and Spencer, B.
The assay of arylsulfatases A and B in human urine.
Clin. Chim. Acta 4, 453-455, 1959.

13) Bauman, E.
Pfluger's Arch. ges. Physiol. 12, 63,69; 13, 285, 1876.

14) Beratis, N.G., Turner, B.M., Weiss, R. and Hirschhorn, K.
Arylsulfatase deficiency in Maroteaux-Lamy Syndrome: cellular studies and carrier identification.
Pediat. Res. 9, 475-480, 1975.

15) Bergmann, W.
In Comparative Biochemistry, 3(A), 136-143, 1962.
Eds. Florkin, M. and Mason, H.S. New York: Academic Press.

16) Bleszynski, W.
Purification and separation of four soluble arylsulfatases from ox brain.
Enzymologia 32, 169-181, 1967.

17) Bleszynski, W. and Dzialoszynski, L.
Purification of soluble arylsulfatases from ox brain.
Biochem. J. 97, 360-364, 1965.

18) Bleszynski, W., Leznicki, A. and Lewosz, J.
Kinetic properties of three soluble arylsulfatases from ox brain, homogenous in polyacrylamide gel electrophoresis.
Enzymologia 37, 314-324, 1969.

19) Blosser, J.C. and Wells, W.W.
Enhanced fragility of neural lysosomes from chicks suffering from galactose toxicity.
J. Neurochem. 19, 1539-1547, 1972.

20) Breslow, J.L. and Sloan, H.R.
Purification of arylsulfatase A from human urine.
Biochem. Biophys. Res. Commun. 46(2), 919-925, 1972.

20A) Brophy, P.H. and Vance, D.E.
Elongation of fatty acids by microsomal fractions from the brain of the developing rat.
Biochem. J. 152, 495-501, 1976.

21) Clausen, J. and Asboe-Hansen, G.
Urinary sulfatase activity in mastocytosis.
Clin. Chim. Acta 16(1), 131-134, 1967.

22) Corner, E.D., Leon, J.I. and Bulbrook, R.D.
Steroid sulfatase, arylsulfatase and β-glucuronidase in marine invertebrates.
J. Mar. Biol. Ass., U.K. 39, 51-61, 1960.

23) Cornet, D. and Jangoux, M.
Arylsulfatases and β-glucuronidase in the digestive system of some echinoderms.
Comp. Biochem. Physiol. 47B, 45-52, 1974.

24) Dawes, C.
Circadian rhythms in human salivary flow rate and composition.
J. Physiol. 220, 529-545, 1972.

25) Derrien, M.
Bull. Soc. Chim. Biol. (Paris) 9, 110, 1911.

26) Dodgson, K., Lewis, J. and Spencer, B.
The arylsulfatase and β-glucuronidase in marine invertebrates.
Biochem. J. 55, 253-259, 1953.

27) Dodgson, K.S. and Spencer, B.
The occurrence of arylsulfatase A and B in human urine.
Clin. Chim. Acta 1, 478-480, 1956.

28) Dodgson, K. and Spencer, B.
Assay of sulfatases.
Meth. Biochem. Anal. 4, 211-255, 1957.

29) Dodgson, K.S., Spencer, B. and Thomas, J.
Studies on sulfatases. 2. The assay of the arylsulfatase activity of rat tissues.
Biochem. J. 53, 452-457, 1953.

30) Dodgson, K.S., Spencer, B. and Wynn, C.H.
Studies on sulfatases. The arylsulfatases of human tissues.
Biochem. J. 62, 500-507, 1956.

31) Dodgson, K.S. and Wynn, C.H.
Studies on sulfatases. 19. The purification and properties of arylsulfatase B of human liver.
Biochem. J. 68, 387-395, 1958.

32) Dolly, J.O., Dodgson, K.S. and Rose, F.A.
Studies on oestrogen sulfatase and arylsulfatase C activities of rat liver.
Biochem. J. 128, 337-345, 1972.

33) Don, M.M., Masters, C.J. and Windsor, D.J.
Further evidence for the concept of bovine plasma arylesterase as a lipoprotein.
Biochem. J. 151, 625-630, 1975.

34) Eto, Y., Kiguchi, K. and Aoki, K.
Studies with soluble arylsulfatase from rat brain.
Transactions of the American Society for Neurochemistry, Vol. 7, No. 1, Abstr. No. 202, 1976.

35) Eto, Y., Rampini, S., Wiesmann, U. and Herschkowitz, N.
Enzymic studies of sulfatases in tissues of the normal human and in metachromatic leukodystrophy with multiple sulfatase deficiencies: arylsulfatases A, B and C, cerebroside sulfatase, psychosine sulfatase and steroid sulfatases.
J. Neurochem. 23, 1161-1170, 1974.

36) Eto, Y., Wiesmann, U. and Herschkowitz, N.
Sulfagalactosylsphingosine sulfatase.
Journal of Biol. Chem. 249(15), 4955-4960, 1974.

37) Farooqui, A.A. and Bachhawat, B.K.
Purification and properties of arylsulfatase A from chicken brain.
Biochem. J. 126, 1025-1033, 1972.

38) Fischer, G. and Jatzkewitz, H.
Hoppe-Seylar's Z. Physiol. Chem. Bd. 356, S. 605-613, Mai, 1975.

39) Fluharty, A.L., Stevens, R.L., Killgrove, A.R. and Kihara, H.
Minor arylsulfatases of human brain.
Transactions of the American Society for Neurochemistry, Vol. 7, No. 1, Abstr. 203, 1976.

40) Fluharty, A.L., Stevens, R.L., Miller, R.T. and Kihara, H.
Sulfoglycerogalactolipid from rat testis: a substrate for pure human arylesterase A.
Biochem. Biophys. Res. Comm. 61, 348-354, 1974.

41) Fluharty, A.L., Stevens, R.L., Miller, R.T. and Kihara, H.
Ascorbic acid-2-sulfate sulfhohydrolase activity of human arylsulfatase A.
Biochim. Biophys. Acta 429, 508-516, 1976.

42) Flynn, T.G.
Ph.D. Thesis, Univ. of Wales, 1966.

43) Fromageot, C.
Adv. Enzymol. 7, 369, 1947.

44) Fromageot, C.
In The Enzymes, Vol. 1, p. 517. Ed. by Sumner, J.B. and Myrback, K. New York: Academic Press, 1950.

45) Fromageot, C.
Ergeln. Enzymforsch. 7, 50, 1958.

46) Fromageot, C., Chatagner, F. and Bergeret, B.
La formation d'alanine par desulfination enzymatic de l'acide L-cysteine-sulfinique.
Biochim. Biophys. Acta 2, 294-301, 1948.

47) Fromageot, C., Wookey, E. and Chaix, P.
C.V. hebd. Seanc. Acad. Sci., Paris 209, 1019, 1939.

48) Fushimi, H. and Tarui, S.
β-glycosidases and diabetic microangiopathy.
I. Decreases of β-glycosidase activities in diabetic rat kidney.
J. Biochem. 79, 265-270, 1976.

49) Fushimi, H. and Tarui, S.
β-glycosidases and diabetic microangiopathy.
II. An insulin-dependent isozyme of β-N-acetylglucosaminidase.
J. Biochem. 79, 271-275, 1976.

50) Gniot-Szulzycka, J. and Dzialoszynski, L.M.
Some properties of human placental soluble arylsulfatases.
Acta Biochim. Pol. 13, 171-176, 1966.

51) Gniot-Szulzycka, J. and Dzialoszynski, L.M.
Separation and purification of soluble arylsulfatases of human placenta.
Acta Biochim. Pol. 14(4), 477-478, 1967.

52) Goldstone, A., Konecny, P. and Koenig, H.
Lysosomal hydrolases: conversion of acidic to basic forms by neuraminidase.
Fed. Europ. Biochem. Soc. 13, 68, 1971.

53) Graham, E.R. and Roy, A.B.
The sulfatase of ox liver.
XVII. Sulfatase A as a glycoprotein.
Biochim. Biophys. Acta 329, 88-92, 1973.

54) Greene, H.L., Hug, G. and Schubert, W.K.
Metachromatic leukodystrophy: treatment with arylsulfatase A.
Arch. Neurol. (Chicago) 20, 147-153, 1969.

55) Harada, T.
Bull-agric. chem. Soc. Japan 21, 267, 1957.

56) Harada, T.
Bull-agric. chem. Soc. Japan 23, 222, 1959.

57) Harada, T.
Mem. Inst. scient. ind. Res. Osaka Univ. 20, 111, 1963.

58) Harada, T.
Bull-agric. chem. Soc. Japan 20, 110, 1956.

59) Harada, T. and Spencer, B.
The effect of sulfate assimilation on the induction of arylsulfatase synthesis in fungi.
Biochem. J. 82, 148-156, 1962.

60) Harada, T. and Spencer, B.
Repression and induction of arylsulfatase synthesis in aerobacter aerogenes.
Biochem. J. 93, 373-378, 1964.

61) Harinath, B. and Robins, E.
Arylsulfatases in human brain: separation, purification and certain properties of the two soluble arylsulfatases.
J. Neurochem. 18, 245-257, 1971.

62) Hiltz, D.F. and Lightle, T.E.
β-glucuronidase and arylsulfatase in some marine invertebrates of the Canadian Atlantic Coast.
J. Fish Res. Bd. Can. 27, 1898-1900, 1970.

63) Ho, M.W. and O'Brien, J.S.
Gaucher's disease: deficiency of acid β-glucosidase and reconstitution of enzyme activity in vitro.
Proc. Nat. Acad. Sci. U.S.A. 68, 2810-2813, 1971.

64) Ho, M.W., O'Brien, J.S., Radin, N.S. and Erickson, J.S.
Glucocerebrosidase: reconstitution of activity from macromolecular components.
Biochem. J. 131, 173-176, 1973.

65) Hook, G., Dodgson, K., Rose, F. and Worwood, M.
Relative distribution of arylsulfatase A and B in rat liver parenchymal and other cells.
Biochem. J. 134, 191-195, 1973.

66) Hopkins, H.A., Bonney, R.J., Walker, P.R., Yager, J.D. and Potter, V.R.
Advances in Enzyme Regulation 11, 169-191, 1974.
Ed. George Weber, Pergamon Press.

67) Horpacsy, G., Nagy, S. and Barankay, T.
Decrease of arylsulfatase A activity in plasma in hemorrhagic shock.
Experientia 15(4), 372-373, 1974.

68) Hors-Cayla, M.C., Henertz, S., Picard, J.Y. and Frezal, J.
Regulation in man x man somatic cell hybrids: possible negative control of arylsulfatase A.
Ann. Hum. Genet., London 38, 57-62, 1974.

69) Huggins, C. and Smith, D.R.
Chromogenic substrates.
III. p-nitrophenyl sulfate as a substrate for the assay of phenolsulfatase activity.
J. Biol. Chem. 170, 391-398, 1947.

70) Jatzkewitz, H. and Mehl, E.
Cerebroside-sulfatase and arylsulfatase A deficiency in metachromatic leukodystrophy (MLD).
J. Neurochem. 16, 19-28, 1969.

71) Jenkins, G.N.
The physiology of the mouth.
Blackwell Scientific Publications, Oxford, 1966, p. 311.

72) Jerfy, A. and Roy, A.
The sulfatase of ox liver.
XII. The effect of tyrosine and histidine reagents on the activity of sulfatase A.
Biochim. Biophys. Acta 175, 355-364, 1969.

73) Jerfy, A. and Roy, A.B.
The sulfatase of ox liver.
XVI. A comparison of the arylsulfatase and cerebroside sulfatase activities of sulfatase A.
Biochim. Biophys. Acta 293, 178-190, 1973.

74) Kaback, M. and Howell, R.
Infantile metachromatic leukodystrophy: heterozygote detection in skin fibroblasts and possible applications to intrauterine diagnosis.
New Eng. J. Med. 282, 1336-1340, 1970.

75) Karlsson, K.A., Samuelsson, B.E. and Steen, G.O.
The lipid composition and $Na^+ - K^+$ - dependent adenosine-triphosphatase activity of the salt (nasal) gland of eider duck and herring gull. A role for sulfatides in sodium-ion transport.
Eur. J. Biochem. 46, 243-258, 1974.

76) Kitchen, B.J., Masters, C.J. and Windsor, D.J.
Effects of lipid removal on the molecular size and kinetic properties of bovine plasma arylesterase.
Biochem. J. 135, 93-99, 1973.

77) Kleinberg, I.
Effect of varying sediment and glucose concentrations on the pH and acid production in human salivary sediment mixtures.
Archs. Oral Biol. 12, 1457-1473, 1967.

78) Koudstall, J.
The histochemical demonstration of arylsulfatase and acid hydrolases in carcinoma of the colon and the breast.
Europ. J. Cancer 11, 479-482, 1975.

79) Larmes, M. and Scheinen, A.
Studies on dog saliva.
III. Observations on enzymes acting on ester bonds and glycosyl compounds.
Acta Odont. Scand. 30, 357-370, 1972.

80) Lee, G.D. and Van Etten, R.L.
Purification and properties of a homogenous arylsulfatase A from rabbit liver.
Arch. Biochem. Biophys. 166, 280-294, 1975.

81) Leon, Y.A., Bulbrook, R.D. and Corner, E.D.
Steroid sulfatase, arylsulfatase and β-glucuronidase in the mollusca.
Biochem. J. 75, 612-617, 1960.

82) Leroy, J.G., Van Elsen, A., Dumon, J.E. and Radermecker, J.
Deficiency of arylsulfatase A in juvenile metachromatic leukodystrophy: fibroblast studies.
Monogr. Hum. Genet. 6, 148-149, 1972.

83) Li, S.C. and Li, Y.T.
An activator stimulating the enzymic hydrolysis of sphingoglycolipids.
J. Biol. Chem. 251, 1159-1163, 1976.

84) Li, S.C., Wan, C.C., Mazzotta, M.Y. and Li, Y.T.
Requirement of an activator for the hydrolysis of sphingoglycolipids by glycosidases of human liver.
Carbohyd. Res. 34, 189-193, 1974.

85) Li, Y.T., Mazzotta, M.Y., Wan, C.C., Orth, R. and Li, S.C.
Hydrolysis of Tay-Sachs ganglioside by beta-hexosaminidase A of human liver and urine.
J. Biol. Chem. 21, 7512-7515, 1973.

86) Lowry, O., Rosebrough, N., Farr, A and Randall, R.
Protein measurement with the folin phenol reagent.
J. Biol. Chem. 193, 265-275, 1951.

87) Makinen, K.K.
Study of oral enzymes.
IV. Fractionation and characterization of various hydrolytic enzymes in human saliva.
Acta Odont. Scand. 24, 709-721, 1966.

88) Manowitz, P. and Shapiro, S.
Ascorbate-2-sulfate levels in metachromatic leukodystrophy patients.
Transactions of the American Society for Neurochemistry, Vol. 7, No. 1, Abstr. No. 262, 1976.

89) Mayo, J.W. and Carlson, D.M.
Protein composition of human submandibular secretions.
Arch. Biochem. Biophys. 161, 134-145, 1974.

90) Mehl, E. and Jatzkewitz, H.
Eine Cerebrosidsulfatase aus schweineniere.
Z. Physiol. Chem. 339, 260-276, 1964.

91) Mehl, E. and Jatzkewitz, H.
Cerebroside 3-sulfate as a physiological substrate of arylsulfatase A.
Biochim. Biophys. Acta 151, 619-627, 1968.

92) Milsom, D.W., Rose, F.A. and Dodgson, K.S.
The specific assay of arylsulfatase C, a rat liver microsomal marker enzyme.
Biochem. J. 128, 331-336, 1972.

93) Mraz, W. and Jatzkewitz, H.
Cerebroside sulfatase activity of arylsulfatases from various invertebrates.
Hoppe-Seyler's Z. Physiol. Chem. Bd. 355, S. 33-44, 1974.

94) Nichol, L.W. and Roy, A.B.
The sulfatase of ox liver.
VIII. The sedimentation of purified arylsulfatase A.
The J. of Biochem. 55(6), 643-651, 1964.

95) Nichol, L.W. and Roy, A.B.
The sulfatase of ox liver.
IX. The polymerization of sulfatase A.
Biochemistry 4, 386-395, 1965.

96) Nichol, L.W. and Roy, A.B.
The sulfatase of ox liver.
X. Some observations on the intermolecular bonding in sulfatase A.
Biochemistry 5, 1379-1388, 1966.

97) Nichol, L.W. and Roy, A.B.
The sulfatase of ox liver.
XV. Changes in the properties of sulfatase A in the presence of substrate.
Biochim. Biophys. Acta 242, 141-151, 1971.

98) Percy, A.K. and Brady, R.O.
Metachromatic leukodystrophy: diagnosis with samples of venous blood.
Science 161, 594-595, 1968.

99) Perumal, A.S. and Robins, E.
Regional and subcellular distribution of aryl- and steroid sulfatases in brain.
Brain Research 59, 349-358, 1973.

100) Pilz, H.
Late adult metachromatic leukodystrophy.
Arylsulfatase A activity of leukocytes in two families.
Arch. Neurol. 27, 87-90, 1972.

101) Polasek, J. and Rotrekl, B.
Arylsulfatases of human blood platelets.
Nature 214, 187-188, 1967.

102) Porter, M.T., Fluharty, A.L., De La Flora, S.D. and Kihara, H.
Cerebroside sulfatase determination in cultured human fibroblasts.
Biochim. Biophys. Acta 258, 769-778, 1972.

103) Porter, M., Fluharty, A. and Kihara, H.
Metachromatic leukodystrophy: arylsulfatase A deficiency in skin fibroblast cultures.
Proc. Nat. Acad. Sci. 62, 887-891, 1969.

104) Porter, M.T., Fluharty, A.L. and Kihara, H.
Correction of abnormal cerebroside sulfate metabolism in cultured metachromatic leukodystrophy fibroblasts.
Science 172, 1263-1265, 1971 (June 18).

105) Pritchard, E.T.
Investigation of lipids and lipid metabolism in submandibular salivary gland of the rat.
Archs. Oral Biol. 12, 1445-1456, 1967.

106) Pritchard, E.T.
Sulfolipid labelling from ^{35}S - sulfate by various rat tissue in vitro.
Archs. Oral Biol. 18, 1337-1340, 1973.

107) Pritchard, E.T.
Variations in sulfolipid metabolism during early growth in some rat tissues.
Int. J. Biochem. 6, 353-359, 1975.

108) Pritchard, E.T. and Rusen, D.R.
In vivo incorporation of ^{35}S-sulfate into submandibular gland sulfolipids in the rat.
Archs. Oral Biol. 17, 1619-1621, 1972.

109) Rammler, D.H., Grado, C., Fowler, L.R.
Sulfur metabolism of Aerobacter aerogenes I.
A repressible sulfatase.
Biochemistry 3, 224-230, 1964.

110) Rinderknecht, H., Geokas, M., Carmack, C. and Haverback, B.
The determination of arylsulfatases in biological fluids.
Clinica Chimica Acta 29, 481-491, 1970.

111) Robinson, D., Spencer, B. and Williams, R.T.
Spectrophotometric determination of phenolsulfatase (arylsulfatase).
Biochem. J. 48, XXVII, 1951.

112) Roche, J., Michel, R., Closon, J. and Michel, O.
Hepatic sulfate conjugation of 3,5,3'-triiodo-L-thyronine and the presence of a sulfuric ester of this hormone in bile and plasma.
Biochim. Biophys. Acta 33, 461-469, 1959.

113) Rotrekl, B. and Polasek, J.
Nitrocatecholsulfatase in human blood platelets.
Acta Haemat. (Basel) 39, 129-138, 1968.

114) Roy, A.B.
The sulfatase of ox liver.
I. The complex nature of the enzyme.
Biochem. J. 53, 12-15, 1953.

115) Roy, A.B.
The sulfatase of ox liver.
II. The purification and properties of sulfatase A.
Biochem. J. 55, 653-661, 1953.

116) Roy, A.B.
The intracellular distribution of sulfatase.
Biochim. Biophys. Acta 14, 149, 1954.

117) Roy, A.B.
The sulfatase of ox liver.
IV. A note on the inhibition of sulfatases A and B.
Biochem. J. 59, 812, 1955.

118) Roy, A.B.
The sulfatase of ox liver.
V. Sulfatase C.
Biochem. J. 64, 651-657, 1956.

119) Roy, A.B.
Comparative studies on the liver sulfatases.
Biochem. J. 68, 519-528, 1958.

120) Roy, A.B.
The sulfatase of ox liver.
XIII. The action of carbonyl reagents on sulfatase A.
Biochim. Biophys. Acta 198, 76-81, 1970.

121) Roy, A.B. and Trudinger, P.
The biochemistry of inorganic compounds of sulfur.
1970. Cambridge University Press, London, p. 135.

122) Roy, A.B.
The Enzymes. Ed. P.B. Boyer 5, 1, 1971. 3rd Edition
Academic Press.

123) Roy, A.B.
L-ascorbic acid 2 sulfate. A substrate for mammalian arylsulfatases.
Biochim. Biophys. Acta 337, 356-363, 1975.

124) Sandham, H.J. and Kleinberg, I.
Effect of glucose concentration on carbon dioxide production in a salivary sediment system.
Archs. Oral Biol. 15, 1285-1302, 1970.

125) Schneyer, L.H., Pigman, W., Hanahan, L. and Gilmore, R.W.
Rate of flow of human parotid, sublingual and submaxillary secretions during sleep.
J. Dent Res. 35, 109-114, 1956.

126) Schroeder, H.R., Lawler, J.R. and Wells, W.W.
Effect of dietary galactose on lysosomal enzymes in the chick.
J. Nutrition 104, 943-951, 1974.

127) Scott, J.M. and Spencer, B.
Sulfate transport in Aspergillus nidulans.
Biochem. J. 96, 78, 1965.

128) Seiguer, A. and Castro, A.
Electron microscopic demonstration of arylsulfatase activity during acrosome formation in the rat.
Biology of Reproduction 7, 31-42, 1972.

129) Sephadex-gel filtration in theory and practice.
Pharmacia Fine Chemicals, Uppsala, Sweden.

130) Snyder, R.A. and Brady, R.O.
The use of white cells as a source of diagnostic material for lipid storage diseases.
Clin. Chim. Acta 25, 331-338, 1969.

131) Spencer, B.
Enzymic cleavage of aryl hydrogen sulfatases in the presence of H_2O.
Biochem. J. 69, 155-159, 1958.

132) Steiner, D.F.
Insulin and the regulation of hepatic biosynthetic activity.
Vitamins and Hormones 24, 1-61, 1966.

133) Stevens, R.L.
Minor anionic arylsulfatases in cultured human fibroblasts.
Biochim. Biophys. Acta 370, 249-256, 1974.

134) Stevens, R.L., Fluharty, A.L., Skokut, M.H. and Kihara, H.
Purification and properties of arylsulfatase A from human urine.
J. Biol. Chem. 250(7), 2495-2501, 1975.

135) Stinshoff, K.
Kinetic properties of the arylsulfatase A from human kidneys.
Biochim. Biophys. Acta 276, 475-490, 1972.

136) Stinshoff, K. and Jatzkewitz, H.
Comparison of the cerebroside sulfatase and the arylsulfatase activity of human sulfatase A in the absence of inhibitors.
Biochim. Biophys. Acta 337, 126-138, 1975.

137) Sunshine, G.H., Williams, D.J. and Rabin, B.R.
Sex specific role in binding of ribosomes to endoplasmic reticulum.
Nature New Biol. 230(13), 133-136, 1971.

138) Weinstein, E. and Mandel, I.D.
Facts and artifacts in acid phosphatase and esterase localization in disc electrophoresis of human saliva.
Archs. Oral Biol. 14, 1-6, 1969.

139) Wiesmann, U.N., Rossi, E.E. and Herschkowitz, N.N.
Treatment of metachromatic leukodystrophy in fibroblasts by enzyme replacement.
N. Eng. J. Med. 284(12), 672-673, 1971 (Mar. 25).

140) Winters, R., Delluva, H., Deyrup, I. and Davies, R.
Accumulation of sulfate by mitochondria of rat kidney cortex.
J. Gen. Physiol. 45, 757-775, 1962.

141) Worwood, M., Dodgson, K., Hook, G. and Rose, F.
Problems associated with the assay of arylsulfatases A and B of rat tissues.
Biochem. J. 134, 183-190, 1973.

142) Yamamoto, L. and Segel, I.
The inorganic sulfate transport system of Penicillium chrysogenum.
Archs. Biochem. Biophys. 114, 523-538, 1966.

143) Yamato, K., Handa, S. and Yamakawa, T.
Purification of arylsulfatase A from boar testes and its activities toward seminolipid and sulfatide.
J. Biochem. 75, 1241-1247, 1974.

144) Yang, C.H., Srivastava, P.N. and Williams, W.
Purification and properties of arylsulfatases from rabbit sperm acrosomes.
Proc. Soc. Exp. Biol. 145, 721-725, 1974.

145) Yang, R.S.H., Pelliccia, J.G. and Wikinson, C.F.
Age-dependent arylsulfatase and sulfotransferase activities in the southern armyworm: a possible insect endocrine regulatory mechanism?
Biochem. J. 136, 817-820, 1973.