"SOME ASPECTS OF METABOLISM IN PERIPLANETA AMERICANA"

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ABSTRACT

"Some aspects of metabolism in Periplaneta americana."

The structure of insect cuticle has been reviewed briefly and an account given of the processes by which hardening (tanning) occurs after the moult. The sclerotin wall of the cockroach ootheca is formed by the intermingling of the secretions of the left and right colleterial glands. This system, because of its comparative convenience, is studied with a view to shedding light on the sclerotin formation in the cuticle.

With reference to their role in sclerotization, the properties of insect blood and cuticle oxidases are reviewed and comparisons made with the animal and plant enzyme groups they most resemble - tyrosinases and laccases. The function of copper in these oxidases is discussed.

A method was developed for determining the protein concentrations, in the presence of phenolic compounds, of the diluted secretion from the left colleterial glands of Periplaneta. This involved digestion (6N - HCl, 6 hours) of the protein followed by estimation with ninhydrin of the total amino acids in the hydrolysate.

Investigation of the properties of the copper-containing

(0.1 - 0.2%) phenoloxidase of the left colleterial gland showed that the enzyme is specific towards diphenols (especially ortho-) and is therefore not a tyrosinase. In the majority of its properties the oxidase resembles a laccase, although it is more specific than the plant enzymes. Phenolic substrates bearing an amino group in the side chain are not active - a property which clearly distinguishes the cockroach phenoloxidase from the blood tyrosinase of insects. Towards certain substrates (catechol, hydroquinone, homoprotocatechuic acid) activity is unexpectedly stable to boiling while towards others (protocatechuic acid and its aldehyde) it is destroyed. Possibly non-enzymic, as well as enzymic, activity exists.

The effect of some substances (detergents, dopa, catechol, tyrosine, ascorbic acid) on the activity of the phenoloxidase towards protocatechuic acid (3 μmoles) is measured. The stimulation caused by ascorbic acid (0.15 μmoles) is related to the possible requirement of cuprous ions for enzyme activity.

The phenoloxidase activity which resides in a particulate fraction of the gland secretion, is (unlike other copper oxidases) not inhibited by metal chelating agents, such as phenylthiourea, diethyldithiocarbamate, ethylenediaminetetracetic acid and 4-chlororesorcinol, in the presence of sodium phosphate buffer (0.1M -, pH 6.8). Cyanide and borate are effective inhibitors, however.

Varied attempts to purify the particulate enzyme and thus to fractionate it from the prosclerotin or structural protein met with little success. The specific activities (Q_{O_2}'s) of the supernatants obtained after high speed centrifugation (100,000 - 182,000 x g) are approximately five to ten times higher than those (3.93±1.15(10)) of untreated enzyme preparations. An examination of this "purification" led to the conclusion that the enzyme activity appeared to be rather dependent on the state of aggregation of the protein particles. Further evidence of this effect was inferred from the stimulating action of anionic (deoxycholate) and neutral ("Tween") detergents (added prior to buffering) upon phenoloxidase activity.

Prior to the institution, as a regular procedure, of dialysis (0.01 M-NaCl) of the colleterial gland secretion (to remove endogenous phenolic compounds), a long induction period (ca. 20-60 mins.) before commencement of oxygen uptake by enzyme and substrate was frequently observed. This long period was minimized by the addition of right gland homogenate or ascorbic acid.

In section II, the phenolic compounds found in insect cuticle and in cockroach colleterial glands were examined with reference to their biosynthesis and to their role in sclerotization. Protocatechuic acid, its 4-o-β-D-glucoside and p-tyrosine were identified (chromatographically and spectrophotometrically) in homogenates of left collet-

erial gland. After aerobic incubation (37°C) of these homogenates substances strongly resembling catechol and tyramine were separated by paper chromatography and identified by their u. v. spectral properties after elution. Protocatechuic acid and p-tyrosine, when added (with and without right gland preparation) to homogenates of left glands, increased the production of catechol and tyramine. respectively. From densitometric measurements of ferric chloride/ferricyanide sprayed paper chromatograms the aerobic conversion by left gland homogenate of p-tyrosine (1 μmole added) into tyramine (0.62 μmoles) was demonstrated. The amine was also separated by absorption on Amberlite-IRC (H^+). The decarboxylase systems responsible for catechol and tyramine production, whether induced or not, were heat labile. There is some manometric evidence that the former ensyme is located in right gland cells while the latter is present in the larger gland tissue. These findings are made uncertain by the retardation of the reactions in presence of inorganic buffers (and an anaerobic atmosphere) and by extraneous gas evolution particularly during incubation of mixed homogenates of both glands. In the light of some evidence, the possibility that tyramine might be ensymically oxidised by homogenates of left gland is weighed. The significance of such a reaction, together with the decarboxylation of p-tyrosine and protocatechuic acid, is discussed with regard to the biosynthesis of tanning quinones from the p-tyrosine of the insect blood.

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Abbreviations

Lh/Lh*	- left gland homogenate/ -do- boiled
Le	- left gland 'exudate'
Le* or Le†	- boiled gland 'exudate' (5 or 10 minutes)
T.C.A.	- trichloroacetic acid
Nin	- ninhydrin sprayed
Fe^2	- 1% ferric chloride/1% potassium ferricyanide sprayed
B.A.W.	- n-butanol: acetic acid: water
BEB	- n-butanol: ethanol: borate
BEW	- n-butanol: ethanol: water
DSA	- diazotized sulphanilic acid sprayed
BAL	-(2,3-dimercaptopropanol)
EDTA	- ethylenediaminetetracetic acid
DDC	- sodium diethyldithiocarbamate
Pcat	- protocatechuic acid
PcatG	- 4-o-β-D-protocatechuic acid glucoside
Cat	- catechol
HQ	- hydroquinone
Dopa	- 3,4-dihydroxyphenylalanine
Tyr	- p-tyrosine
Tyram	- p-tyramine
Dopam	-3-hydroxytyramine
u.v.	- ultraviolet
$NADH_2$	- nicotinamide adenosine dinucleotide (reduced)
-G-C^{14}	- generally labelled C^{14} compound

CHAPTER ONE

GENERAL INTRODUCTION

Despite the numerical dominance of animal life on earth by insects and despite their economic importance to man, our knowledge of their biochemical processes, as opposed to their biology and ecology is still very incomplete. It is to be expected that effective measures of control of the pests among insects, whether these measures be chemical or of an environmental nature, will more and more depend on an understanding of the physiology of the arthropod concerned. Progress in the design of rational insecticides, which formerly relied for their development on chance findings, may be based increasingly on the growing knowledge of the biological function and chemical processes of insect cells and tissues (Hurst 1945, Peterson 1959).

Only in recent years have biochemists begun systematically to turn their attention to invertebrate forms of life other than micro-organisms. During the last 130 years, experimental biologists have thrown much light on the function of insect tissues. The opportunity may now exist that the limited tools of the cytologist and histochemist can be augmented or replaced by biochemical techniques.

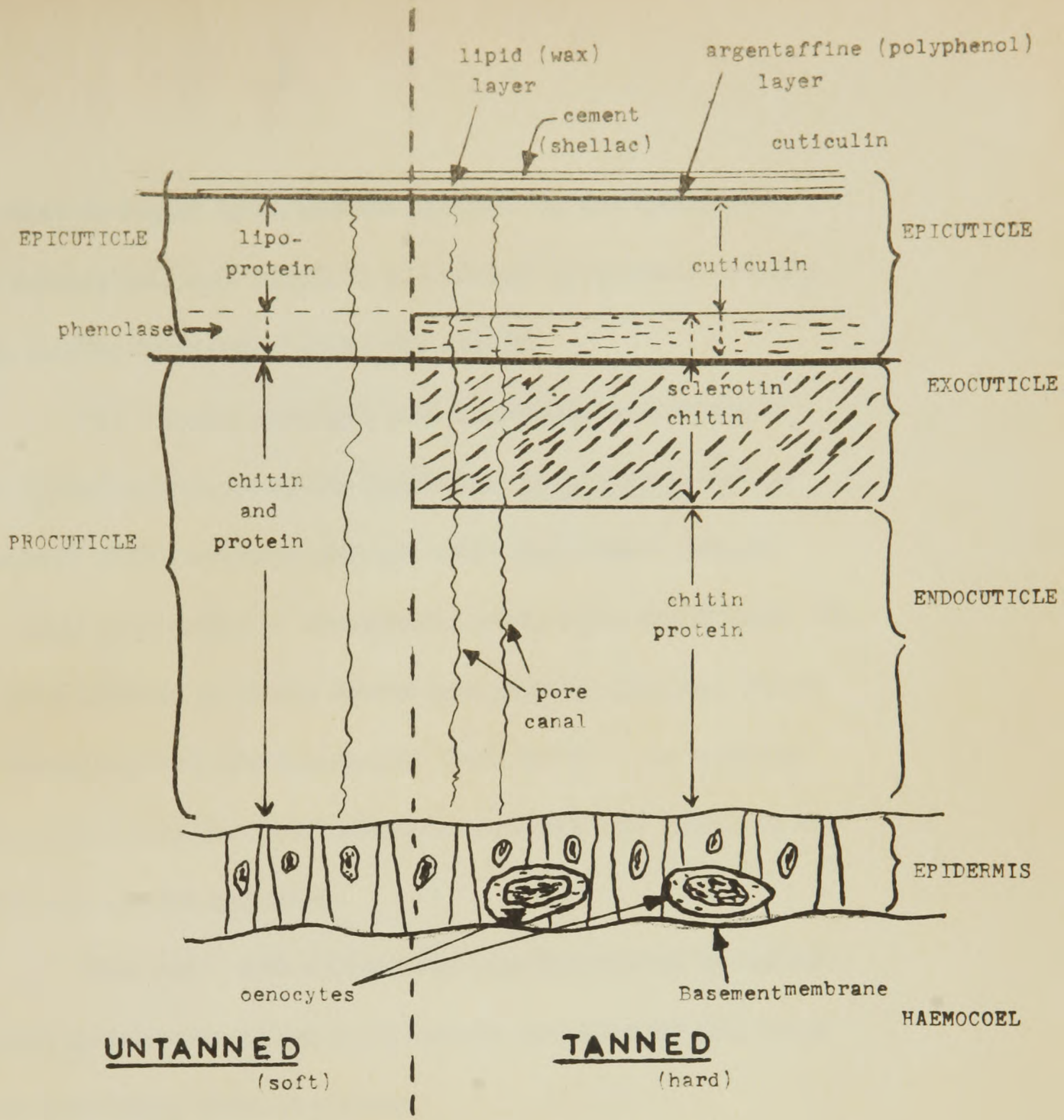
The immediate problems, confronting the biological chemists in this field, include ones of procuring sufficient material of a uniform nature and of correlating experimental results with those of other investigators who have used different tissues. Thus the building up of an overall picture of insect metabolism will undoubtedly present some difficulties.

A. THE CUTICLE

The properties of the integument of arthropods influence their existence and growth very largely. The study of this structure and its mode of formation is paramount to an understanding of the phylum.

(i) Structure and composition

That diminutive terrestial insects are able to exist is dependent on the relative strength and impermeability of their cuticles. This impermeability to water, which is independent of thickness and rigidity is a property conferred by the wax (lipid) layer, of the epicuticle, which is itself usually overlayed with a hard, shellac-like cement (Wigglesworth 1953, 1957). The rest of the relatively thin epicuticle (fig. 1) is a non-chitinous, lipoprotein



FIG, 1. LONGITUDINAL SECTION THROUGH TANNED AND UNTANNED CUTICLE (model)

region, which is layed down before ecdysis by the epidermal cells and oenocytes, and which is converted by quinone tanning into hard, amber 'cuticulin' after the moult.

The tensile strength and flexibility of cuticle is imparted by the structure of the laminated procuticle, which later becomes differentiated into the exo- and endo- layers (fig. 1). This procuticle is composed, when fully developed, of a cross-grid system of chitin fibres and protein chains, which exhibits birefringence (Richards and Pipa 1958). An analogy to the functional relationship between cellulose and lignin in the plant cell wall can be inferred.

The pro- and epicuticles are traversed by spiral cytoplasmic processes called pore canals and also by the ducts of cement-secreting dermal glands.

Greater rigidity of the fully developed cuticle appears to be imparted by phenolic (quinone) tanning of the proteins in the epi- and exocuticles, but more will be said of this process later.

The variation in the structure and composition of the integument of different orders, genera and even species is so great that the description of the cuticle given above only represents a

generalised model picture. The cuticles of larvae, pupae and adults of the same insect often differ widely in their make-up also. Figure 2 illustrates the changes undergone in the larval cuticle of the blowfly Sarcophaga falculata during the formation of the puparium (Richards, pp 185, 1951). For further comparison with the model cuticle (fig. 1), a longitudinal section through the fully developed cuticle of the dorsal abdominal tergite (anterior region) of Periplaneta americana (L) is shown in figure 3a (Dennell and Malek 1954).

Since such wide divergence between different cuticles is possible (see Wigglesworth 1953) it is not surprising that the reviews of the literature on the formation, structure and chemical composition of the integument (Richards 1951, 1958; Wigglesworth 1948a, 1953, 1957; Hackman 1953a and b, 1959) differ on certain points.

Problems of the precise demarcation, nature and function of different layers (e.g. cement, lipid and polyphenol regions (see fig. 1)) cannot be definitely resolved by the histochemical techniques available. Therefore different interpretations have arisen in the literature.

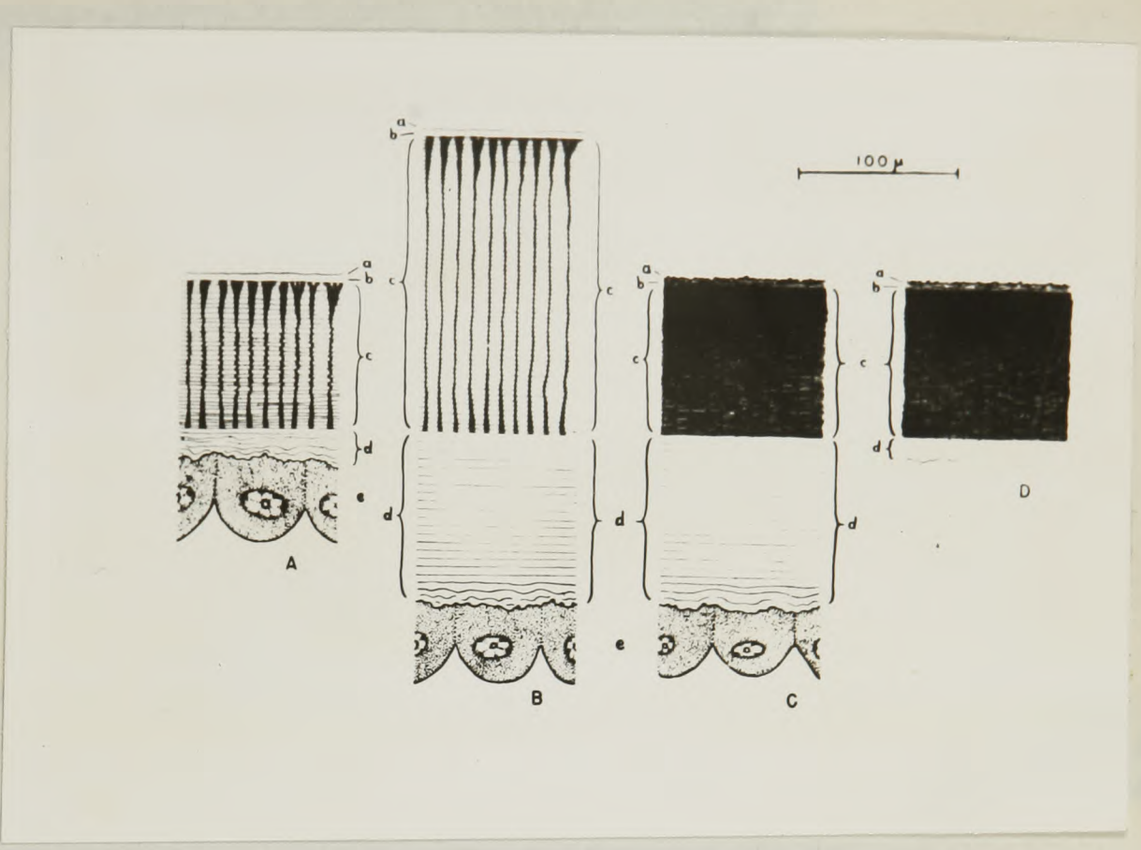


FIGURE 2.

Changes undergone by the larval integument of the blowfly Sarcophaga falculata during the formation of the puparium

a) lipid or wax layer
b) inner epicuticle
c) outer procuticle (prospective exocuticle)
d) inner procuticle (prospective endocuticle)
e) epidermal cells

A. Larval cuticle ± 2 days after hatching

B. Larval cuticle fully mature

C. Mature larval cuticle immediately prior to separation of puparium from epidermis.

D. Cuticle of old puparium.

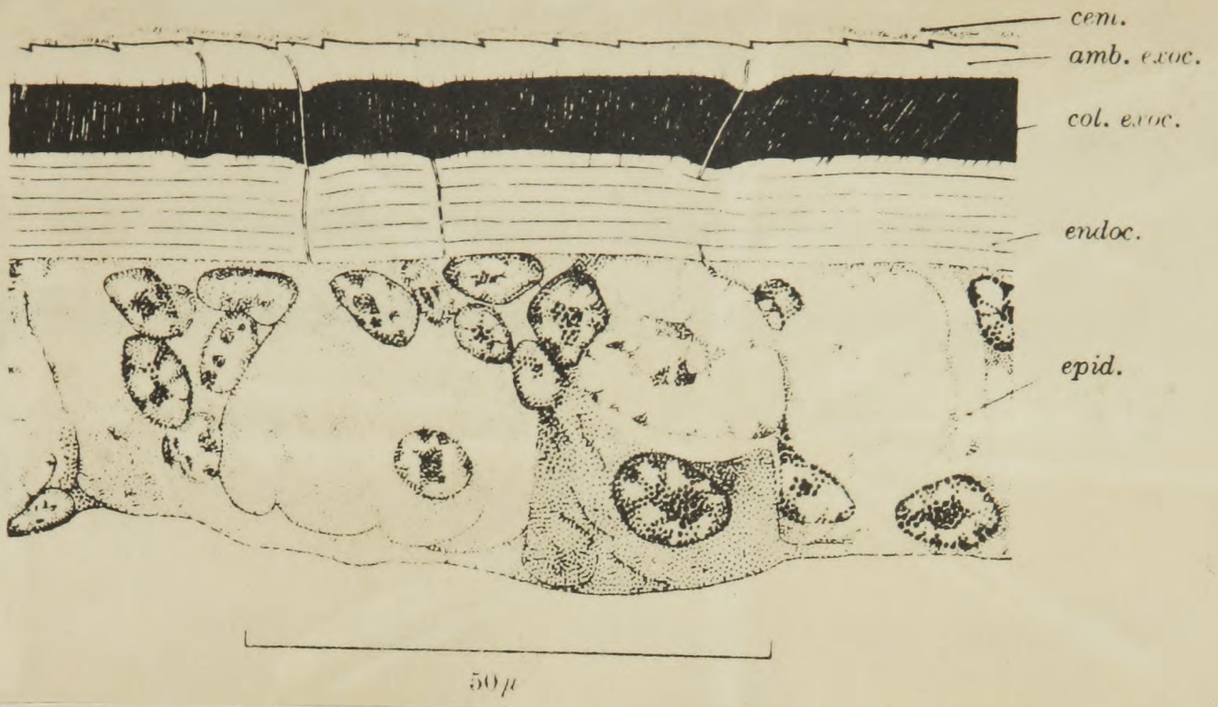


FIGURE 3a: Longitudinal section through the anterior region of a sclerite (tergite) of Periplaneta americana (Dennell and Malek 1954). amb.exoc: amber exocuticle; cem: cement layer; col.exoc: colourless exocuticle; endoc: endocuticle; epid: epidermis; solid black region indicates stained with haemotoxylin.



FIGURE 3b: Change in colour accompanying tanning of the cuticle of the adult cockroach (Periplaneta americana) after moult from final instar shown.

Although it is certain that there is a basic similarity in the process of tanning of insect cuticles, it is not yet clear to what extent this tanning may differ in detail in different insects.

(ii) Tanning of the cuticle (see fig. 3b)

This is the process mentioned earlier which results in the hardening of the integument. It is accompanied by the darkening of some layers (e.g. the exocuticle). This is brought about by the interaction, and finally attachment, of quinones -- formed from dihydroxyphenols -- with the free amino groups of protein chains (Pryor 1940a, b). The subject has been reviewed by Mason (1955), Dennell (1958), Hackman (1953c, 1959) and Wigglesworth (1957). The complexity of the fundamental biochemistry of the cuticle is emphasised by the fact that the mode of the tanning process is lent different interpretations in the literature.

Pryor (1940a, b) gave the name "sclerotin" to the coloured, quinone-tanned protein of the cockroach oöthecal wall and insect integument. The water-soluble, heterogeneous (Hackman 1953a) protein fraction of 'soft' (untanned) cuticle, which is capable

of being tanned artificially and is believed to give rise to 'sclerotin' in vivo, was called 'arthropodin' by Fraenkel and Rudall (1947). Malek (1952) observed that in situ arthropodin of Periplaneta, like the precursor of cuticulin, may be a lipoprotein.

In fly larvae, the tanning process, which takes place in a series of steps, is heralded by a rise in the tyrosine and tyrosinase content of the blood (Dennell 1958a). It is then set in motion by the release of the pupation hormone, which appears to be responsible for raising the redox potential of the blood (Dennell 1947). This rise in potential encourages the action of the tyrosinase, with the eventual result that dihydroxyphenols, precursors of the tanning quinones, are formed from tyrosine. These diphenols diffuse outwards through the integument to the epicuticle where they are oxidized by the polyphenoloxidase present there (fig. 1). o-Quinones so formed (or p-quinones according to Dennell (1958b)) diffuse inward tanning the epicuticle, to form cuticulin, and the procuticlar proteins to give sclerotin, as stated earlier. The chitin fibres, instead of being randomly dispersed as in the soft cuticle, become orientated in planes parallel to the surface of the integument. There is reason to believe that 'sclerotin' in the exocuticle can be attached, and thus

aligned to the chitin chains, through quinone nuclei by primary valence linkages (Mason 1955).

(iii) The chemistry of tanning.

According to Mason (1955), an o-quinone reacts first with a terminal amino group of a protein chain to form a colourless aminohydroquinone (fig. 4a) capable of being further oxidised to the corresponding quinone by excess of the reacting o-quinone. The quinonoid protein could then react with another terminal amino group of arthropodin (with which these proteins are well endowed (Hackman 1953a)) so forming a disubstituted, cross-linked derivative (fig. 4b) if such a second position on the quinone molecule is available. This requirement is met only, because of ready decarboxylation, by (see fig. 4c) 4-carboxy -1:2-benzo quinone (from protocatechuic acid) states Mason (1955). Hackman (1959) pointed out that he and Todd (1953) found that as many as three aniline residues could be substituted in the quinone nucleus of oxidized 4-methylcatechol and 3,4-dihydroxyphenyl propionic acid. Therefore by analogy, Hackman (1959) says that

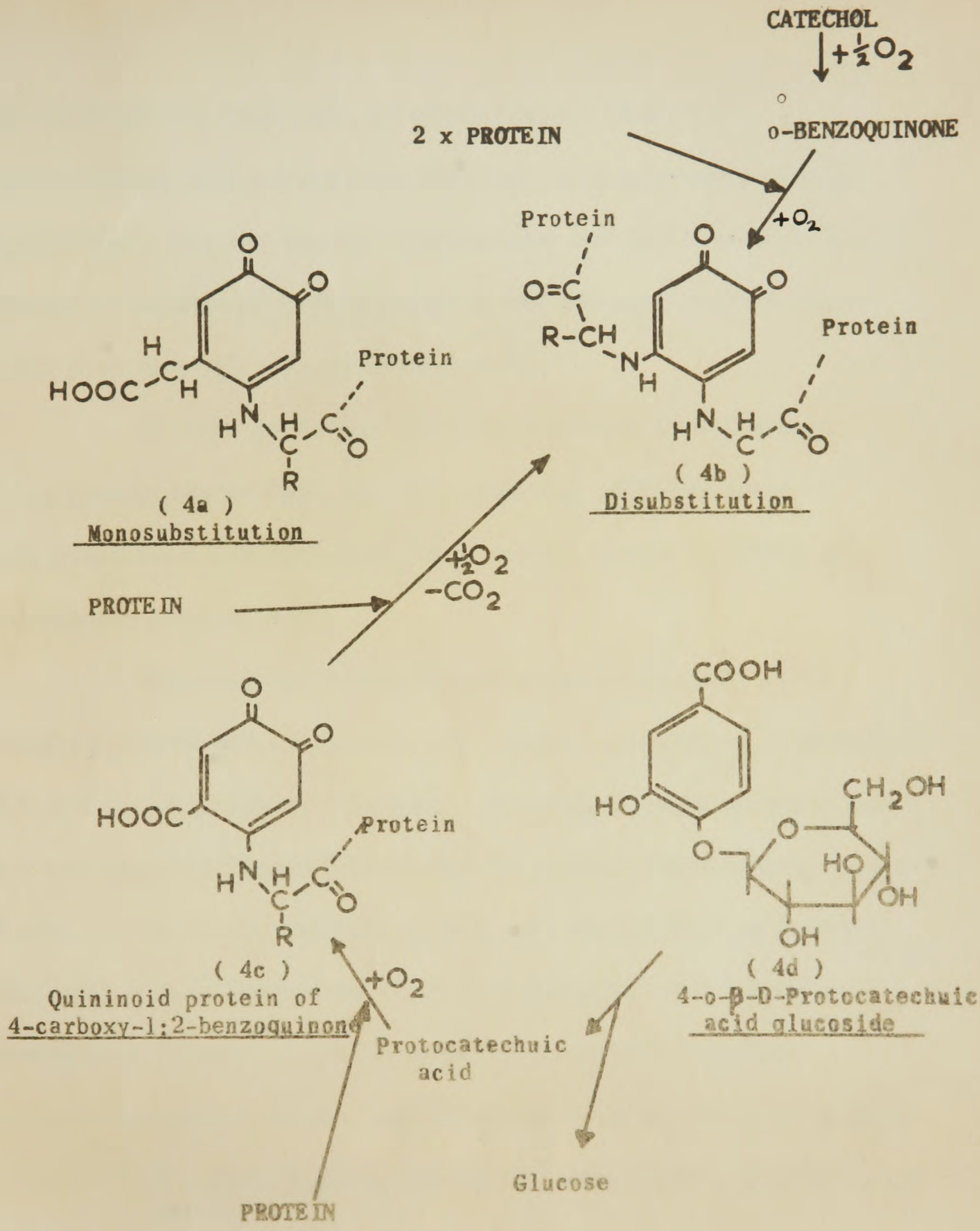


FIGURE 4. REACTION OF TERMINAL AMINO GROUP OF A PROTEIN WITH THE o-QUINONE FORMED FROM a) 3,4-DIHYDROXYPHENYLACETIC ACID b) CATECHOL & c) PROTOCATECHUIC ACID. (Hackman and Todd (1953, Mason 1955).

any 4-substituted diphenols, of which three - other than protocatechuic acid - have been isolated from arthropod cuticles (Mason 1955, and see page 147 chapter six, section II of this thesis), "should be capable of linking together two or three protein chains in the presence of a polyphenoloxidase".

Be this as it may, once the terminal amino groups of the protein chains have reacted, quinones will then form monosubstituted products with the ε-amino groups of lysine in arthropodin (Mason 1955).

The final darkening stages of tanning appear to be brought about by polymerization and copolymerization of o-quinones with protein or quininoid proteins. Secondary cross-linkages are also envisaged by Mason (1955) between carbonyl and amide groups. Finally, in the sclerotized exocuticle, the strong bonding between chitin and protein (Richards 1951) may be explained if quinone tanning sets up cross-linkages between unacetylated amino groups in the chitin chains, and quininoid proteins. Hackman (1959) considers that a large fraction of the cuticular protein may be bound to chitin by covalent bonds - forming a glycoprotein which is stable and polydisperse, - possibly though aspartyl and histidyl residues, the

linkbeing in the form of N-acyl-glucosamine.

Two other possibilities in connection with sclerotization have been mooted. The first, which is not fully substantiated (Hackman 1959) is that the proteins to be tanned in the cuticles of the cockroach and blowfly exist as sterol complexes (Dennell 1958). Secondly, in some invertebrates, it is thought possible that the phenolic residues of proteins (e.g. tyrosine) may themselves serve as substrates for polyphenoloxidases (Brown 1950, Mason 1955).

Why some hard, tanned cuticles are colourless is not fully understood (Hackman 1959). Mason (1955) suggests by implication that the ratio of o-quinones to protein is much lower during the tanning of colourless cuticles than in that of dark ones i.e. polymerization of quinones with quininoid proteins would be limited. Malek (1957) has claimed that the darkening (in this case called melanization) process is separate from and independent of the hardening process in cuticle of the last nymphal instar of the desert locust, Schistocerca gregaria (F). The two processes he says appear to involve their own separate phenoloxidases. In the dark patches of the cuticle, sclerotization may be delayed until darkening is complete. Lack of pigmentation is explained somehow by the accumulation of "reducing substances" in the parent epidermal cells, which apparently respond by withholding the required chromogen

in the cuticle.

Our knowledge of the nature of the polyphenol oxidase involved in tanning will be briefly reviewed in Chapter three, Section I and the identity and properties of the dihydroxyphenols found in cuticle will be dealt with in Chapter six, Section II.

B. THE COCKROACH OOTHECA

The theory of the tanning of cuticular proteins outlined above is derived directly from the observations made by Pryor (1940a) on the formation of the ootheca (egg capsule) of Blatta orientalis (L). Previously, Campbell (1929) -- possibly taking into account the work of Ito (1924) -- had shown that the walls of the ootheca, unlike procuticle, did not contain chitin. It had long been recognized (Duchamp 1878, Kadyi 1879) that the "carpet bag"-shaped, (Pryor 1940a) oothecal case (± 7 mm. long) was the product of the secretion of two sexual accessory (colleterial) glands. Both glands open through single ducts on the dorsal side of the genital vestibulum, into which their secretions are extruded and intermingled when the ovaries deposit their eggs there (fig. 5b) prior to "egg-laying". Figure 5a shows the formation of the ootheca and its extrusion from the vestibulum, and illustrates the changes undergone in the oothecal case as a result of tanning.



FIGURE 5a: "Laying" of the ootheca by the female cockroach and changes undergone in the appearance of the ootheca during tanning of the proteinaceous walls.

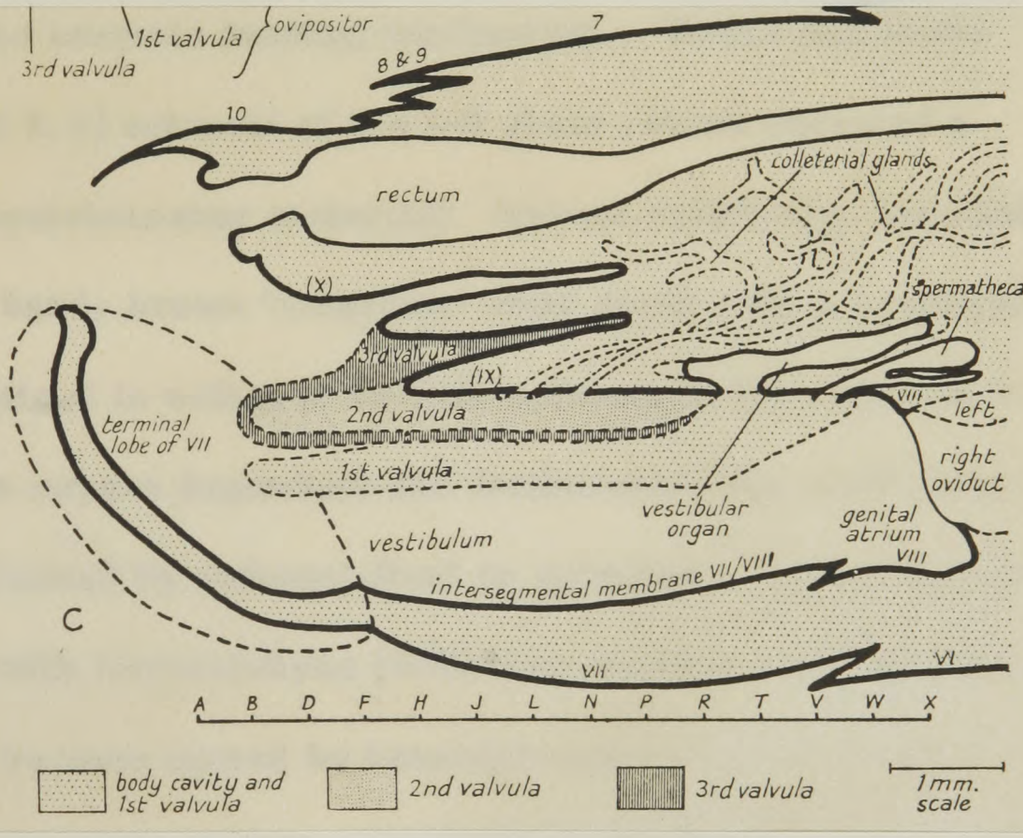


FIGURE 5b: Sagittal section of the posterior of the abdomen of the female cockroach showing the relation between colleterial glands, oviducts and vestibulum. Solid line — surface cut by section: broken line — tissue not lying in medium plane. (Brunet 1952 — with permission).

Pryor (1940a) examined in great detail the mode of formation of the proteinaceous fabric of the egg capsule (of Blatta) by the colleterial glands. He concluded that the larger, whiteish, left gland of the assymetric pair secreted the structural protein, while the translucent right gland produced the dihydroxyphenolic substances, found in the oothecal fluid, which were required as substrates for the enzymic tanning mechanism. Pryor had found that buffered (pH 8.0) extracts of the left gland (which secreted a white, viscous, proteinaceous material) formed relatively, insoluble and unreactive, hard, brown 'sclerotin' when stood with p-benzoquinone, or the fluid contained in oothecae or with extracts of the right gland. The reaction was oxygen dependent and furthermore the right gland extract was not tanned by oothecal fluid or quinones. The left gland, after treatment with formaldehyde (40%) then washing and buffering (pH 8.0) did not become tanned by benzoquinone or by the extract of right gland, andsince formaldehyde dichromate-fixed (12hours) right and not left gland, gave a positive (dihydroxyphenol specific) chromaffine reaction (Lison 1936) the natural source of the quinone precursor was thought to be the smaller gland.

In 1931, Schmalfuss and Barthmeyer had shown that the ootheca fluid contained an o-dihydroxyphenol, but it was only in 1946 that Pryor, Russell and Todd identified it as 3,4-dihydroxybenzoic (protecatechuic) acid.

After the publication of the results of Pryor's research (1940a and b), workers interested in insect cuticle formation soon realised that the continuously secreting, easily dissected, colleterial glands of the cockroach were convenient material (as opposed to the epidermis) upon which to carry out studies on the mechanism of sclerotization. (Brunet 1951, 1952).

Some consternation was caused when, despite confirmation of the weakly positive chromaffine reaction described, the presence of protocatechuic acid or its precursors could not be definitely demonstrated in the right colleterial gland. The reason for this discrepancy was later made apparent by Brunet and Kent (1955). They found that, together with protein and a polyphenoloxidase (Brunet 1952) the left gland of Periplaneta and of Blatta actually secreted the diphenol, but conveniently masked as the 4-O-β-D-glucoside (fig. 4d) (Kent and Brunet 1959). The right gland secretion possessed β-glucosidase activity and was responsible for freeing the diphenol, ready for enzymic oxidation to the quinone, once the

gland secretions intermingled in vitro or in the vestibulum.

Brunet and Kent (1955) showed chromatographically that the protocatechuic acid glucoside present in homogenized left gland was completely hydrolysed in two hours to the free phenolic acid and glucose upon adding right gland homogenate, or a preparation of emulsin, to it and incubating at 37°C. This evidence of hydrolysis was supported by ultraviolet absorption spectroscopy and by chemical tests with chromaffine, Fontana's, ferric chloride/sodium carbonate, and aniline hydrogen phthalate reagents.

The glucoside was later synthesised (Kent and Brunet 1959) and shown to exhibit all the properties of the naturally occurring masked diphenol in Periplaneta left colleterial gland.

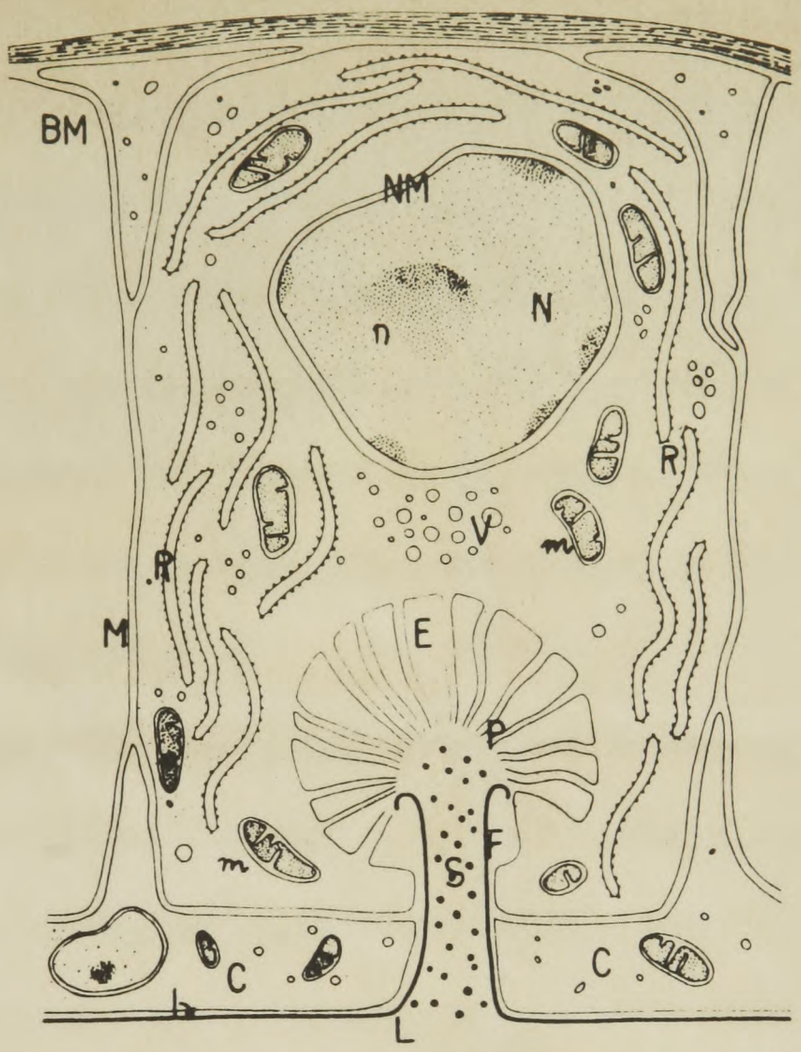
C. THE LEFT COLLETERIAL GLAND

Bordas (1909) first described the histology of the colleterial glands. Later, four regions of cytologically different cells were described in the left gland (Brunet 1951, 1952) which was followed by an electron microscopical study of these individual cells (Mercer and Brunet 1959).

The most numerous "type 4" squat cells, which lie at the innermost ends of the tubules, secrete the "structural protein" (c.f. arthropodin), required for the fabric of the oothecal walls, together with calcium oxalate (Duchamp 1878, Kadyi 1879). The oxalate, which crystallizes in the lumen of the tubules, appears to have the sole function of reinforcing the sclerotin walls of the ootheca. A diagrammatical representation of a "type 4" cell, showing the remarkably specialised, secretory system of the plasma membrane (end apparatus) is given in figure 6, (Mercer and Brunet 1959).

"Type 2" cells, which are located at the posterior branches of the tubules, also appear to secrete structural proteins copiously but the continuous secretion may be effected by mitochondria and not by a membranous system such as in "type 4" cells. The diphenol-oxidizing enzyme could possibly be secreted by these "type 2" cells (Mercer and Brunet 1959).

Cells of "type 3", which secrete less and are taller (15-50μ) than those in the regions on either side of them (2 and 4), most probably are responsible for the synthesis of the polyphenoloxidase system. These cells are positive in reaction to 'Nadi' reagent and leuco methylene blue and they are cyanide (0.002 M, pH 7.3) sensitive (Brunet 1952).



TEXT-FIG. 6. A greatly simplified reference diagram of a type 4 secretory cell embodying the combined results of light and electron microscopy. *N*, nucleus; *n*, nucleolus; *NM*, nuclear membrane; *R*, reticulum; *m*, mitochondrion; *E*, end-apparatus; *P*, processes of end-apparatus; *F*, funnel of end-apparatus; *C*, chitinogenic cell; *L*, lumen containing secretion; *I*, intima; *V*, small vesicles; *S*, secretion; *M*, cell membrane; *BM*, basement membrane.

It is not known in which cells the protocatechuic acid glucoside is synthesised. The structural protein is, however, stained by ammoniacal silver hydroxide (argentaffine (Lison 1936)) and gives a positive chromaffine test, attributable possibly to 3, 4-dihydroxyphenyl alanine(dopa)residues. The protein stains with phosphotungstic acid and appears as a mass of droplets of variable size (figs. 7 and 9) whose diameter ($\bar{+}$ 700 Å) is similar to that of the tubules of the end apparatus (fig. 8) (Mercer and Brunet 1959).

The present author set out to investigate the nature of the polyphenoloxidase secreted by the left colleterial gland of Periplaneta -- described by Brunet (1952) - and, if possible, to purify this enzyme system by separating it from the structural protein fraction.

Investigation of the possible biosynthetic pathways of the glucoside of protocatechuic acid, precursor of the quinone responsible for tanning, was to be undertaken by studying the specificity of the polyphenoloxidase and by attempting to isolate any metabolic intermediates occurring in incubated extracts of the cells of the gland.

FIGURE 7: Photoelectronmicrograph of the end apparatus and a portion of the cytoplasm (stained) with phosphotungstic acid) of a "type 2" cell from left colleterial gland tubule (Mercer and Brunet 1959 — with permision). The cytoplasm is tightly packed with mitochondira (m). S is the secretion in the lumen which is produced by the end apparatus.



(Mercer and Brunet: Left colleterial gland of cockroach)

FIGURE 8: An enlargement of a photoelectronmicrograph of the tips of the processes of the end apparatus (stained with phosphotungstic acid) of "type 4" cell of left colleterial gland tubule. (Mercer and Brunet 1959 -- with permission) Droplets of secretion at S; possibly within the processes at D; and emerging at O.

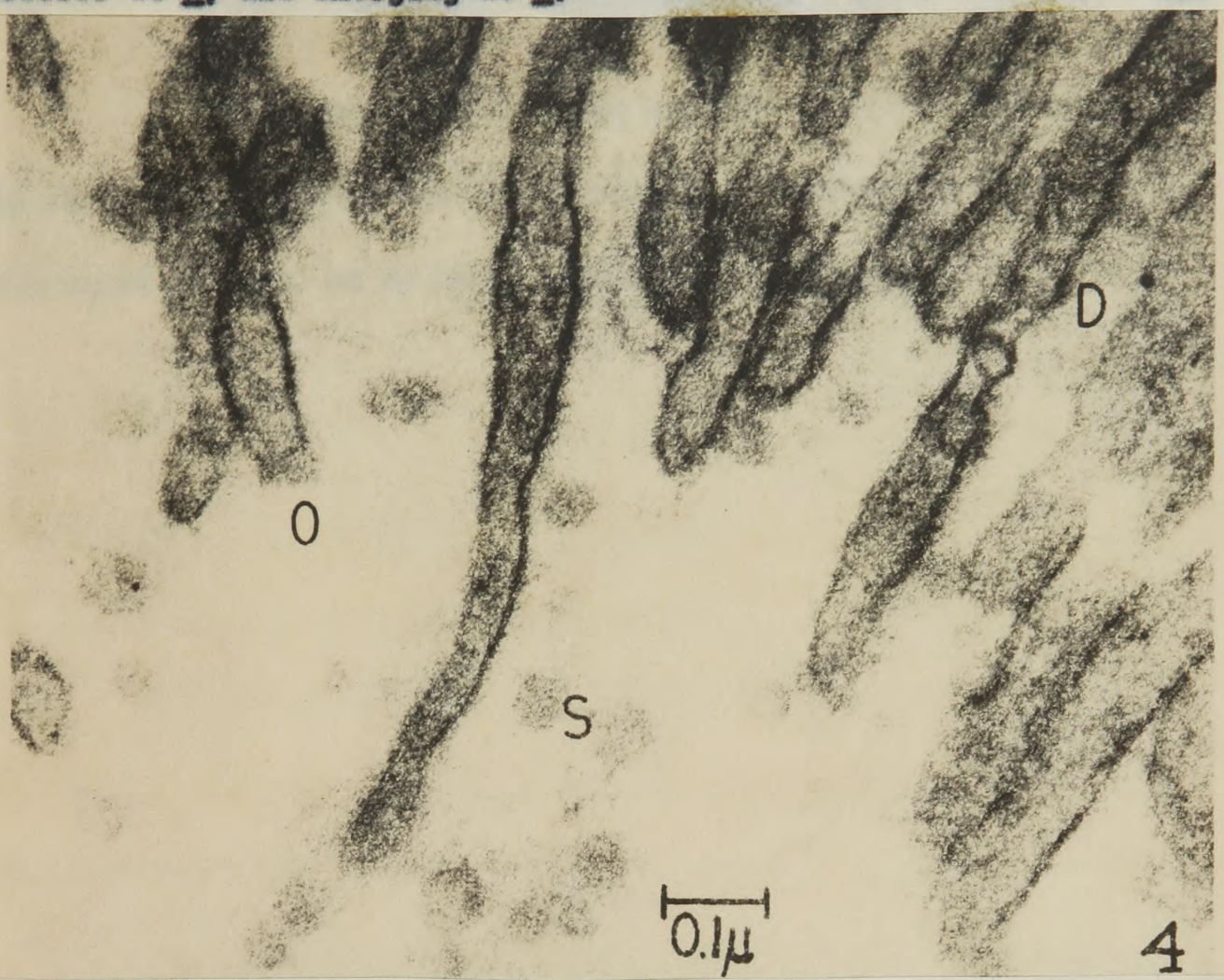
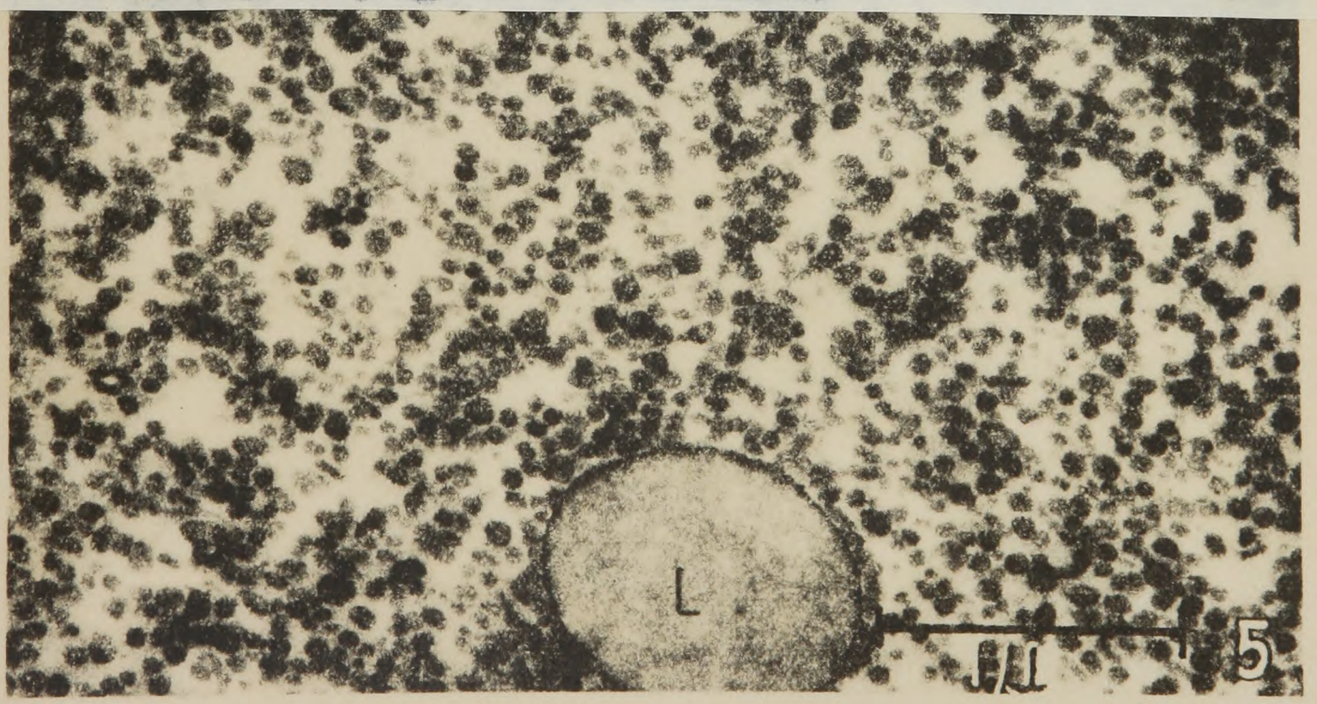


FIGURE 9: Secretion of "type 4" cells (i.e. the structural protein and phenolase to which the phenolic glucoside adheres) as seen in the lumen of of the tubule. Large body (L) — not identified.



(Mercer and Brunet: Left colleterial gland of cockroach)

It was to be hoped that the results of these studies would shed some light on the biochemistry of sclerotinization in arthropod cuticle, or at least in that of Periplaneta.

CHAPTER TWO

EXPERIMENTAL METHODS AND MATERIALS

A METHODS

(i) Culture of Periplaneta americana (L)

The cockroaches were reared in a dark room, controlled at 28°C, in large glass tanks, containing an inch-deep layer of vermiculite. The insects were fed weekly on powdered standard rat-cake ration supplemented with bread, fruit and raw vegetable kitchen waste which had been saturated with water and pulped by hand. To control the humidity in the tanks and avoid infestation by parasitic mites, the fresh water supply was strictly limited to that contained in the pulped food.

The tanks were partitioned with strips of hardboard 8" wide and closed with lids made of metal mesh while the upper sides of the walls were greased with vaseline to prevent the insects escaping.

(ii) Dissection

All dissections were performed in cold isotonic saline (0.9% sodium chloride + 0.01% calcium chloride in glass distilled water). The abdomen of an adult female cockroach was severed from the thorax with sharp scissors, and the alimentary system (mid-gut) carefully extracted with forceps. Two longitudinal cuts were made along the edge of the full length of the abdomen, which allowed the terga to be raised up. These cuts were continued at right angles so as to meet below the anus, after the abdomen had been secured under the saline. Care had to be taken that the rectum was not punctured at this juncture. The terga were then lifted from the posterior end and removed while the retaining tracheoles were severed with a fine scalpel. The ovaries, which are attached anteriorly to the dorsal wall of the abdomen, were removed with the terga (by severing their ducts to the vestibulum) to avoid their rupture during subsequent dissection of the colleterial glands. The fat body was now cleared away from the accessory glands and the visibly distinct left and right colleterial glands (Brunet 1952) were carefully parted, while still attached by their main ducts to the dorsal wall of the vestibulum.

This separation involved untangling the intertwined tubules under a low-power binocular microscope and severing the interposed tracheoles as before. The two colleterial glands could now be removed individually and in toto by their 'stalks'. A final check in saline under the microscope was made to ensure the absence of tubules of the opposite gland and of fat body tissue, nerve cord or malphigian tubes. Each dissection occupied about fifteen minutes.

(iii) Preparation of enzymes from the glands

(a) Whole glands

In preliminary experiments, the intact excised left glands were blotted, weighed and then each suspended in 1 ml. of an insect ringer solution (pH 7.3) containing:-

14 g.	NaCl
0.2 g.	$CaCl_2$
0.2 g.	KCl.
0.2 g.	$MgSO_4$
0.04 g.	$NaHCO_3$

10 mls. of buffer (4 parts 0.1M-Na_2HPO_4 and 1 part 0.1M-NaH_2PO_4) made up to one litre with distilled water.

(Griffiths and Tauber 1943, Roeder 1948)

(b) 'Exudate'

To obtain the secretion of the left colleterial gland for phenoloxidase experiments, the gland was blotted, after dissection, on absorbing tissue paper, rinsed twice in fresh saline and again blotted. The gland tissue was then rinsed twice in glass distilled water and blotted again.

The glandular secretion contained in the lumen of the tubules was obtained by twice immersing (and occasionally agitating) the tissue in glass distilled water (0.5 mls. and 0.2 mls.) for 15 minutes each. The resulting milky suspension was transferred to a tube and immediately chilled to 0° - 3°C.

When sufficient secretion had been harvested for an experiment (about 12 mls.) it was centrifuged (1000 r.p.m. for 20 mins.) in a tapered tube to remove tubule debris. The supernatant was dialysed twice (in cellophane) for 4 and 12 hours at 3°C against 2 l. and 5 l. of 0.01M- sodium chloride solution respectively. This treatment removed endogenous substrates from the enzyme preparation. Consequently the first dialysate was stored at 3°C if the phenolic compounds were to be extracted from it. Inorganic ions were then dialysed from the 'exudate' with 2 l. and 5 l. of chilled, glass distilled water for 4 and 12 hours respectively, as before.

This final preparation was stored at 3°C and used directly as a source of phenoloxidase for assay or for further fractionation as described later.

(c) Homogenate of glands

Left colleterial glands were dissected, rinsed and blotted as described above, then stored in weighed, glass, Potter-type homogenizers chilled in ice. These homogenizers were thick, bulbed tubes of 1.0 ml. working capacity (bulb half-full when plunger inserted) with matched, close-fitting, glass plungers, which had been ground into the tubes themselves. When enough glands (5 to 7) were collected per tube and their combined wet weight recorded, 1 ml. of cold glass-distilled water was pipetted into each homogenizer. The plungers were then inserted, while being slowly rotated, and the glands ground under hand pressure by twenty brisk turns. The homogenized tissue was transferred (Pasteur pipette) to a graduated tube standing in ice and the procedure repeated on the debris until the required volume/gland was forthcoming (usually 0.5 - 0.6 mls. i.e. 11-12 mls. / 18-20 glands).

Unlike the dialysed 'exudate' described above, the homogenate could not be stored for longer than a few hours for the

endogenous phenols present would be oxidized enzymically. The homogenized tissue was centrifuged at 1000 r.p.m. for 5 minutes before being used.

A similar procedure was carried out when homogenates of combined left and right glands or of right colleterial gland alone were required. In the latter case, smaller glass homogenizers (0.1 ml. working capacity) were used.

(iv) Enzyme assay by manometry

(a) Phenoloxidase activity

This enzyme system was studied manometrically in an atmosphere of oxygen at 37°C using Warburg-type apparatus with flasks of 5 to 7 ml. capacity, shaken at 100 oscillations/ min. Krebs' (1951) fluid was used in the manometers.

Each flask contained 1 ml. of enzyme preparation ('exudate' or homogenate) and 0.25 ml. of 0.3 M-sodium phosphate buffer (pH 6.8). Substrates (0.25 ml.), prepared in concentrations of 12 mM- or 60 mM- in the phosphate buffer used above, were added from the side bulb after equilibration. In certain experiments, additions of antibiotics (0.25 ml, 0.6% (w/v) and/or of inhibitors (0.01 - 10 mM) were made together with the substrate or buffer from the side arm. The centre well (rim greased with lanolin) of

each flask contained filter paper (Whatman No. 40) moistened with 2 N- sodium hydroxide (0. 1 ml.). The vessels were equilibrated by being shaken in the bath at 37°C for 15 mins. before tipping. Each bank of flasks and manometers (14) included a thermobarometer i. e. one of the flasks contained 1 ml. of water and 0.5 mls. of buffer.

With each enzyme preparation, as a result of preliminary experiments, it was found necessary to include the following controls:- Measurement of (a) the "endogenous oxygen uptake" (due to enzyme in buffer alone), (b) the "autoxidation" of introduced substrate in the absence of enzyme, and (c) the oxidation of substrate by boiled enxyme (10 mins. at 98°C).

The oxygen uptake measured for from 3 to 6 hours, was expressed in µls/hour/mg. wet weight of gland (Q_{O_2}) or/mg. of trichloroacetic acid (T. C. A.) precipitate or/mg. of protein estimated by the ninhydrin method (page 43).

After incubation the contents of the flasks were frequntly subjected to further analysis (for phenolic components, presence of bacteria or copper content). Oxygen uptake could be also expressed in terms of µls. /hour/µg of copper present ($q_{O_2}^{Cu}$).

(b) Decarboxylase activity

Homogenates (1 ml.) of left colleterial gland, or of left with right glands, were investigated manometrically using the identical system described above (see (a)) except that all solutions were prepared in boiled, glass-distilled water, equilibrated with the nitrogen - 5% carbon dioxide gas mixture used as the atmosphere in these experiments. The substrates (0.06 M) to be tested were introduced from the side bulb in sodium bicarbonate buffer (3.67×10^{-3} M, pH 6.8 at 37°) containing 10 µg. of pyridoxal-6-phosphate, while the centre well contained 0.5 M- pyrogallol (0.1 ml.) absorbed on filter paper. Control incubations were as before. The results ($Q_{CO_2}^{N_2}$) were expressed in µls. of CO_2 liberated/hour/mg. wet weight of gland used.

(v) Paper Chromatography

(a) Descending partition chromatography of the phenolic and amino compounds on Whatman No. 1 paper (2 l. x 50 cms.) was performed overnight at room temperature in all-glass tanks with n-butanol: glacial acetic acid: water (BAW), (4 : 1 : 1) as the usual solvent mixture. Other solvent systems employed were:-

n-butanol: glacial acetic acid: water (BAW), (4: 1: 5)

n-butanol: ethanol: water (BEW), (4 : 1: 1)

n butanol: ethanol: borate (BEB), (1:1:1:) using paper previously buffered in the same sodium tetraborate solution (9.54 g/l.) (Coulson and Evans 1958).

2% glacial acetic acid in water:

benzene: glacial acetic acid water: (20:5: just sufficient to saturate the organic solvents).

Phenol saturated with water (74g. : 24g.) was used chiefly to separate amino compounds.

The chromatographic paper was allowed to hang and equilibrate for 6-12 hours in the tank, (at the bottom of which was a beaker of the solvent to be used,) before the trough was filled through a hole in the air-tight glass lid.

(b) Ascending chromatograms were run on Whatman No.1 paper in lipless 2 l. and 4 l. beakers fitted with airtight glass lids. The same solvents, as employed for descending chromatography, were used.

Samples (0.01 - 0.20 mls.) of the solutions, together with reference compounds for chromotography were applied to the base line on the paper (which was raised on glass rods) by calibrated standard or Carlsberg pipettes.

Samples (0.20 - 1.0 ml.) were applied as bars (2-8 cms.), instead of spots, when component substances were to be eluted from the paper after chromatography.

Phenols and amino compounds (synthetic or from insects) were applied to the chromatograms in water or 50% aqueous ethanol. Before chromatography of incubation mixtures, proteins and some inorganic materials were precipitated by adding one volume of 96% ethanol and centrifuging at 3000 r.p.m. for 20 minutes.

(c) Detection of compounds

(1) Some phenols could be detected on the dried papers by contact printing (Markham and Smith 1949) on Kodak, Duostat 13, reflex paper for 20-35 seconds with the light (98%, 253.7 mμ) emitted by a mercury-arc lamp (British Thermal Syndicate). Catechol and tyrosine did not absorb strongly at the wavelength used and therefore could not be readily detected by this method. The phenolic compounds could also be suitably detected on the chromatograms by spraying them on test strips with a freshly prepared mixture of equal volumes of 1% (w/v) ferric chloride and 1% (w/v) potassium ferricyanide (Fe^2) (Barton, Evans and Gardner 1952), or with Fontana's ammoniacal silver nitrate solution (Lison 1936). In the case of the former spray the spots were allowed to develop for 4 - 5 mins, before the 'background'

was eluted with dilute HCL (0.01N) and the paper rinsed in tap water and dried in air. Both sides of the paper were sprayed to achieve the best result, but care had to be taken not to super-saturate the chromatogram with the spray. Other phenol developing sprays employed were aqueous 1% ferric chloride and Pauly's (Erspamer and Boretti 1951) diazotised sulphanilic acid reagent (DSA), as modified by Acheson & Dearnley (1960), but these reagents were less sensitive and more specific than the ferric chloride/ferricyanide mixture.

(2) Amino acids and amines were detected by spraying the dried chromatograms with a solution of 0.4% (w/v) ninhydrin in n-butanol saturated water (Nin) and heating them for 10-15 mins. at 100 - 110°C. The purple spots which developed could be preserved for a while, if need be, by adding collidine (0.2 ml.) to the ninhydrin solution (100 mls.) developing at 80°C for 2 - 3 mins., and storing the papers at 2°C in polythene envelopes in the dark (Woiwod 1960)

(3) Glucose was detected on paper chromatograms by spraying them with "aniline hydrogen phthalate" reagent and heating for 5 mins. at 105°C. (Partridge 1949).

(d) Elution of compounds from paper chromatograms.

The region carrying the component to be studied further was excised together with a piece of similar dimensions from an adjacent free part of the same paper. Both pieces, shaped to a point at one end, were continuously eluted in an all glass apparatus with 2 - 3 mls. of solvent -0.01 N-HCL, distilled water, or 50% aqueous ethanol (spectrographically pure, BDH Ltd.) The eluates were then read against each other in the ultraviolet region, using a Unicam SP 500 spectrophotometer, before being taken to dryness under vacuum over phosphorus pentoxide and sodium hydroxide. The residues could be redissolved in water or 50% aqueous ethanol and submitted to (further) chromatography if evidence of their identity was required.

(e) Quantitative examination of chromatograms

Samples (0.01 - 0.05 mls.) were applied as bars (1 cm.) chromatographed on Whatman No.1 paper in BAW (4 : 1 : 1) and developed with ferric chloride/ferricyanide (Fe^2) reagent. The chromatograms were cut lengthwise into strips (3 cms.) which were cleared in high-boiling paraffin and pressed between glass plates. The optical density of the blue zones were determined and recorded (Sunvic potentiometric recorder) by continuous, automatic

traverse at 1.2 inches/minute through a densitometer built in this laboratory by Dr. P.W. Kent. The light used (yellow filter) passed through a slit of width 0.05 inches.

This instrument was primarily employed to check the identity and recovery of the phenolic compounds eluted from the cellulose column described below. The results were quantitated by running standards of known substances on the same chromatograms and by comparing the ratio of areas under the curves plotted on the chart.

(vi) Column chromatography

Phenols, amines and amino acids were separated by partition chromatography on a column (3 x 55 cms.) of Whatman cellulose powder (standard grade ashless) using BAW (4 : 1 : 1) as the fluid phase (Awapara, Davis and Graham 1960). The powder was purified by washing with 2.1 of the following liquids, in the order:-

distilled water, 0.1 N - HCl, distilled water, methanol, n-butanol, BAW (4 : 1 : 1).

The powder was packed, as a slurry in n-Butanol, into the column under gravity and the column liquid-volume was determined by

running the BAW (4 : 1 :1) mixture through (flowrate 7-8 mls./ 10 mins.) while the eluate was tested with an indicator (screened methyl red – 3 parts bromocresol green (0.1%), 2 parts methyl red (0.1%)) for the first sign of the appearance of the acidified phase. The column, which had a sintered glass (porosity No.1) plate 4 cms. above the outlet, had an outer jacket filled with water maintained at 23°C. The inner walls of the column were previously covered with a film of silicone oil (0.5 mls.) introduced in diethyl-ether (10 mls.) while the column (horizontally inclined) was rotated until the ether had evaporated to leave a thin film of the oil.

A sample (0.5 - 1.0 ml.) of the aqueous solution to be chromatographed was mixed with 4 parts by volume of n-butanol and 1 part of glacial acetic acid and was filtered through a sintered-glass (porosity 2) funnel before being applied to the column. After most of the "dead volume" of solvent (500 mls.) had emerged from the column, 150-170 fractions (5 ml.) were collected with a Towers, Model B fraction collector. The partition curve and the nature of the eluted substances were established by recording the ultraviolet absorption of most fractions and by chromatography of some samples (0.01 -0.1 ml.) on paper as before. Larger samples (0.1 - 1.0ml.) were concentrated in hot air before application to the paper.

(vii) Ion exchange

Amines, in incubated homogenates (0.5 - 1.0 ml.) largely deproteinized by centrifugation (40,000 r.p.m. for 1 hour) were separated from phenols and amino acids by absorption on Amberlite IRC (H^+), type[1] (passing 200 mesh) resin (0.1 g.) by the method of Awapara, Davis and Graham (1960). The resin, which was shaken with the solution for 1 hour, was poured on to a column (0.5 x 5-6 cms) of the same resin (1 g.), eluted with glass distilled water (20-30 mls.) -- to remove all non-amines -- and then with 4 N-- acetic acid or 1 N- HCl (20 mls.) to recover the amines for further investigation. The resin was purified by the method of Partridge and Davis (1958).

Dowex 50 (Na^+) (200 mesh x 8 crosslinkage) purified by the method of Hirs, Moore and Stein (1953), was used to separate each of the amines and amino acids from each other (Bertler and Rosengren 1959). Proteins were removed from the incubated homogenates (0.5 ml.) of gland by adding 0.4 N- $HClO_4$ (0.5 ml.), and centrifuging (3,000 r.p.m. for 30 minutes) at 0°C. The supernatant was neutralized (pH 4.0), with 2 N- K_2CO_3 solution and centrifuged (1,000 r.p.m. for 10 minutes) again, at 0°C, then introduced to the top of the column (1.5 x 6 cms.) which had been

prepared by the method of Bertler, Carlson, Rosengren and Waldeck (1958). Subsequently a step-wise elution under slight air pressure (2 cms. Hg) with the following concentrations (N-) of HCl (100 mls.) was performed:

0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, and finally 3.0N- .

A Towers Model B fraction collector was used to collect the samples (5ml.) of the elute. The adsorption of each fraction at 280 mμ was recorded with a Unicam SP 500 spectrophotometer.

(viii) Paper electrophoresis

Dialysed 'exudate' of left colleterial gland was subjected to electrophoresis on (7 x 34 cm.) strips of Whatman No. 1 or No. 3 paper held between glass plates (9 x 25 cms.) using a Shandon type perspex tank. The following buffers (pH 8.0) were employed:

sodium phosphate (0.1M-); tris (hydroxymethyl) amino ethane-HCl (0.05 M-); and sodium diethyl barbiturate-HCl (0.05M) (Gomori 1955). The current was supplied by two, 120 V dry cell batteries in series. The current was reversed for each successive run in the same buffer.

The strips of paper were dipped in the buffer solution to be used and blotted before the 'exudate' (0.01 mls) and bovine serum albumen were applied by pipette. Proteins detected on the dried paper by development for 10 mins. with a solution of 1% (w/v bromophenol blue in ethanol saturated with mercuric chloride, excess of which was removed from the paper by washing in 0.5% acetic acid.

Phenolic compounds were separated by electrophoresis in sodium tetraborate (9.54 g./l.) (Coulson and Evans 1958) and in 0.1 M- sodium phosphate buffer (pH 6.0). The methods used to detect these substances on the dried paper strips have been described under section (c) p.34.

(ix) Ultraviolet absorption spectra.

Spectral properties of phenolic and amino-compounds (both reference and those eluted from paper chromatographs, ion-exchange or cellulose columns) were measured in matched 1 cm. silica cuvettes using the Unicam SP 500 spectrophotometer or the Carey recording spectrophotometer. The optical density (in 0.01N-HCl, distilled water, spectrographically pure ethanol or BAW (4:1:1)) was determined against a solvent blank at wavelengths between 200 and 310 mμ.

(x) Centrifugation

Enzyme preparations were centrifuged in tapered tubes using a Gallenkamp or MSE instrument at 1000 - 3000 r.p.m. to separate the tubule debris present after preparation.

Attempts to fractionate the 'exudate' were made using the International refrigerated model, with belt-driven high-speed attachment, at speeds up to 18,000 r.p.m. (26,000 g.). The Model L Spinco preparative ultra-centrifuge was used to achieve separation at forces up to 125,000 and 105,000 g. (Average R.) respectively (40,000 r.p.m.) in plastic tubes.

The Svedberg, oil turbine, analytical ultracentrifuge with the optical system of Baldwin (1953), was used (6 cm. head, 900 revs/second (182,000 g.) at 28°C) to attempt the resolution of proteins in concentrated 'exudate'.

(xi) Protein determinations

(a) Trichloroacetic acid (TCA) method

An equal volume of 10% TCA was mixed with 1 ml. of gland 'exudate' and allowed to stand for 10 minutes. The resulting precipitate was filtered off on weighed, dried discs of Whatman No. 1 paper, washed with glass distilled water (10 mls.) dried at 105°C for six hours and re-weighed.

The result was expressed in mgs. of TCA precipitate.

(b) Ninhydrin method.

1 ml. of 'exudate' solution (or fraction of) was hydrolysed with 12 N- HCl (1 ml.) in a sealed pyrex tube for 6 hours at a 100°C. The tube was opened when cool and the contents quantitatively transferred into a 10, 25 or 100 ml. volumetric flask with distilled water (preferably) or with 50% ethanol. 35% (w/v) sodium carbonate solution (2 mls.) was added carefully to neutralise (pH 5) the hydrolysate before the solution was made up to the mark. The total amino acids present in a sample (1, 2 or 4 mls.) of the diluted hydrolysate were determined by the methods of Moore and Stein (1954) using 1 ml. of a reagent of Ninhydrin-hydrindantin in methoxy-ethanol buffered with 4 N- sodium acetate (pH 5.5).

The colour was developed by heating the tubes in a boiling water bath for 20 minutes, and cooling them (with occasional shaking) in the dark with a current of air for 15 minutes. (It was found desirable to pass oxygen into each tube for 30-40 seconds before shaking to oxidize the residual hydrindantin.) Each fraction was then diluted to 5 or 10 ml. with 50% ethanol. The optical density at 570 mμ.

was determined with a Unicam SP 500 spectrophotometer. DL-leucine (0.05 umoles/ml.) in 0.1 M- citrate buffer (pH 5.0) and bovine serum albumen (2-10 mg. in 2 mls. of 6N-HCl, hydrolysed and diluted as described above) were used as standards in the ranges 0.01 - 0.1 µmoles and 1 -12 µg. respectively. The calibration curves for these standards are shown in figure 10.

(xii) Copper estimation

The copper content of 'exudate' proteins was determined by adding 1 ml. of 2,2' - diquinolyl in glacial acetic acid (0.5 mg./ml) (Kertesz 1957) to 2 mls. of the resuspended (in water or 10% acetic acid) residue of protein (or TCA precipitate) which had been digested with concentrated nitric acid (5 - 10 drops) in a muffle furnace at 180°C. Ashing at higher temperatures would involve correcting for the loss of volatile, covalent $Cu(NO_3)_2$ (Addison and Hathaway 1958). A crystal of hydroxylamine was added (to reduce the copper to the cuprous form), and the colour was estimated (after four hours) at 540 mµ with the Unicam SP 500 instrument. Copper sulphate solutions (10µg./ml.), similarly 'digested' with nitric acid, were used as standards - the results were expressed as µg of copper/mg. of TCA precipitate (or as a %).

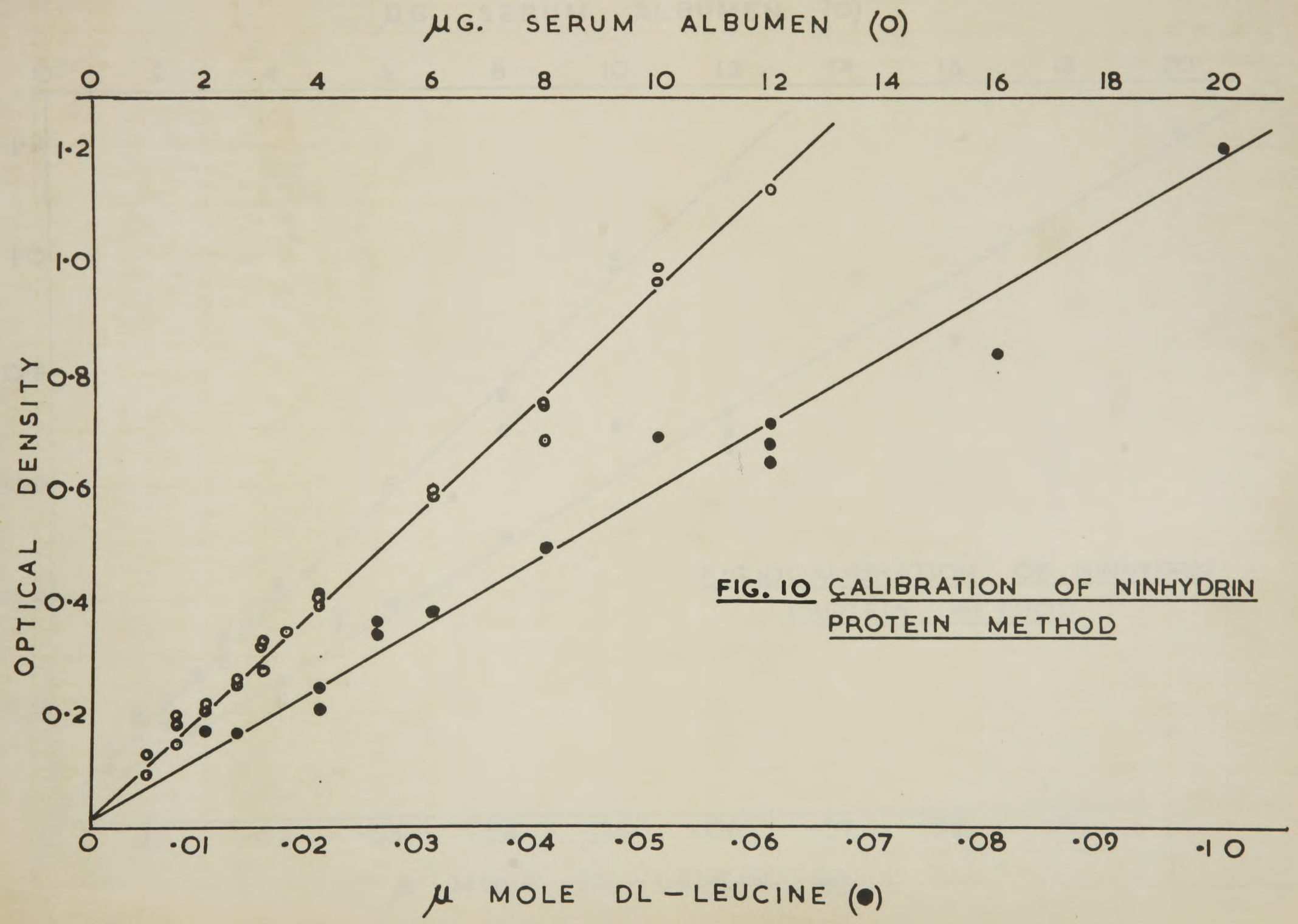


FIG. 10 CALIBRATION OF NINHYDRIN PROTEIN METHOD

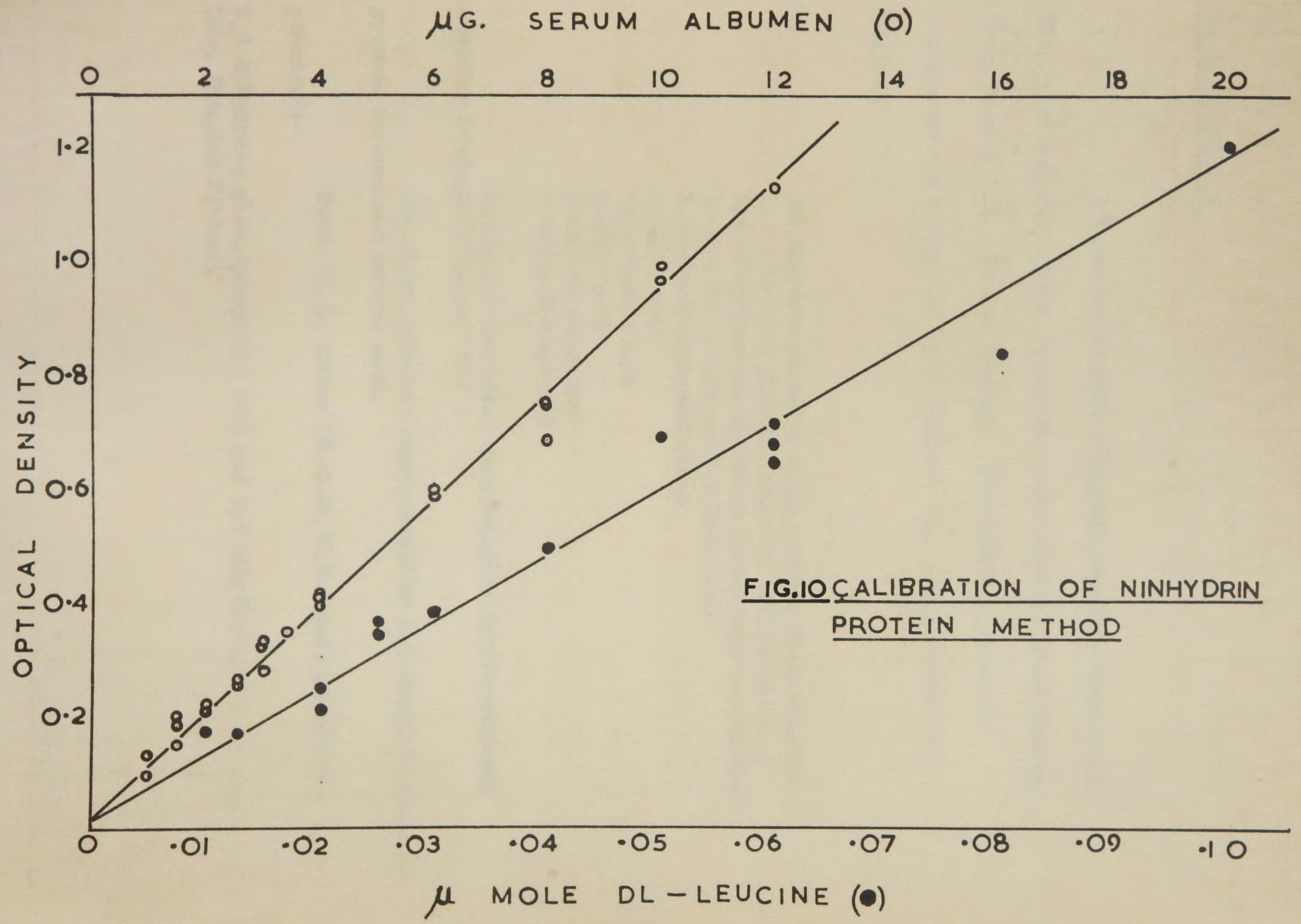


FIG.10 CALIBRATION OF NINHYDRIN PROTEIN METHOD

B. MATERIALS

Wherever possible AR grade reagents were used. The great majority of the chemicals were obtained from British Drug Houses Ltd., Poole, Dorset. The following phenolic compounds were supplied by L. Light & Co. Ltd., Colnbrook, Bucks:-

All dihydroxybenzoic acids (except Protocatechuic)
3,4-dihydroxyphenylethylamine HCL(dopamine)
3,4-dihydroxybenzaldehyde (protocatechualdehyde)
L- and DL-noradrenaline bitartrate
2,3-dihydroxynaphthalene
vanillic acid
chlorogenic acid
caffeic acid
4-chlororesorcinol
o-and p-flurophenol

L. Light & Co. Ltd., also supplied polyoxyethylene sorbitan trioleate ("Tween" 85)

Dr. R.M. Acheson kindly supplied 2,5-dihydroxyphenyl-pyruvic lactone and ferulic acid.

Prof. W.C. Evans (U.C.N.W. Bangor) generously provided:-

3,4-dihydroxy phenylpropionic acid and 3,4 dihydroxyphthalic acid (Mr. Douglas Ribbon).

From Roche Products Ltd., Welwyn Garden City, Herts, were obtained:-

L-3, 4-dihydroxyphenylalanine (L-dopa) and L (+) - ascorbic acid.

Calmic Ltd., Crewe, Cheshire, supplied polybactrin of which 1 g. contained:-

neomycin sulphate:	500 mg.
polymyxin 'B' sulphate:	100,000 units
zinc bacitracin:	25,000 units

Armour Hess Ltd., Chiswell Street, London, supplied bovine serum albumen (containing 15. 12%N, and 4% H_2O), penicillin and streptomycin.

The melting points of the phenolic compounds used were determined as a check on purity. It was found necessary to re-crystallise the samples of:-

protocatechuic acid - (water)
3,4-dihydroxybenzaldehyde - (water)
catechol - (toluene)
DL-dopa - (10% ethanol)
caffeic acid - (water)
chlorogenic acid - (water)

from the appropriate solvents shown.

SECTION I

THE PHENOLOXIDASE OF LEFT COLLETERIAL GLAND

CHAPTER THREE

INTRODUCTION.

Many reviews have been written on the important subject of phenolases (Dawson and Tarpley 1951, Lerner 1953, Mason 1955, Bonner 1957, Massart and Vercauteren 1959).

This group of oxidases all appear to contain the element copper - which is by and large responsible for their activity - and they promote the direct, aerobic oxidation of their respective substrates without the concomitant formation of hydrogen peroxide.

A. TYROSINASE

The most widely occurring and most investigated of these copper oxidases is tyrosinase or polyphenoloxidase (Dawson and Tarpley 1951). This enzyme is capable of the oxidation of both monohydroxy- and o-dihydroxyphenolic compounds.

(i) General properties

In spite of much research and continued interest in the polyphenoloxidases throughout a great many years, the knowledge today of the precise nature and fundamental properties of this enzyme group, and their role, if any, in cellular respiration, is very incomplete. Controversy still exists whether the monophenolase ("cresolase") and o-dihydroxyphenolase ("catecholase") activities can be attributed to the same enzyme, but in view of the evidence it is generally agreed that this is most likely so (Bonner 1957).

However, it is recognised that, although minor differences (e.g. colour and copper content) exist, the tyrosinase found in a wide variety of animals, insects, plants and micro-organisms is a similar type of enzyme. The enzyme from plants and lower animals appears to be less specific than mammalian tyrosinase (Lerner 1953). In general, tyrosinase from all sources - in so much as a comparison can be made of preparations with differing degrees of purity - show a relatively long induction or lag period during incubation with suitable monophenolic substrates. This lag is reduced or removed by presence of trace amounts of some o-diphenols, which appear to act by raising the redox potential of the system; and which can themselves, when in quantity, be oxidised much more efficiently than monophenols

(Lerner, Fitzpatrick, Calkins and Summerson 1949, 1951).

The products of this o-diphenol oxidation can inactivate the usually soluble plant phenoloxidases, whereas the not readily-purified, mammalian tyrosinase appears to be more stable (in the presence of dopa) (Lerner 1953).

Mason (1955) and Bonner (1957) have published excellent reviews on soluble plant phenoloxidases, the most common source of which are the edible mushroom, Psalliota campestris, and the white potato, Solanum tuberosum.

The main purpose of the enzyme in plants would not appear as in most animals, to be to produce melanin (though plant seeds can contain it) but it has long been speculated that its chief function may be as a terminal oxidase in energy yielding respiratory processes (Mason 1955, Fowlks and Peterson 1955, Bonner 1957). However, the polyphenoloxidase of the orchard grass is not thought to function as a terminal oxidase (Tookey 1959). Bonner (1957) considers the oxygen affinity of the copper enzymes to be too low to catalyze the direct transfer of electrons to oxygen. Polyphenols, rather than dopa and tyrosine, are the main substrates for the enzyme in plants.

The function of tyrosinase in melanization (Raper 1928, Mason 1955) as a whole will not be considered here, since melanin is not formed in the oothecal walls (Brunet 1952) and cuticle of Periplaneta (Dennell and Malek 1955c) nor in the normal, intact cuticle of some other insects (Malek 1957). The functioning of tyrosinase is known to be influenced by the presence of o-diphenols, by the redox potential, temperature, pH and the concentration of electrolytes (Lerner and Fitzpatrick 1950). Sulphydryl groups whether in a protein or reagent inhibit tyrosinase, but this effect can be readily counteracted (e.g. by iodoacetamide) (Flesch and Rothman 1948). Other reducing agents such as ascorbic acid and reduced DPN (i.e. NAD. H_2) increase the activity of the enzyme most probably by coupled oxidation-reduction with the o-quinones formed, (thus resupplying the tyrosinase with diphenols at the reductants expense) (Dawson and Tarpley 1951, Krueger 1958). (But the vitamin has been recovered unchanged after reaction according to Anderson, McElroy and Gray (1958).) If vitamin C is witheld from the diet of locusts, the growth and cuticle formation of the last nymphal instar and of the adult is found to be seriously impaired (Dadd 1960).

Ascorbic acid cannot reverse the inhibition of some tyrosinases, once caused, by the products of aerobic oxidation of

diphenols (Scharf and Dawson 1958), as it was once thought possible but it can protect liver p-hydroxyphenylpyruvic acid oxidase in vivo and in vitro from inactivation by substrate, if added initially (Zannoni and LaDu 1959, 1960). The implications of these observations on the activity of the tyrosinases in vivo are not clear.

Plant polyphenoloxidase, especially that of the cultivated mushroom, can oxidize the tyrosine bound in the peptide chain of proteins (Sizer 1953, Yasunobu and Wilcox 1958, Lissitzky, Rolland and Lasry 1960), whereas the mammalian enzyme appears to oxidize the amino acid only if it is terminal in the protein molecule (Lerner 1953). Tyrosinase from mammals is temporarily inhibited (i.e. the lag period is greatly lengthened) by high concentrations of tyrosine in the absence of diphenol. Miyamoto and Fitzpatrick (1957) found that phenylalanine competitively inhibited the enzyme.

Commercially prepared mushroom phenoloxidase was not inhibited by 5-hydroxytryptamine (serotonin) but the enzymes in homogenized larvae of meal worm (Tenebrio molitor) and in Harding-Passey mouse melanoma were (i.e. non-competitively). Mansour (1958) also found that the particulate phenoloxidase of homogenized liver fluke (Fasciola hepatica) could be inhibited (competitively) by serotonin. This enzyme oxidizes catecholamines and tyramine more

rapidly than dopa, catechol and tyrosine ethyl ester. On the other hand, pig pancreas tyrosinase is inhibited by tyramine, but the enzyme can only oxidize tyrosine and 3-fluorotyrosine (Schweet and Allen 1958).

Brown and Ward (1951, 1958, 1959) have succeeded in preparing a soluble mammalian tyrosinase (by chromatography on ion exchange, diethylaminoethyl cellulose (DEAE)) which they were able to purify fifty fold. Plant polyphenoloxidase has long been regarded as soluble, but there is some evidence that the enzyme is partly associated with cytoplasmic inclusions, the cell wall or even with mitochondria (Bonner 1957), but, unlike the particulate tyrosinase of mammals, it can be readily removed into solution. Kertesz and Zito (1957) have prepared soluble, electrophoretically pure enzyme (m. wt. 34,500) from edible mushrooms and more recently (Frieden and Ottesen 1959) it has been purified on DEAE cellulose.

(ii) Insect phenolases

(a) Protyrosinase

Tyrosinase occurs in some insects and lower animals as the inactive form (protyrosinase) (Bodine 1945a) which can be activated by detergents, heat, urea or solutions of some salts. The enzyme produced from grasshopper (Melanoplus differentialis) diapause eggs shows a variation in the ratio of monohydroxyphenolase and

dihydroxyphenolase activities and in stability, depending on the method of activation (Bodine 1945b) Monohydroxyphenols and o-dihydroxyphenols but not meta- or para-diphenols, are oxidized by this enzyme.

(b) Blood tyrosinase

The blood (Raper 1926, Bhagvat and Richter 1938) and tissues of most insects contain tyrosinase and tyrosine which react to form dopa and eventually melanin if the animal is wounded (Raper 1928). However, they are prevented from reacting in the intact, animal - until the onset of pupation - probably by the low redox potential of the haemolymph (Pryor 1955, Wigglesworth 1953). This effect may be caused by the presence of a dehydrogenase system, according to Dennell (1947), but Pryor (1955a) suggests that enzyme and substrate are kept apart due to the structure of the cytoplasm.

It is interesting to note that Heller and Szarkowska (1958) consider that in the cells of the hawk-moth (Celerio euphorbiae) a specific quinone reductase exists together with the polyphenoloxidase. A similar system in wheat-stem rust uredospores ensures that the phenolic substances are held in a reduced state until germination, after which they are released together with the polyphenoloxidase (Farkas and Ledingham 1959).

Onishi (1954c) found that brei of Drosophila tissue

could activate the inert pro-tyrosinase. He claimed that the activator was a non-dialysable protein, which could be destroyed by heat or urea treatment. The blood tyrosinase of Drosophila, produced by the oenocytes (localized with 'Nadi' reagent) which increase in number and then die away shortly before pupation (Dennell 1947), resembles the mushroom polyphenoloxidase in its properties (Onishi 1954a). Use of the 'Nadi' reagent has been criticized (for its non-specificity) by Dercleir, Aerts and Vercauteren (1960) who demonstrated aheat labile o-dihydroxyphenolase in haemolymph cells of Tenebrio molitor larvae .

Some 5 - 10 hours before pupation, depending on the insect, the levels of both tyrosinase and tyrosine reach a maximum (Daneel 1943, Onishi 1953). The pupation or moulting hormone released at this time from the Weismann's ring shaped gland (in larvae of Diptera) by raising the blood redox potential, in some way induces the enzymatic formation of dopa and some melanin (Dennell 1947). In caterpillars the hormone is produced by the prothoracic gland (Wigglesworth 1953, Karlson 1959).

Onishi (1954b) claims, however, that there is no rise in blood enzyme and substrate level during pigmentation of adult Drosophila, as in the larvae and pupae. The significance of this-

apparent exception is not clear, but he did state also that the adult cuticle phenolase was different in properties from that of the pupal cuticle.

(c) Cuticle oxidase

Comparatively little is known about this phenolase for knowledge of the enzyme and its localization has been, until recently, based almost entirely on histological tests, such as that employing the 'Nadi' reaction (dimethyl-p-phenylenediamine and alpha-naphthol oxidized to indophenol blue) (Mason 1955).

The oxygen needed for the enzymatic oxidation of diphenols in the cuticle is supplied not from the outside but from within by way of the tracheal system, (Dennell 1958c). Before moulting has taken place in Calliphora larvae the diphenols in procuticle are protected from oxidation possibly by low oxygen tension and lack of enzyme, for according to Dennell (1958c) the oxidase, which may be a sterol-protein complex, itself migrates inwards from the epicuticle. The polyphenol layer of the epicuticle (Fig.1) is due, he thinks in this case, to protein with high tyrosine content, and not to free diphenols.

In 1944, Nelson and Dawson considered that there was some doubt whether insect blood and cuticle copper oxidases were similar in

nature. The phenolase of (final instar) Drosophila virilis larval cuticle did not appear to resemble typical insect blood tyrosinase because, for one thing, according to Onishi (1954a), the cuticle enzyme alone could oxidize p-phenylenediamine. The enzyme in "blood and guts" of Calliphora larvae is often inactive towards tyramine whereas the "skins" or cuticles are very active (Pryor 1955a). In larvae of Protophormia terrae-novae and Calliphora vomitoria it appears that the blood enzyme can be inhibited by 0.01 M-phenylthiourea but the cuticle oxidase is unaffected, for tanning still takes place (Dennell 1958a). Because of this, Dennell suggests that the diphenol produced in the blood is not the precursor of the tanning quinone, but this is not the popular view (Mason 1955).

Pryor's (1940b) suggestion that cuticle oxidase utilizes protocatechuic acid directly has been recently challenged by implication by Aerts, Vercauteren and Decleir (1960), who claim that the phenolase of Tenebrio Molitor oxidizes monophenols (p-hydroxyphenyl-pyruvic, p-hydroxyphenylacetic and p-hydroxycinnamic acids) preferentially to o-diphenols (e.g. caffeic acid). They claimed that this phenolase differs from the blood tyrosinase in its temperature coefficient and its sensitivity towards iodoacetamide. These workers were unable to repeat Dennell's (1958) non-specific hydroxylation

(of phenylalanine) experiments using Calliphora erythrocephala larvae (last instar) preparations.

B. LACCASE

This copper oxidase, discovered in the latex of lacquer trees (Bertrand 1895) is responsible for the formation of the black lac, by the oxidation of polyphenols. It is present in a variety of plant leaves, fruit, vegetables and fungi (Bonner 1957).

The enzyme has been extracted and investigated by Keilin and Mann (1939), Bertrand (1945), and Gregg and Miller (1940), who showed, among other things, that the activity did not depend on a manganous ion, as Bertrand (1897) originally believed.

Although it occurs together with tyrosinase in plants, laccase is distinguishable from it (Dawson and Tarpley 1951) because it oxidizes a variety of di- and polyphenols but not resorcinol or any monophenol; it oxidizes L-ascorbic acid, p-phenylene diamine and hydroquinone, it is apparently insensitive to carbon monoxide inhibition; in the manner of ascorbic acid oxidase; it possesses a blue colour which can be removed but at the expense of the activity of the enzyme and the enzyme is associated with a polysaccharide component (45%) (Nakamura 1958a).

However, Lindberg (1948, 1950) has shown that there is a relationship between laccase and phenoloxidase in Hymenomycetes; for the latter is found in the mycelia and the former in the fruiting bodies. This distribution is reversed in the mushroom Psalliota bispora, albida (Lindberg 1950). Bonner (1957) suggests that there might be free interconversion between these two enzymes and between ascorbic acid oxidase. Keilin and Mann's (1938) observation on the "cresolase" and "catecholase" activities of phenoloxidase is cited by Bonner (1957) when he suggests that this enzyme, laccase and ascorbic acid oxidase are identical and only show different specificities because of the presence or absence of certain co-factors, but tangible evidence of this theory is lacking so far.

Laccase is inhibited by cyanide, azide and other metal chelating agents, such as diethyldithiocarbamate (Bonner 1957) but it is not as sensitive to 2,3-dimercaptopropanol (BAL) as a polyphenol-oxidase (Bertrand 1948).

The laccase of the Chinese lacquer tree (Rhus vernicifera) has been purified (checked by sedimentation studies) by zone electrophoresis and column chromatography on an ion-exchange (Amberlite) resin (Nakamura 1958a). The purified enzyme showed the usual protein absorption maximum at 280 mμ but it also exhibited a maximum at 615 mμ and a shoulder at 330 mμ which both disappeared

upon adding a reducing agent (ascorbic acid or ferricyanide). Contrary to earlier statements by Tissieres (1949) and others (Dawson and Tarpley 1951), purified laccase was unable to oxidize reduced heart muscle cytochrome C (Nakamura 1958a). The enzyme was not precipitated by 5% T.C.A. or by heating at 100°C for two minutes at neutral pH, but it was inactivated and lost its blue colour irreversibly by these treatments. (Nakamura 1958a).

Malmstrom, Fahraeus and Mosbach (1958) found that the formation of laccase (in mycelia of Polyspora versicolor) could be induced by 2,5- Xylidene. The enzyme which was secreted into the culture medium was homogeneous (determined by ultra-centrifugation, electrophoresis, end group determinations and copper analyses).

C. THE ROLE OF COPPER IN PHENOLASES

In 1937, Kubowitz demonstrated that the phenoloxidase of potato (50% pure) contained copper (0.12%) which was proportional to the "catecholase" activity of the preparation.

He was able to show that the enzyme lost most of its activity when the element was removed by dialysis after cyanide treatment. Readdition as $CuSO_4$ of ten times the amount of copper removed, and not the addition of iron, zinc manganese, cobalt or nickel, restored the activity.

Allen and Bodine (1941) found that twice as much copper originally present in grasshopper egg protyrosinase was necessary to restore complete activity and Lerner, Fitzpatrick, Calkins and Summerson (1950) showed that replacement of the copper that had been removed from mouse melanoma tyrosinase by cyanide treatment restored 90% of the enzyme activity towards dopa. Before and after the cyanide treatment the latter enzyme contained 0.19 and 0.09% copper respectively.

James (1953) has reviewed the subject of copper oxidase inhibitors and Dawson and Tarpley (1951) have discussed those specific to phenol oxidase. (Richter (1934)) suggested the use of various phenolic substances as competitive inhibitors of phenolase activity.)

The best preparation of Keilin and Mann (1938) of highly purified polyphenoloxidase from Agaricus campestris contained 0.3% copper, was free of haematin and very sensitive to KCN, H_2S and CO. Dalton and Nelson (1939), crystallised some of their purified enzyme (10%) from the wild mushroom (Lactarius piperatus) and found that the monophenolase and diphenolase activities were both proportional to the copper content (0.25%).

In vivo the tyrosinase of the common mushroom is believed to contain 0.1% copper, which is thought responsible for both the equivalent mono and di phenolase activities (Malette and Dawson 1949). Upon purification, however, fragmentation of the protein complex with no loss of copper apparently took place resulting in a high "catecholase" type of enzyme with four copper atoms (i.e. 0.25% (w/w)) per molecule of m.wt. 100,000).

On the basis of carbon monoxide inhibition studies, Kubowitz (1937, 1938) suggested that tyrosinase copper is first reduced to the cuprous state by the diphenol and is subsequently reoxidized by molecular oxygen (equation 1).

$$4\ Cu^{++}\text{-Enzyme} + 2\ \text{catechol} \longrightarrow 4\ Cu^{+} - \text{Enzyme} + 2.\text{o-quinone} + 4H^{+}$$

$$4\ Cu^{+} - \text{Enzyme} + 4H^{+} + O_2 \longrightarrow 4\ Cu^{++} - \text{Enzyme} + 2H_2O$$

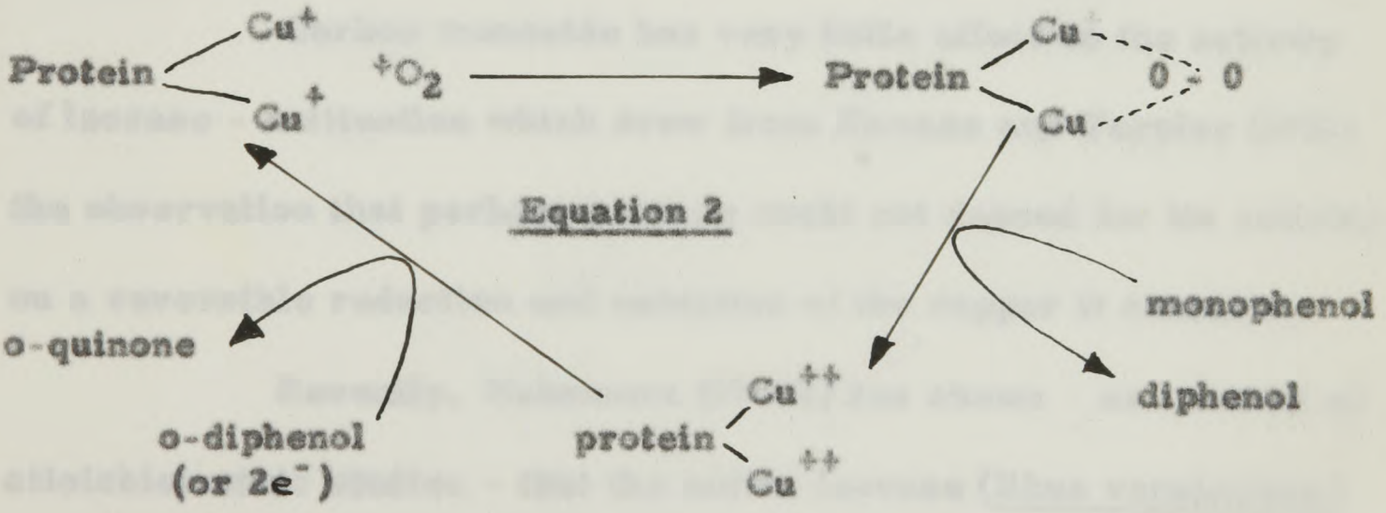
Equation 1

Carbon monoxide inhibits the enzyme only after addition of substrate and, unlike cytochrome oxidase, this inhibition is not by light reversible.

Mason (1955) believes that oxygen may play a more direct role in the tyrosinase system, since the enzyme, in the presence of

diphenols, activates oxygen (0^{18}) for hydroxylation of the aromatic ring of monophenols (Mason et al 1955).

Because of this and because Kertesz (1957) found that oxidized polyphenoloxidase of mushroom contained cuprous ions, Ingraham (1959) suggests - and leads evidence to the effect - that this type of phenolase may not function through a cuprous-cupric couple as Kubowitz (1937, 1938) had thought. Kinetic studies indicate that the enzyme reversibly reacts with oxygen before it combines with a diphenol (Ingraham 1957). This is in agreement with Mason's et al (1955) "oxygen transferase" definition for polyphenoloxidase, the dual action of which may be as follows:-



There is some evidence that the copper of oxidised cytochrome C oxidase is also in the cuprous form (Wende and Wainio 1960) but that of the purified cytochrome a of Takemori, Sekuzu and Okunuki (1960) appears to be in the cupric form.

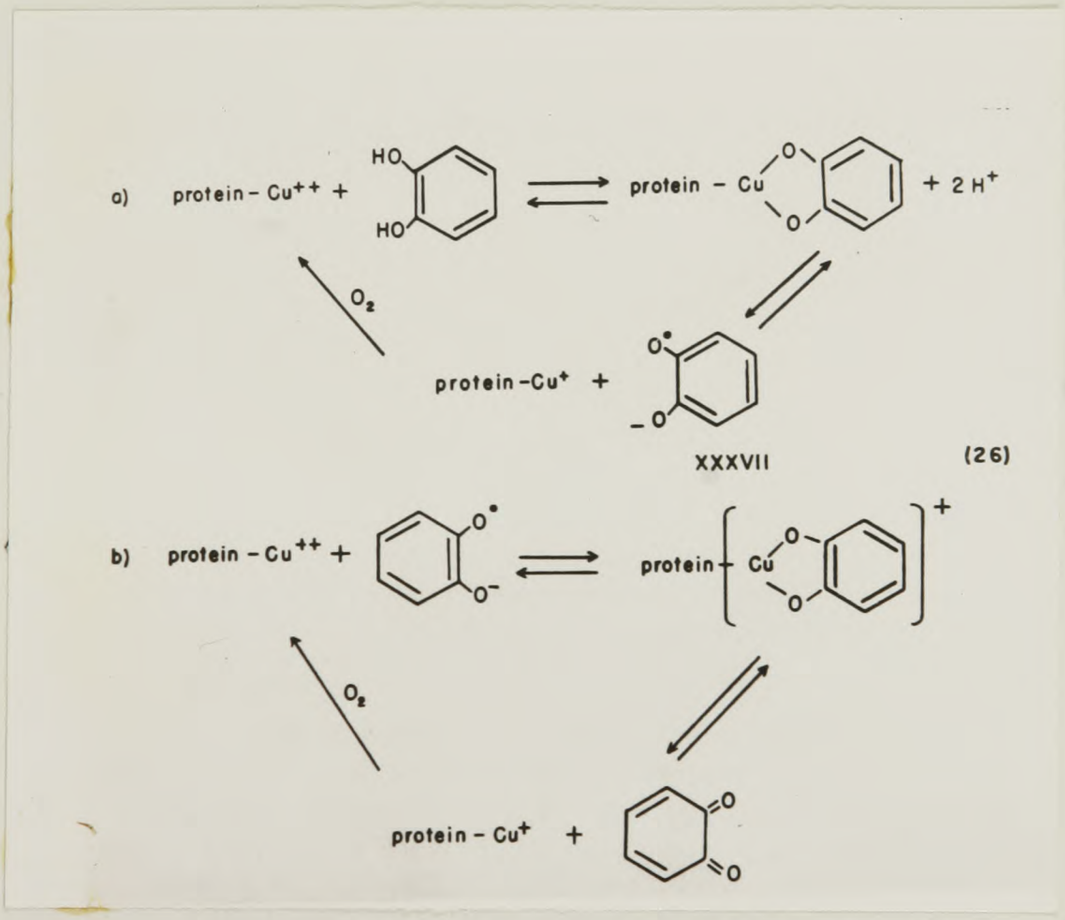
The possibility that the cuprous enzyme is the phenol o-hydroxylase of the phenolase complex has been discussed (Mason 1955). The cupric enzyme would first have to be reduced by o-diphenols before monophenolase activity would be possible, as found in practice (Lerner et al 1949). Frieden (1958) warns that as well as diphenols, proteins themselves can reduce cupric ions.

Concerning laccase, Tissieres (1948) was able to remove the copper from a partially purified enzyme (Rhus succidanea) by dialysis against cyanide. He found that the enzyme could be reactivated after this treatment only by the addition of cupric ions.

Carbon monoxide has very little effect on the activity of laccase - a situation which drew from Dawson and Tarpley (1951) the observation that perhaps laccase could not depend for its activity on a reversible reduction and oxidation of the copper it contains.

Recently, Nakamura (1958b) has shown - as a result of stoichiometric studies - that the native laccase (Rhus vernicifera) is reduced by hydroquinone or ascorbic acid to the cuprous enzyme and subsequently reoxidised by molecular oxygen. He also demonstrated (1958c) that the ascorbic acid-reduced, native enzyme had a magnetic moment (measured by susceptibility with a Gouy balance) compatible with the

the presence of cuprous ions in the protein (which are less paramagnetic than the oxidized state of copper). The magnetic moment reverted to the original (cupric, native enzyme) value upon reoxidation with molecular oxygen. Studies by spectrophotometry and magnetometry on the kinetics of the reaction of reduced laccase and oxygen suggest that a $Cu^{+} - 0_2$ complex (suggested for phenoloxidase (Mason 1955)) does not exist in this reaction (Nakamura 1960).



EQUATION. 3 Oxidation of diphenols involving semiquinones (Mason 1955)

Finally, it was demonstrated that the copper content of purified native laccase was directly proportional to the optical density at 615 mμ (Nakamura 1958 b), and upon reduction of the enzyme this absorption band completely disappeared (1958a).

CHAPTER FOUR

RESULTS

A. PRELIMINARY EXPERIMENTS

(i) A preliminary survey designed to test for the presence of oxidative pathways linked to the tricarboxylic acid cycle, in the cells of intact, excised left colleterial glands met with negative results. The oxygen uptake of the glands suspended in an 'insect' ringer solution (1 ml.) was not significantly affected by the addition of D-glucose (10 mg.) or of the sodium salts of pyruvic, succinic or fumaric acids (1 mg. each) or of sodium fluoracetate (1.5 mg.)

The explanations advanced for this lack of effect were:

(a) that diffusion of the substrates into the cells was impaired by their thick basal membranes (see fig. 5)

or

(b) that the oxidation by the phenoloxidase of the relatively large amount of diphenolic compounds present in the secretion of the cells was sufficient to mask that of other metabolic pathways in the cells. This explanation seems feasible because the protocatechuic acid glucoside is known

to hydrolyse slowly after dissection of the gland and more so if any small piece of right gland tubule was enmeshed in the larger gland and had escaped detection.

As the introduction of protocatechuic acid (77 μmoles) to the ringer solution (1 ml.) bathing the whole gland did not increase the specific activity (oxygen uptake (μls) per mg. dry weight of gland) either, the decision was made to homogenize the gland and so disrupt the cellular, basal membranes. Hydroquinone and protocatechuic acid in 0.1 M-sodium phosphate buffer (pH 6.8) were, in fact, oxidized by homogenates of left gland. Therefore, the enzyme system thought to be responsible was subjected to further study in an attempt to characterize it.

(ii) The results of the initial survey of the specificity requirements of the phenoloxidase system present in the 'exudate' of left glands, which had been dialysed (cellophane) against glass distilled water at 3°C, are presented in Table 1 below. In these experiments the Q_{O_2} (μls O_2/mg. TCA precipitate) values were corrected only for the oxidation of the various substrates in the absence of 'exudate' i.e., for 'autoxidation' ($q_{O_2}^{auto}$) (See Table 1).

TABLE 1 THE IN VITRO OXIDATION OF SUBSTRATES [1] BY THE PHENOLOXIDASE SYSTEM CONTAINED IN THE SECRETION OF LEFT COLLETERIAL GLAND.

Substrates [1]	Protein [2] Concentration mg/ml.	Q_{O_2} Corrected for $q^{auto}_{O_2}$	Relative Activity	auto q_{O_2}
3 4-dihydroxy benzoic (protocatechuic)acid		4.47	100	0.59
3,4-dihydroxyphenylactic(homoprotocatechuic) acid	2.4	2.75	58	1.46
3,4-dihydroxyphenylpropionic acid	1.0	2.7	57	0.93
3,4-dihydroxybenzaldehyde (protocatechualdehyde)	1.0	2.11	44.5	0.12
3 4-dihydroxyphthalic acid	2.4	2.52	53.2	0
3,4-dihydroxycinnamic (caffeic) acid	3.0	3.44	72.5	1.25
2.5-dihydroxybenzoic (gentisic) acid	2.8	1.71	36.1	0.3
2 5-dihydroxyphenylacetic (homogentisic) lactone	1.0	3.32	70	20.9
hydroquinone	2.4	5.37	113.2	1.13
catechol	2.4	0.69	14.55	0.60
o-aminophenol	3.4	2.56	54	0.55
p-phenylenediamine	3.0	17.25 [3]	364	22.9
3-methoxy-4-hydroxycinnamic (ferulic) acid	4.0	0.85	17.9	0
chlorogenic acid	1.0	3.12	65.8	11.06

1. 3 µmoles
2. determined by the T.C.A. method
3. determined from slope between 10 and 20 mins. (see fig 12).

Fig: 12 Rate of oxidation of p-phenylenediamine (3 µmoles) with 'exudate' (●—●—●) and without (○—○—○).

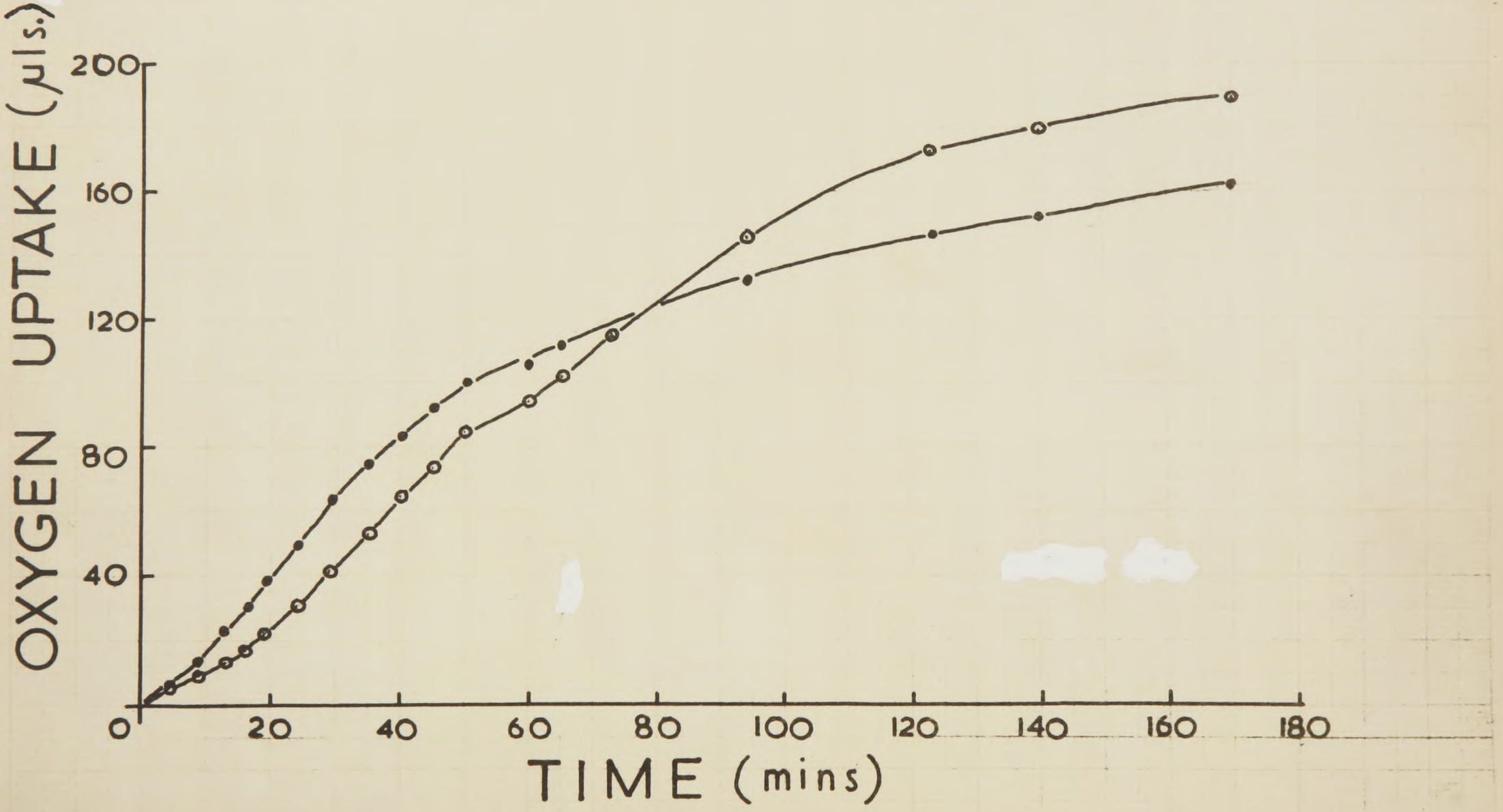


TABLE 2 THE COMPOUNDS [1] NOT OXIDIZED IN VITRO BY THE PHENOLOXIDASE SYSTEM OF LEFT COLLETERIAL GLAND.

Substrate	$q^{auto}_{O_2}$	Substrate	$q^{auto}_{O_2}$
L-p-tyrosine	0.47	phenylalanine	0.77
L-o-tyrosine[2]	0.26	phenylacetic acid	0
p-hydroxybenzoic acid	0	p-aminophenol[2]	17.6
salicylic acid	0	shikimic acid	0.5
resorcinol	1.1	vanillic acid	+0.7
pyrogallol[2]	29.0	L(+) ascorbic acid[2]	18.4
phloroglucinol[2]	1.2		
4-hydroxycinnamic acid	1.0		
L-3,4-dihydroxyphenylalanine[2] (dopa)			5.54
3,4-dihydroxyphenylethylamine (dopamine)			3.8
2,5-dihydroxyphenylpyruvic lactone			0
L- noradrenaline bitartrate			2.57
2,3-dihydroxynaphthaline			4.3
2,3-dihydroxybenzoic acid			0.04
2,6- " "			+2.0
2,4- " "			+1.7
3,5 " "			0.35

1. 3 µmoles in 1.5 mls. of 0.1 M-sodium phosphate buffer (pH 6.8)

2. Autoxidation partially inhibited by the presence of 'exudate' (see also for p-phenylenediamine-fig.12).

In the majority of cases a lag or "induction period" (during which no oxygen uptake occurred) of between 10 to 30 minutes was observed. Therefore the Q_{O_2} values (with the exception of that for p-phenylenediamine (see fig. 12) were determined between 40 - 100 or 60 - 120 minutes. The specific activity of each separate 'exudate' preparation used was determined with respect to 3 µmoles of protocatechuic acid each time and was found to be 3.93 ± 1.15 (10). This variation may be inherent or in part due to the error introduced by the method (T. C. A.) of protein estimation.

For the purpose of comparing the activities of substrates tested on different 'exudate' preparations, the specific activity for protocatechuic acid was taken as 4.74 (see Table 3).

Table 2 above lists the compounds tested that were found to be unoxidized by the phenoloxidase system in 'exudate' preparations. In most cases, the slight oxygen uptake found (Q_{O_2} of 0.2 - 0.6) was attributable to the oxidation of endogenous substrates, which appear to persist in the suspension in spite of prolonged dialysis against distilled water. No correction was possible here, but subsequent experiments included a suitable control.

The values for the autoxidation ($q_{O_2}^{auto}$) of the substrates (3 µmoles) are shown. (See Table 2).

(iii) The elimination of endogenous phenolic compounds from 'exudate' of left gland was thought to be necessary before the results found in the survey of the specificity requirements of the enzyme system could be substantiated. The preparations lost as much as half of their activity during storage, at 3°C for 2 to 3 days, possibly as a consequence of the auto- or enzymic- hydrolysis of the protocatechuic acid glucoside.

Chromatographic examination, of ethanolic extracts of 'exudate' revealed that the protocatechuic acid glucoside which was present in comparatively large quantities, was being hydrolysed during storage and even during incubation of fresh preparations.

In an experiment, which was carried out to attempt to eliminate the glucoside from freshly prepared 'exudate' 14 cockroaches were dissected. The secretion obtained from the left glands was subjected to the treatments shown in figure 13.

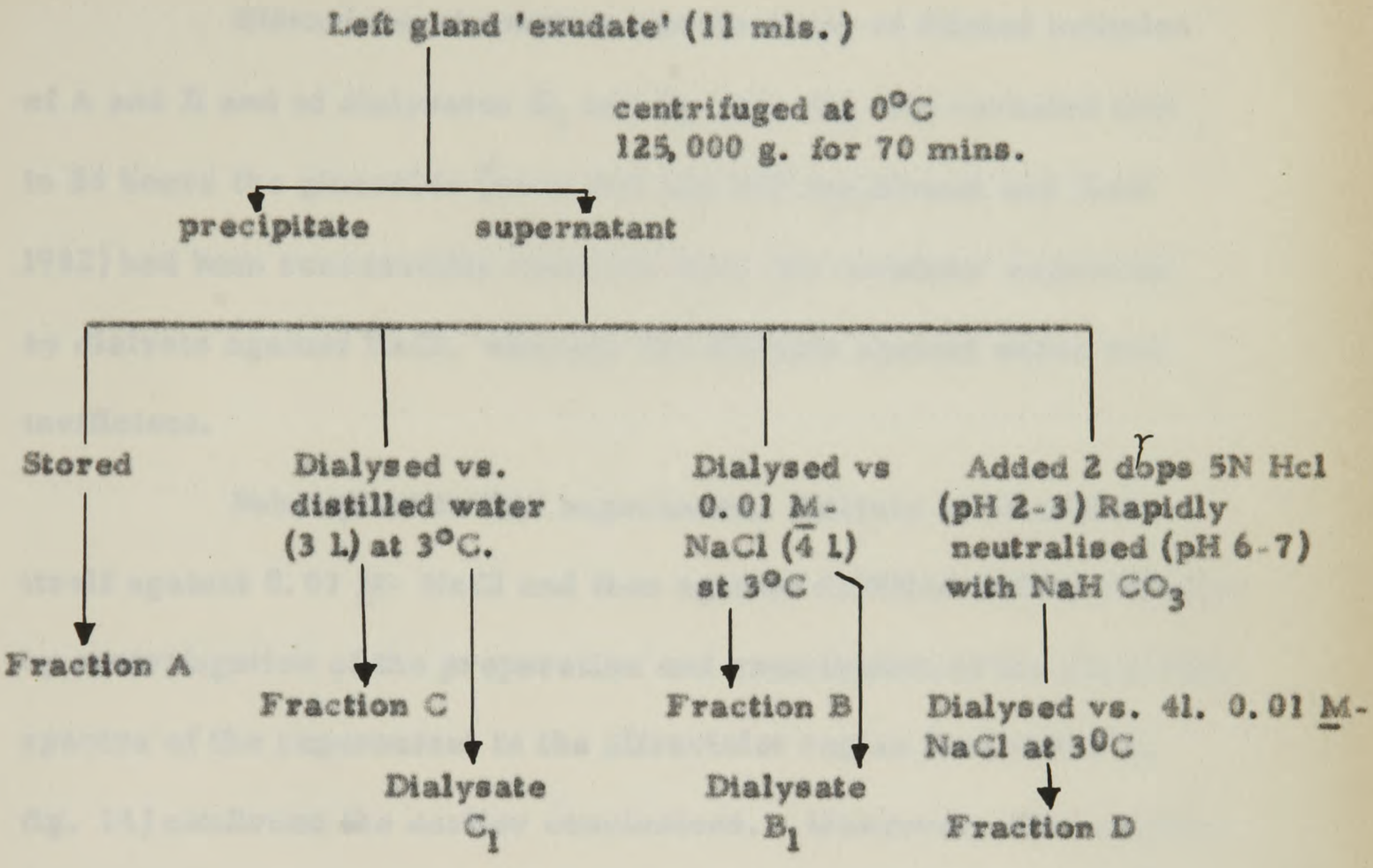


FIG: 13 Test of the effects of various treatments on removal of phenolic compounds from 'exudate'.

Chromatography of ethanolic extracts of fractions C, B and D (0.5 mls. extracted every 12 hours) showed that the treatment of fraction B was superior to treatments D and C in removing the glucoside. (See fig. 13).

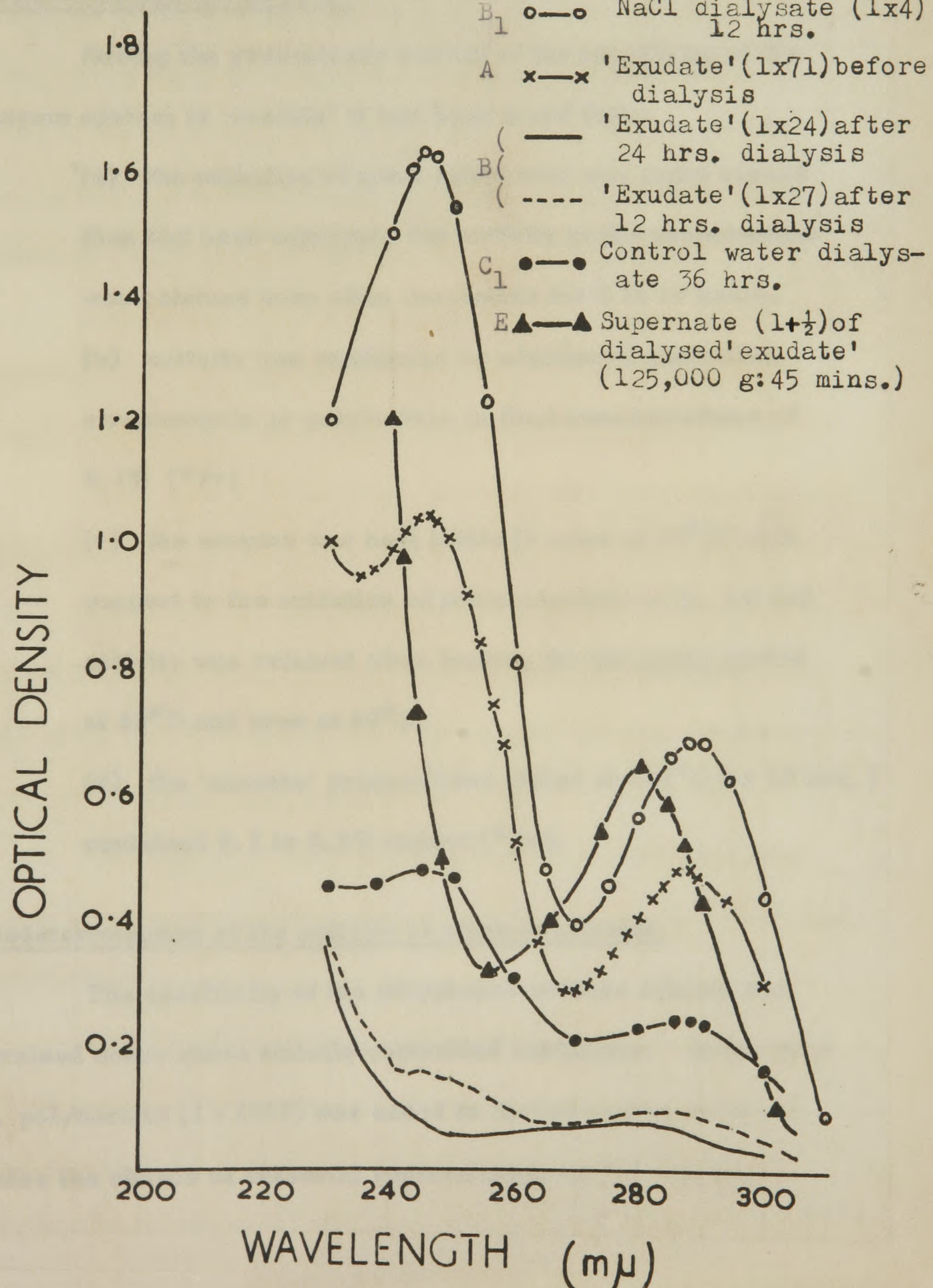
Ultraviolet absorption spectroscopy of diluted samples of A and B and of dialysates B_1 and C_1 (see fig. 14) revealed that in 24 hours the glucoside (λmax 245 and 288 mμ, Brunet and Kent 1952) had been successfully removed from the 'exudate' supernate by dialysis against NaCl, whereas the dialysis against water was inefficient.

Subsequent to this experiment, dialysis of 'exudate' itself against 0.01 M- NaCl and then against distilled water, followed by centrifugation of the preparation and examination of the absorbtion spectra of the supernatant in the ultraviolet region (see curve E, fig. 14) confirmed the earlier conclusions. Moreover, the 'exudate' preparations showed only 20% loss of activity after storage at 3°C or -15°C for a month.

The endogenous phenolic substances could be extracted from the NaCl dialysates (see Chapter 7).

Attempts to develop a spectrophotometric method to measure disappearance of protocatechuic acid as a means of assaying the phosphate buffered (pH 6.8) cockroach phenolase were unsuccessful.

FIG.14 EFFECT, ON 'EXUDATE', OF DIALYSIS AGAINST 0·01 M SODIUM CHLORIDE



B. EXAMINATION OF THE PROPERTIES OF THE LEFT GLAND PHENOLOXIDASE SYSTEM

During the preliminary survey of the specificity of the the enzyme system in 'exudate' it had been found that:-

(a) the oxidation of most substrates was much slower than had been expected, but activity of the preparations was retained even after incubation for 6 to 10 hours.

(b) activity was unaffected by addition of penicillin, streptomycin or polybactrin in final concentrations of 0.1% (w/v)

(c) the enzyme was heat labile (5 mins at 98°C) with respect to the oxidation of protocatechuic acid, but full activity was retained after heating for the same period at 50°C and even at 60°C.

(d) the 'exudate' preparations (dried at 110°C for 10 hrs.) contained 0.1 to 0.2% copper (w/w).

(i) Redetermination of the activity of some substrates.

The specificity of the polyphenol-oxidase system was reappraised under more strictly controlled conditions. In the first place, polybactrin (1 : 1000) was added to the dissecting saline to minimise the chance of bacterial contamination of the 'exudate'

preparations. Secondly, the rate of autoxidation - tested in the presence and absence of boiled 'exudate' (10 mins. at 98°C) - was determined for each substrate. Further, the endogenous oxidation of 'exudate' (Le) and boiled 'exudate' (Le*) (i. e. in the absence of substrate) was checked in case the removal of phenolic compounds by dialysis against NaCl had been inefficient.

The amount of substrate added to the enzyme preparation was increased to 15 μmoles in order that a greater, and more accurately measurable, rate of oxidation could take place.

The concentration of protein in the secretion suspensions was determined by the ninhydrin method described in chapter two page 43. The reliability and accuracy of the former T. C. A. method were suspect (because the proteins might not be wholly precipitated (see Hackman 1953 a, b) and because the method involved estimating small amounts of precipitate gravimetrically).

The results of redetermining the specific activities of those substrates shown earlier to be readily oxidizable are presented in Table 3 below.

The noteable features of the results of the experiment summarised in Table 3 are:

(a) that the oxidase activity toward catechol is heat stable, while the supposed "enzymic" oxidation of homoprotocatechuic acid

TABLE 3 DETERMINATION OF THE SPECIFIC ACTIVITY (Q_{O_2}) OF THE PHENOLOXIDASE SYSTEM OF LEFT GLAND 'EXUDATE' (Le) TOWARDS CERTAIN SUBSTRATES (S)

Column	1	2	3	4	5	6	7
	$q^{auto}_{O_2}(b)$		$q^{auto}_{O_2}(b)$				
Substrate (15 µmoles)	S-θ	Le-θ	LeS-S	LeS-Le	Le*S minus Le*	(LeS-Le) minus (Le*S-Le*)	LeS minus Le*S
protocatechuic acid	0.2	14.2	14.0	15.1	0.19	14.91	14.0
protocatechuic acid (a)				4.74			
catechol	6.0	6.82	0.8	7.74	7.37	0.37	0
hydroquinone	10.2	17,27	6.9	19.45	10.15	9.3	8.1
homoprotocatechuic acid	11.4	10.48	+0.9	11.7	13.89	+2.2	+3.27
protocatechu -aldehyde	1.3	3.2	1.9	4.14	1.89	2.07	1.79
caffeic acid	4.7	7.21	2.5	-	-	-	2.79
gentis ic acid	0.28	0.92	0.6	-	-	-	+0.18

Le* – boiled exudate (10 mins. at 98°C)

(a) – 3 µmoles

(b) – c.f. Table 1

θ – thermobarometric control

1 ml. of 'exudate' containing 2.9 mg. of protein (Nin hydrin method), was incubated at 37°C in 0,1 M sodium phosphate buffer (pH 6.8) Total volume 1.5 ml.

is actually increased (18.8%) by boiling the 'exudate' preparation (column 7).

(b) that a five-fold increase in the amount of protocatechuic acid introduced to the 'exudate' results in an increase of 320% in the specific activity (column 4). The activity toward catechol and hydroquinone (cf. column 3 with Q_{O_2} Table 1) appears to be correspondingly only slightly increased, whereas that of homoprotocatechuic acid, protocatechualdehyde caffeic acid and gentisic acid actually shows a decrease when more substrate is added!

(c) that the rate of oxidation of substrate in buffer alone (cf. column 1 and $q_{O_2}^{auto}$ Table 1) shows a greater, than five-fold increase for catechol, hydroquinone, homo-protocatechuic acid and protocatechualdehyde when 15 μmoles of substrate are incubated in buffer. There is a decrease in the autoxidation rate of protocatechuic and gentisic acids, but some increase, although not five-fold, for caffeic acid.

(ii) The effect of certain substances on the enzymic oxidation of protocatechuic acid and catechol.

Table 4 shows that DL-dopa and catechol have some inhibitory effect on the enzymic oxidation of protocatechuic acid. Presence of a small amount (0.15 µmoles) of DL-dopa completely inhibits the activity of an enzyme preparation (supernatant D, fig. 17) towards protocatechuic acid (3 µmoles) (see also fig.15).

On the other hand p-tyrosine and ascorbic acid (0.15 µmoles each) both stimulate the oxidation of protocatechuic acid; the latter markedly so. (Table 4d, and fig.15).

The addition of one right colleterial gland, homogenized in 0.1 ml. of water, to protocatechuic acid in presence of enzyme appears to increase the activity of the preparation three-fold and to reduce the moderately long lag-period, characteristic of normal oxidation of substrate (fig. 15).

This induction period is not decreased significantly by the presence of concentrated 0.01M- NaCl dialysate of gland 'exudate'.

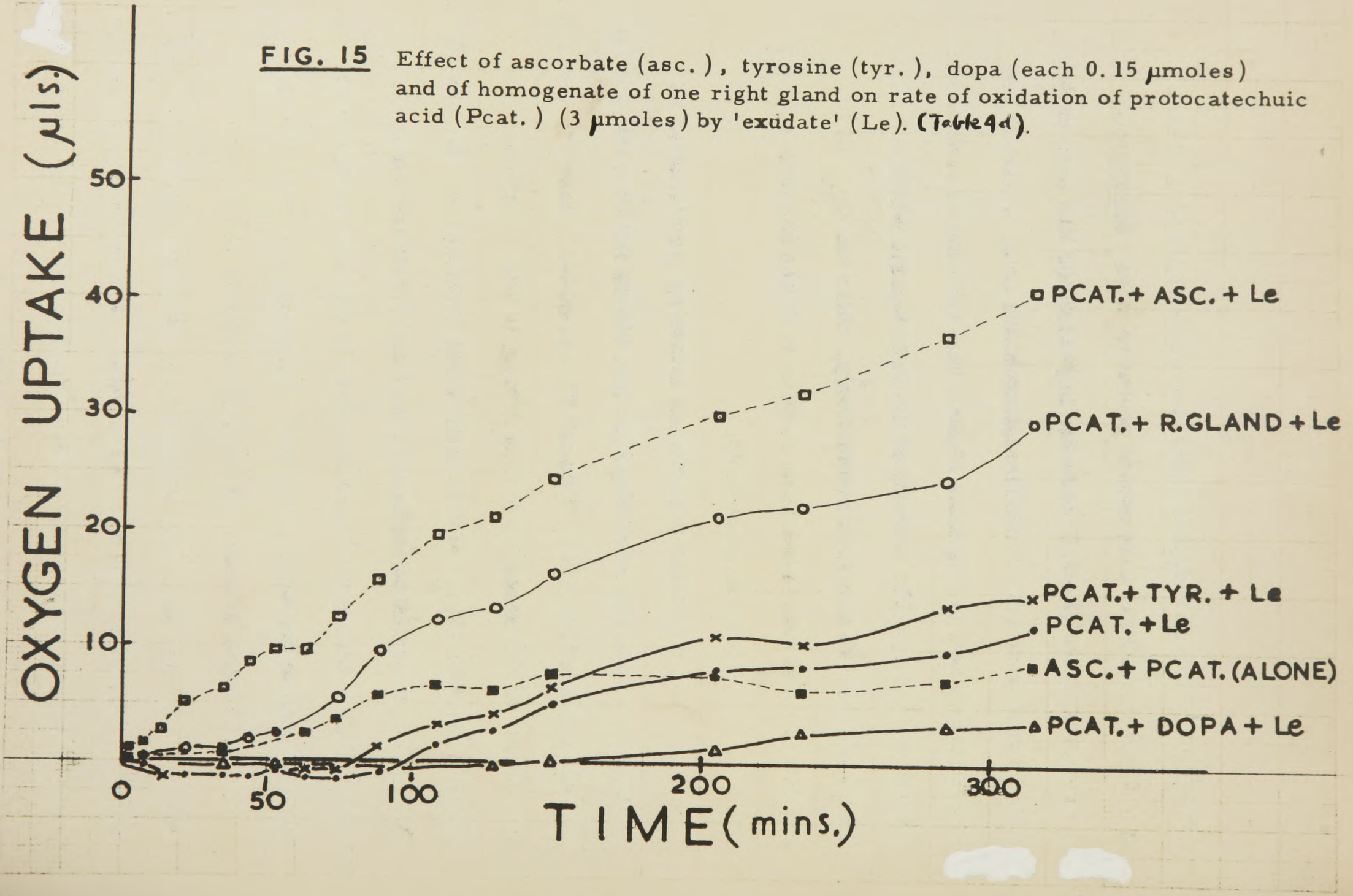
Detergents also influenced the activity of 'exudate' preparations. In one experiment 'exudate' was divided into samples (1.5 mls.) to one of which was added sodium deoxycholate (0.04 mls.

TABLE 4 THE EFFECT OF SOME SUBSTANCES ON THE OXIDATION OF PROTOCATECHUIC ACID AND CATECHOL BY LEFT GLAND PHENOLOXIDASE

	Substrate	µmoles	Substance added	µmoles	Protein T.C.A. mg/ml.	Le Q_{O_2}	Le* Q_{O_2}	$q^{auto}_{O_2}$	Corrected Q_{O_2}	% Inhibition(-) or stimulation(+)
	protocatechuic acid	3	-	-	3.0	5.84	(2.65)?	-	3.19	-
	protocatechmic acid	3	DL-dopa	3	3.0	6.37	3.69	-	2.68	- 16%
(a)	DL-dopa	3	-	-	3.0	1.26	2.66	-	+1.4	-
	catechol	3	DL-dopa	3	3.0	5.77	2.87	-	2.90	-
	catechol	3	-	-	3.0	4.70	1.77	-	2.93	-
(b)	protocatechuic acid	3	-	-	1.0	4.4	-	0	4.4	-
	protocatechuic acid	1.5	L(+) ascorbic acid	1.5	1.0	9.54	-	16.25	+6.71	+(± 100%) ?
	ascorbic acid	3	-	-	1.0	5.0	-	17.9	+12.9	-
(c)	protocatechuic acid	15	-	-	1.2				15.3	-
	protocatechuic acid	7.5	catechol	7.5	1.2				2.73	-(± 41%) ?
	catechol	15	-	-	1.2				+ 1.00	-
	protocatechuic acid	3	-	-	0.6	5.1	-	0.1	5.0	-
	protocatechuic acid	3	L-p-tyrosine	0.15	0.6	7.17	-	0.19	6.98	+39.6%
	protocatechuic acid	3	DL-dopa	0.15	0.6	5.6	-	5.68	+0.08	-100%
(d)	protocatechuic acid	3	L(+) ascorbic acid	0.15	0.6	18.3	-	7.83	10.47	+209.4%
	protocatechuic acid	3	right gland (1) homogenate	-	0.6	18.52	-			+364%
	protocatechuic acid	3	0.25 mls. of 20x concentrate of 0,01 M- NaCl dialysate		0.6	5.55	-			+ 8.8%

Le - Left gland exudate
Le* - boiled left gland exudate

FIG. 15 Effect of ascorbate (asc.), tyrosine (tyr.), dopa (each 0.15 μmoles) and of homogenate of one right gland on rate of oxidation of protocatechuic acid (Pcat.) (3 μmoles) by 'exudate' (Le). (Table 4d).



20% (w/v)). Both samples were then centrifuged (31,000 x g for 30 mins) and the sedimented particles were resuspended in distilled water (1 ml.), once the supernatants had been drawn off. Oxidase activity tests (Protocatechuic acid, 3 µmoles) on supernatants and precipitates showed that addition of cholate had increased the specific and total activity of the supernatant fraction from 1.44 and 1.44 to 3. 16 and 7.96 respectively, while the Q_{O_2} of the precipitate had increased from 1.03 to 1.77 and the total activity had decreased from 5.15 to 4.75.

In a similar experiment involving fractionation of 'exudate' (3 mls.) by centrifugation (63,000 xg for 40 mins.) to one sample 1 ml. of 5% (v/v) "Tween" 85 was added before fractionation. The effect of the presence of this detergent was to increase the specific activity (Q_{O_2}) of the supernatant fraction from 60.6 to 163.0 and of the resuspended sediments (in 1 ml.) distilled water) from 8.6 to 18.5 - with protocatechuic acid (15 umoles) as substrate. The lag-period for each of the four fractions was only 10 minutes.

After 4½ hours of incubation (Table 4a) 20% sodium deoxycholate (0.1 ml.) was added to each flask (containing 1.5 ml. of exudate, substrate and buffer as usual). During the next 90 minutes of resumed incubation at 37°C, the uncorrected and corrected specific activities were as shown in Table 5 below:-

TABLE 5: THE EFFECT OF SODIUM DEOXYCHOLATE ON THE SPECIFIC ACTIVITY OF 'EXUDATE' ALREADY INCUBATED IN THE PRESENCE OF SUBSTRATE (TABLE 4(a))

Substrate(s)	Le Q_{O_2}	Le* Q_{O_2}	(Corrected Q_{O_2}) Le - Le* Q_{O_2}
Protocatechuic acid	6.09	0.4	5.7
protocatechuic acid + dopa	4.57	1.83	2.74
dopa	1.38	1.74	+0.36
catechol + dopa	3.58	1.35	2.23
catechol	4.06	1.35	2.71

The results (Table 5) show that there was a significant increase in the oxidation rate (corrected) of protocatechuic acid, but only a very slight change for other substrates. In all cases, the oxygen uptake for substrates alone was decreased during this period (cf. Tables 5 and 4(a))

In a similar experiment to that described above, a non-ionic detergent or surface active agent ("Tween" 85) was added

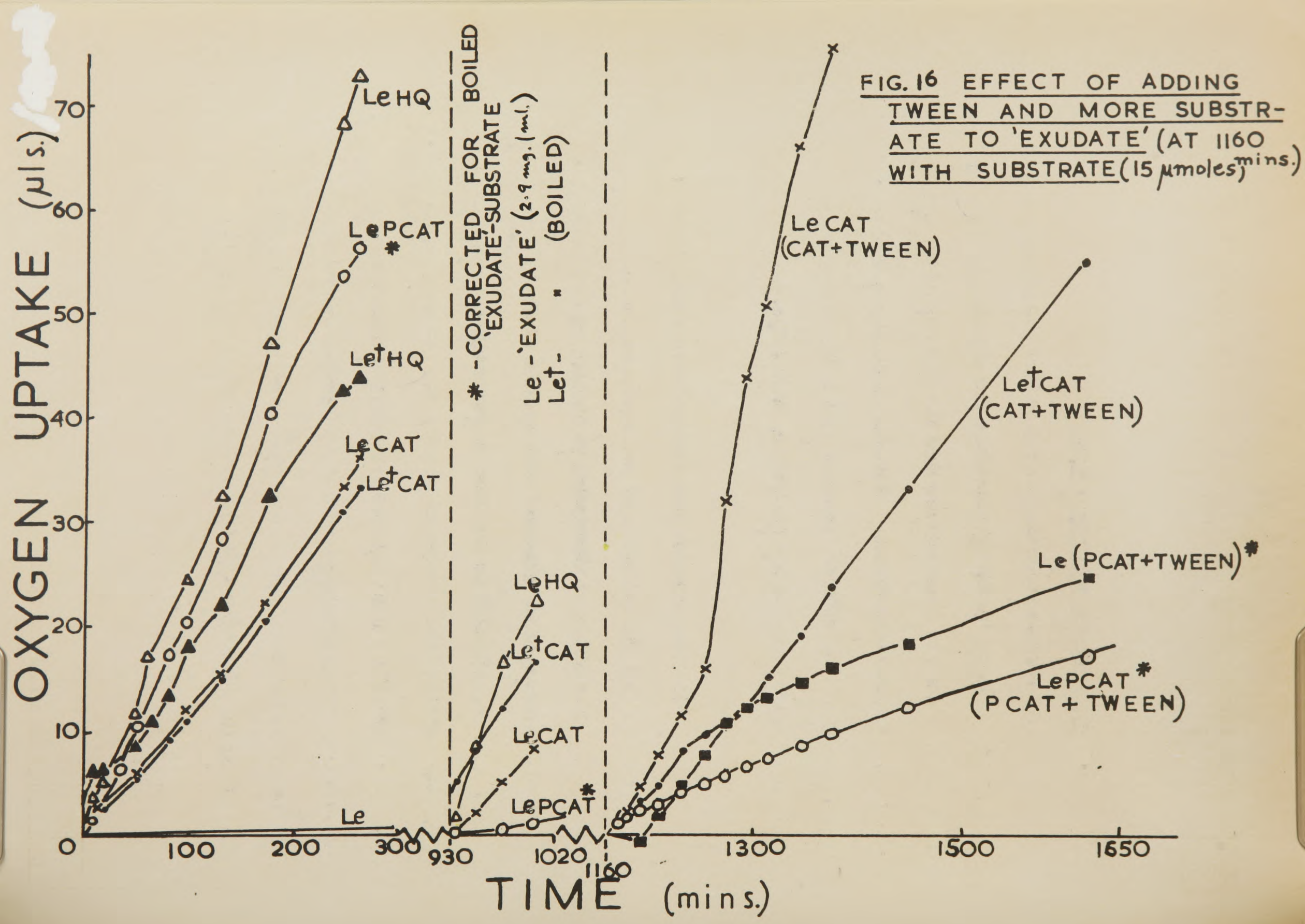
(0.25 mls. of 5%(v/v)) together with more substrate (15 µmoles) in phosphate buffer (0.25 mls. 0.3M-) to each flask containing 'exudate' (1 ml.), substrate (15 µmoles) and phosphate buffer (0.5 ml., pH 6.8) after incubation at 37°C had been in progress for 19 hours and 20 mins. (Fig. 16).

The oxygen uptake, corrected for the rate of oxidation of 'exudate' alone and boiled (10 mins at 98°C) 'exudate' alone, is shown in fig. 16 before and after the addition of "Tween" 85 and substrate. For comparison "Tween" 85 and protocatechuic acid were also added to 'exudate' which had been incubated, up until 1160 minutes in the absence of substrate (fig. 16).

Figure 16 shows that there was a marked increase in oxygen uptake by 'exudate' (2.9 mg. protein/ml. - ninhydrin method) with catechol, but no increase in that of the boiled 'exudate'-catechol control. There was a very slight increase in the rate of oxidation of protocatechuic acid by 'exudate' (corrected for boiled 'exudate'-protocatechuic acid oxygen uptake).

Sodium hexametaphosphate (6 µmoles) does not increase the rate of oxidation of protocatechuic acid (3 µmoles) by 'exudate' (1 ml.) in phosphate buffer (0.5 mls. 0.3M pH 6.8).

FIG. 16 EFFECT OF ADDING TWEEN AND MORE SUBSTRATE TO 'EXUDATE' (AT 1160 mins.) WITH SUBSTRATE (15 µmoles)



(iii) Inhibition of the enzyme system

As there was some indication that the phenoloxidase system of left gland 'exudate' contained the element copper, an attempt was made to demonstrate that the activity of the oxidase was in fact copper-dependent. Several chelating agents were introduced into the buffered 'exudate' (pH 6.8), in presence of protocatechuic acid of hydroquinone, with little result (Table 6).

Potassium cyanide and sodium borate did, however, effectively inhibit the oxidation of protocatechuic acid.

The effect of attempting to chelate and so inactivate the copper contained in 'exudate' with sodium diethyldithiocarbamate (DDC) was complicated by the slow decomposition (James 1953; Hallaway, 1959) of the compound at pH 6.8. Carbon disulphide was generated with the result that the true oxygen uptake was obscured. If anything, DDC stimulated oxidation of protocatechuic acid and hydroquinone but exact measurements of the effect were not made, because of the unexpected (little publicised (Hallaway 1959)) decomposition of the reagent.

TABLE 6 THE EFFECT OF SOME COMPOUNDS ON THE PHENOLOXIDASE SYSTEM IN LEFT GLAND EXUDATE WHEN INCUBATED[2] IN THE PRESENCE OF A SUBSTRATE

'Exudate' protein content [1] mg/ml.	Substrate	µmoles	Original Q_{O_2}	Inhibitor	µmoles	% Inhibition
2.0	protocatechuic acid	3	4.04	KCN	1.5	- 90
2.0	protocatechuic acid	3	5.55	sodium borate	3.0	- 70.5
3.0	protocatechuic acid	6	6.90	EDTA	1.5	0
2.0	protocatechuic acid	3	4.14	4-chlororcinol	3.0	+ 12.1
3.4	protocatechuic acid	3	4.26	p-fluorophenol	3.0	- 2.3
3.4	protocatechuic acid	3	4.26	o-fluorophenol	3.0	+ 4.2
3.0	protocatechuic acid	3	4.72	phenylthiourea	0.03	- 4
3.0	protocatechuic acid	3	4.72	sodium diethyl-dithiocarbamate	0.3	+ 10 ?
3.0	hydroquinone	3	6.87	"	0.3	0 ?
2.4	hydroquinone	3	7.42	boric acid	300	0

1. determined by the T.C.A. method

2. in oxygen at 37°C, total volume 1.5 mls.

In order to investigate the role of copper in 'exudate' (previously dialysed against 0.01 M-NaCl for 36 hours and then against distilled water (12 hours)) an attempt was made to remove the element by dialysis against water after reaction (20 hours) at 0°C of the left gland preparation with an equal volume (2 mls.) of potassium cyanide (0.2M-) (Lerner, et al 1950).

For the purposes of comparison and control sodium diethyldithiocarbamate (2 ml. 0.2 M) and potassium carbonate (2 ml. 1.0M) were also added to samples (2 ml.) of 'exudate' and allowed to stand; after which the three samples were buffered (pH 6.8) with sodium phosphate (1 ml. 0.6 M) and centrifuged (125,000 xg for 1 hour). The precipitates were resuspended in distilled water (2 ml.) and together with the supernatants, were dialysed against 5 l. of water - changed three times at 6 hourly intervals. The activity of the precipitates and supernatants were tested in the normal manner.

To 1 ml. samples of each of the resuspended precipitates, 5 µg. of copper (as CU SO_4) was added and their activity tested too. The results of this experiment are recorded in Table 7 below.

TABLE 7 :

THE EFFECT OF ATTEMPTED REMOVAL AND REPLACEMENT OF COPPER ON THE ACTIVITY OF 'EXUDATE' PHENOLOXIDASE

Sample	Q_{O_2}[3]
0.2M- KCN reacted, precipitate[1]	0.66
0.2M- KCN reacted, precipitate + 5 ug $C\mu^{++}$	1.05
0.2M- KCN reacted, supernatant[2]	5.5
0.2M- DDC reacted, precipitate[1]	0.22
0.2M- DDC reacted, precipitate + 5 ug $C\mu^{++}$	0.63
0.2M- DDC reacted, supernatant[2]	0
1.0M- K_2CO_3 reacted, precipitate [1]	0.75
1.0M- K_2CO_3 reacted, precipitate + 5 ug $C\mu^{++}$	0.70
1.0M- K_2CO_3 reacted, supernatant[2]	9.55
Unreacted 'exudate' (1)(Stored at 0°C)	2.03

1. 2.0mg. T.C.A. precipitate/ml.

2. 0.2mg. T.C.A. precipitate/ml

3. protocatechuic acid (3 μmoles)

C. FRACTIONATION OF THE 'EXUDATE'

Various attempts were made to purify the phenoloxidase in left gland secretion and thus separate it from the other protein fraction - structural protein, the precursor of sclerotin - thought to be present. The results of these fractionations are shown in figures 17 to 22 and 25. The specific activity of the various fractions (1.0 ml.) was determined manometrically (total volume 1.5 mls, atmosphere oxygen) in presence of phosphate buffer (0.5 mls. 0.3 M, pH 6.8) and protocatechuic acid (3 µmoles in fractionations shown in figures 17 - 20 and 15 µmoles in figures 21, 22 and 25).

(i) Differential centrifugation

In a preliminary experiment it was noticed that the supernatant of 'exudate' centrifuged at 20,000 r.p.m. (27,000 x g) for half an hour had a higher specific activity than the resuspended precipitate. This supernatant, containing 0.8 mg. of T.C.A. precipitate/ml., had a Q_{O_2} of 17.08 for 3 µmoles of protocatechuic acid, whereas the precipitate, when washed and taken up in 2 mls. of distilled water, contained 12 mg/ml. and had a Q_{O_2} of only 1.88.

Similarly, supernatant D is more active than precipitate A or B (Fig. 17). Moreover, it was noted that the water washings C

Fig. 17

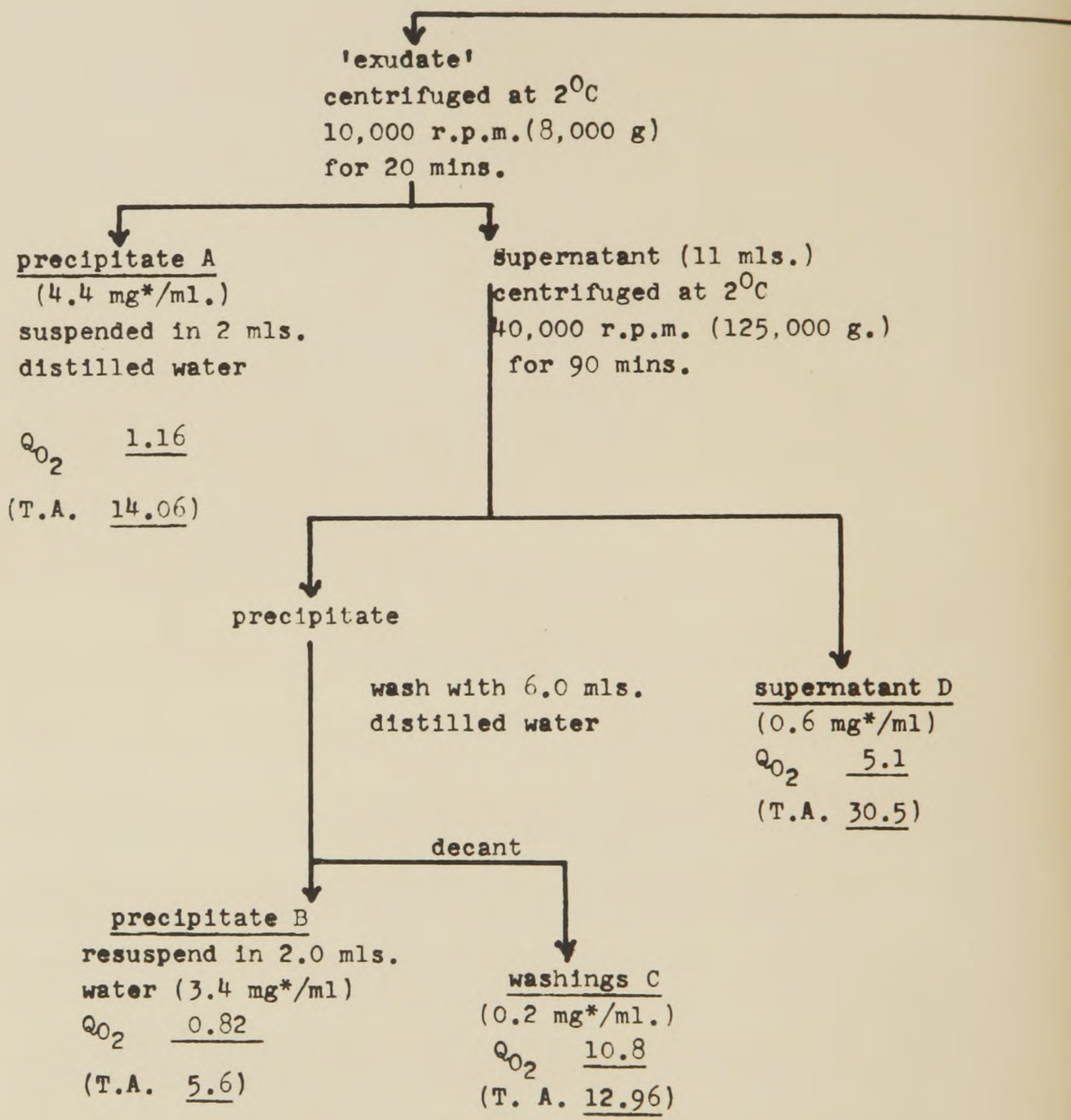
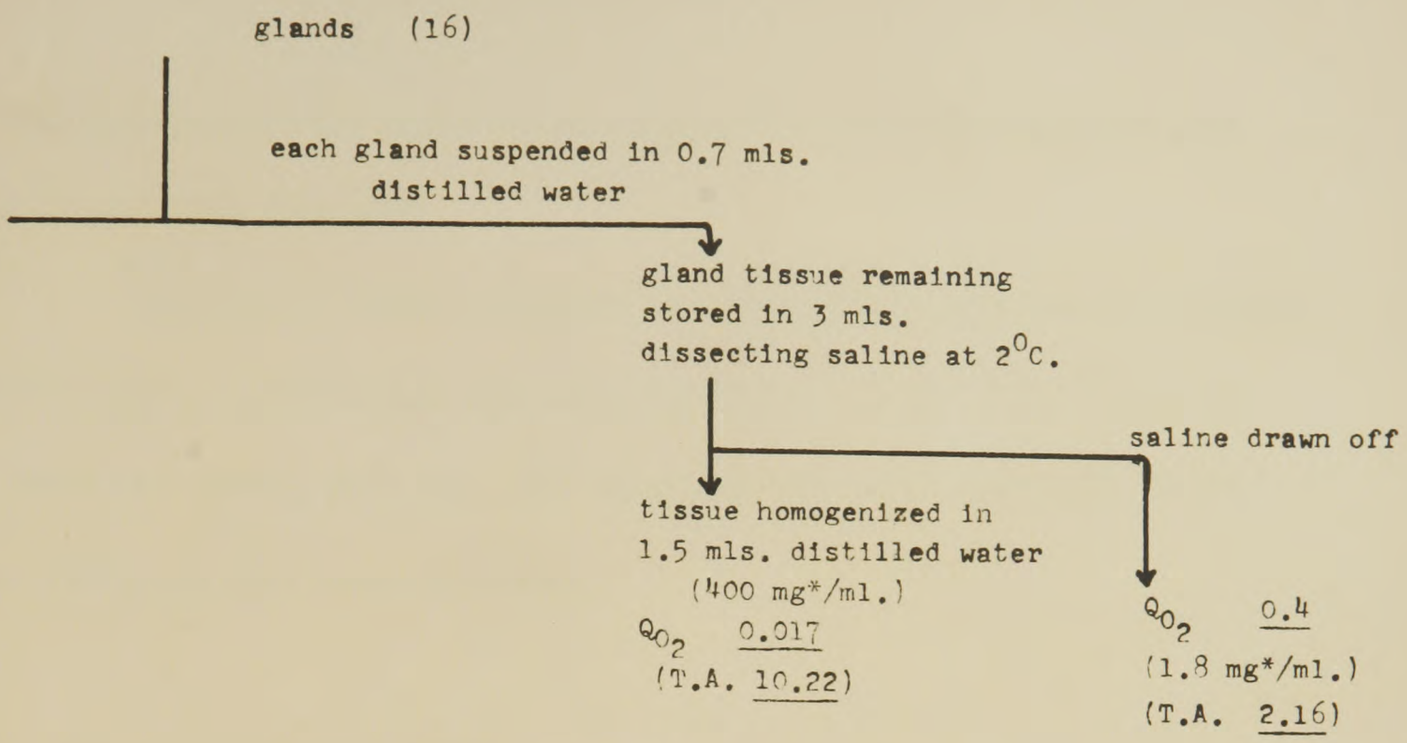


FIG: 17 THE ACTIVITY OF EXUDATE CENTRIFUGATION) AND

* T.C.A. precipitate

T.A. Total activity



FRACTIONS, (SEPARATED BY DIFFERENTIAL
OF GLAND DEBRIS.

of the comparatively inactive precipitate B had high specific and total activities (fig. 17).

However, when 'exudate' is centrifuged and the precipitate (C) is twice resuspended and centrifuged the final supernatant (4) is not very active (see fig. 18) whereas the first supernatant (1) has 24 times this total activity.

(ii) Use of inorganic salts.

Further fractionation of 'exudate' supernatants, by employing sodium chloride, sodium phosphate, and ammonium sulphate as precipitants, was attempted in the experiments illustrated in figures 18 and 19. In general it again appeared that the supernatants fractions were more active than the sedimented material.

In the experiment shown in figure 19 homogenate of left glands was investigated, instead of 'exudate' in an attempt to procure the maximum amount of cell secretion per gland. It was known (see fig. 17) that the gland tissue remaining after extraction of 'exudate' in distilled water retained some oxidase activity. The filtered and dialysed homogenate (fig. 19) was subjected to three fractionation procedures with increasing concentrations of sodium chloride.

In an earlier experiment, samples (1 ml.) of left gland exudate were treated with NaCl such that final concentrations of

Fig. 18

(8 mls.) left

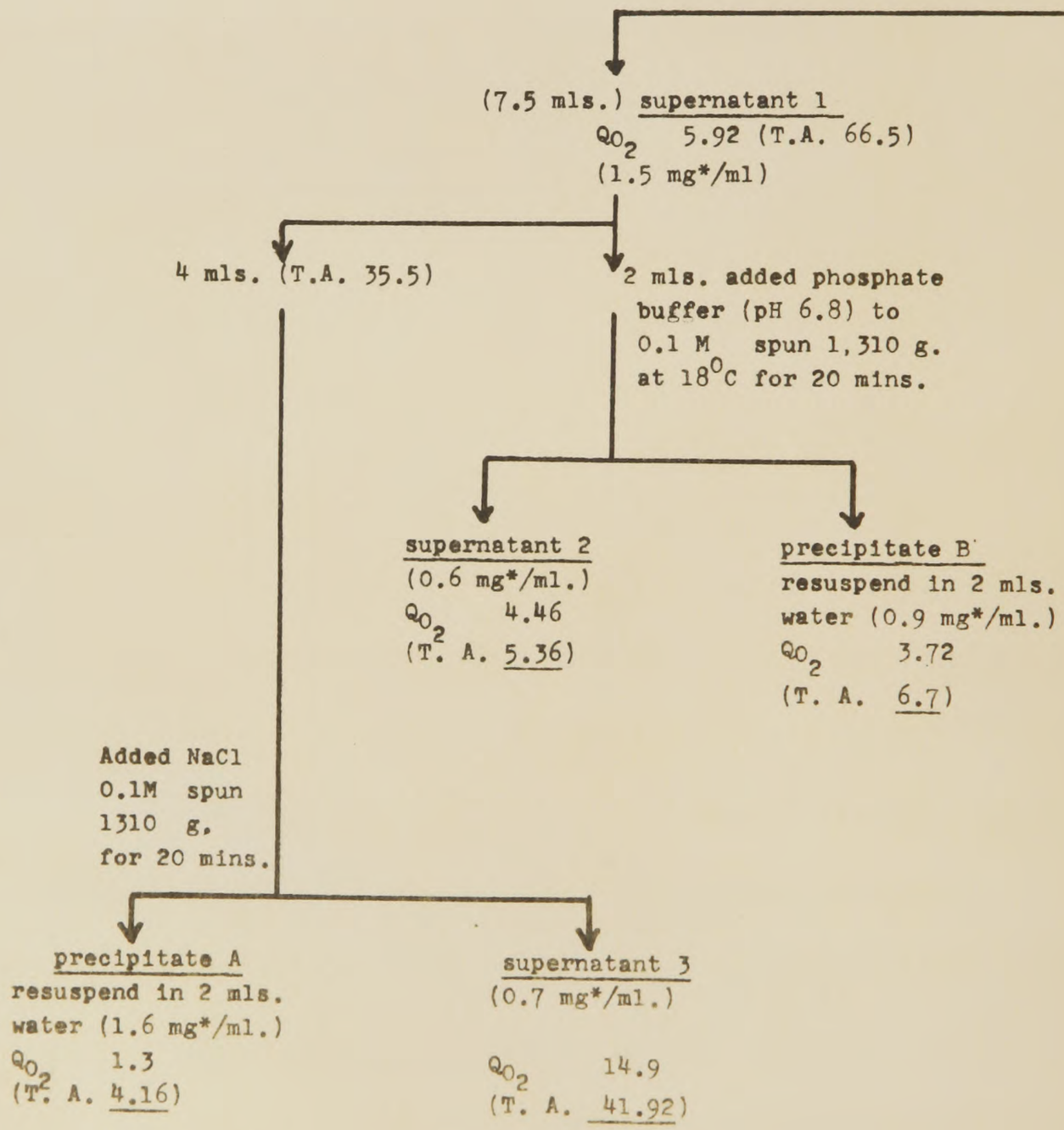


FIG: 18 PRECIPITATION OF SUPERNATANT OF

* T.C.A. precipitate

T.A. Total activity

gland exudate

(3.3 mg*/ml.) dialysed vs.distilled water at 2°C for 18 hours centrifuged at 2°C 27,200 g. for 30 mins.

precipitate C
resuspended in water (8 mls.)

spun 27,200 g. at 2°C for 30 mins.

supernatant
(0.8 mg*/ml)

precipitate D
resuspended in water(8 mls.)

spun 27,200 g.at 2°C for 25 mins.

precipitate
discard

supernatant 4
(0.5 mg*/ml)

Q_{O_2} 0.68
(T.A. 2.72)

'EXUDATE' WITH NaCl AND PHOSPHATE

Fig. 19

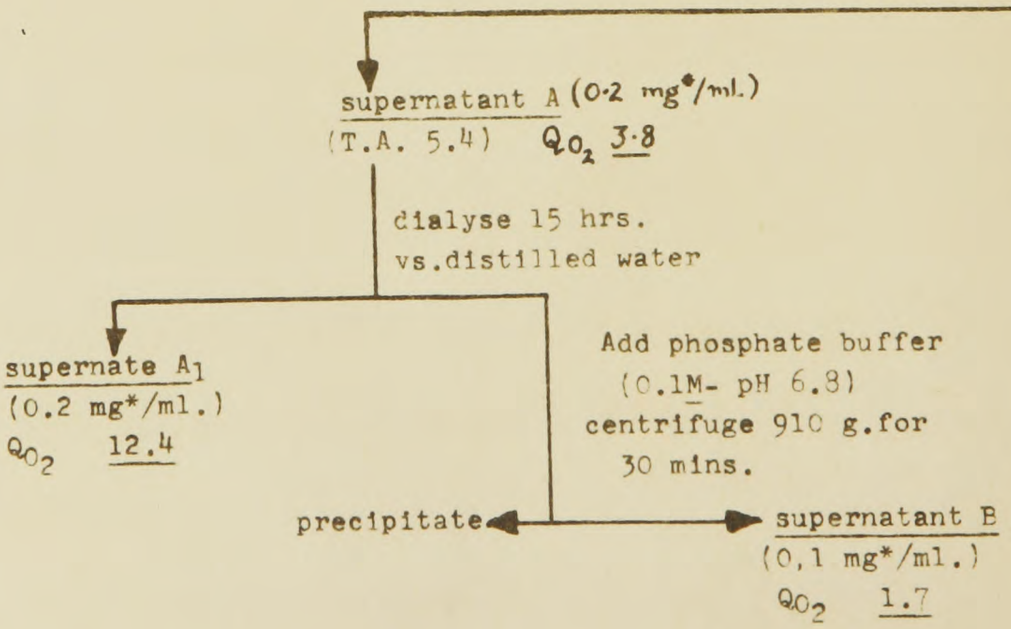
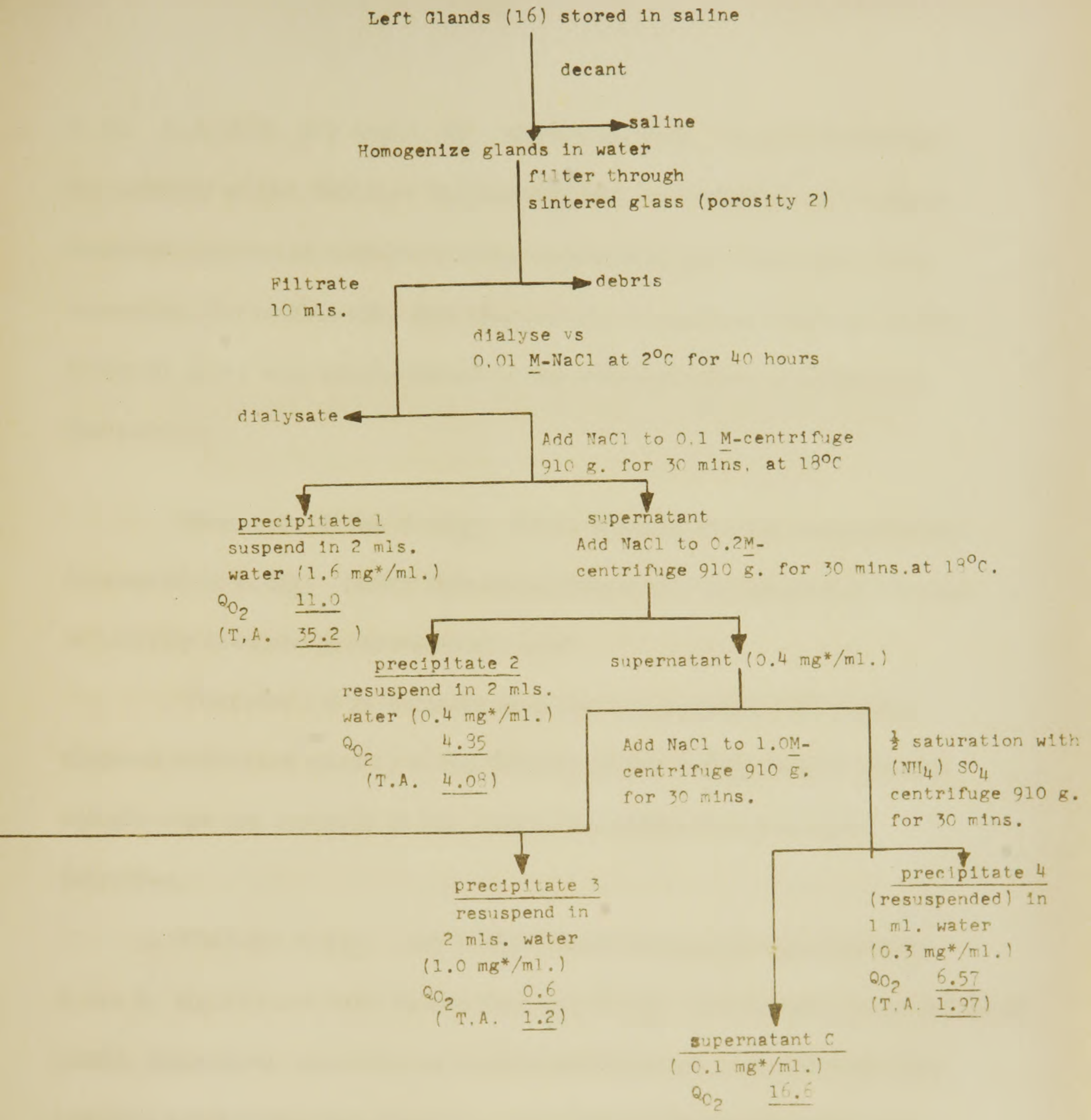


FIG: 19 FRACTIONATION OF HOMOGENATE

* T.C.A. precipitate

T.A. Total Activity.



(FILTERED) WITH NaCl AND $(NH_4)_2$ SO_4

0.05, 0.1, 0.2, 0.5 and 1.0M were obtained. It was found that the activity of the filtrates (sintered glass, porosity No. 2) of these samples decreased with increasing concentration of the salt, thus conveying the impression that the amount of enzyme retained on the sintered glass was proportional to the concentration of sodium in the 'exudate'.

However, if the 0.05M- NaCl filtrate had its concentration increased to 1.0M- (after filtration) there was an apparent 45% loss in activity towards protocatechuic acid.

Therefore it is unlikely that the low activity (43% of the original untreated sample) of the filtrate of the 1.0M- NaCl treated sample was due entirely to the loss of an active fraction upon filtration.

That the 0.2M- and 1.0M- NaCl sediments (precipitates 2 and 3, fig. 19) are less active than the 0.1M- precipitate (precipitate 1) could, therefore, possibly be explained if sodium and chloride ions actually inhibit the phenoloxidase oxidation of protocatechuic acid. Further evidence of this temporary inhibition is shown by the fact that the activity of the 1.0M- NaCl supernatant (A) is increased 3.26-fold following dialysis against distilled water (c.f. supernatants A and A_1 fig. 19).

Addition of sodium phosphate to (dialysed) supernatant A (fig. 19) followed by centrifugation, yields a supernatant (B) of low specific activity. This is surprising in view of the result of phosphate fractionation of supernatant 1 shown in figure 18.

(iii) Activity-copper content correlations of various fractions.

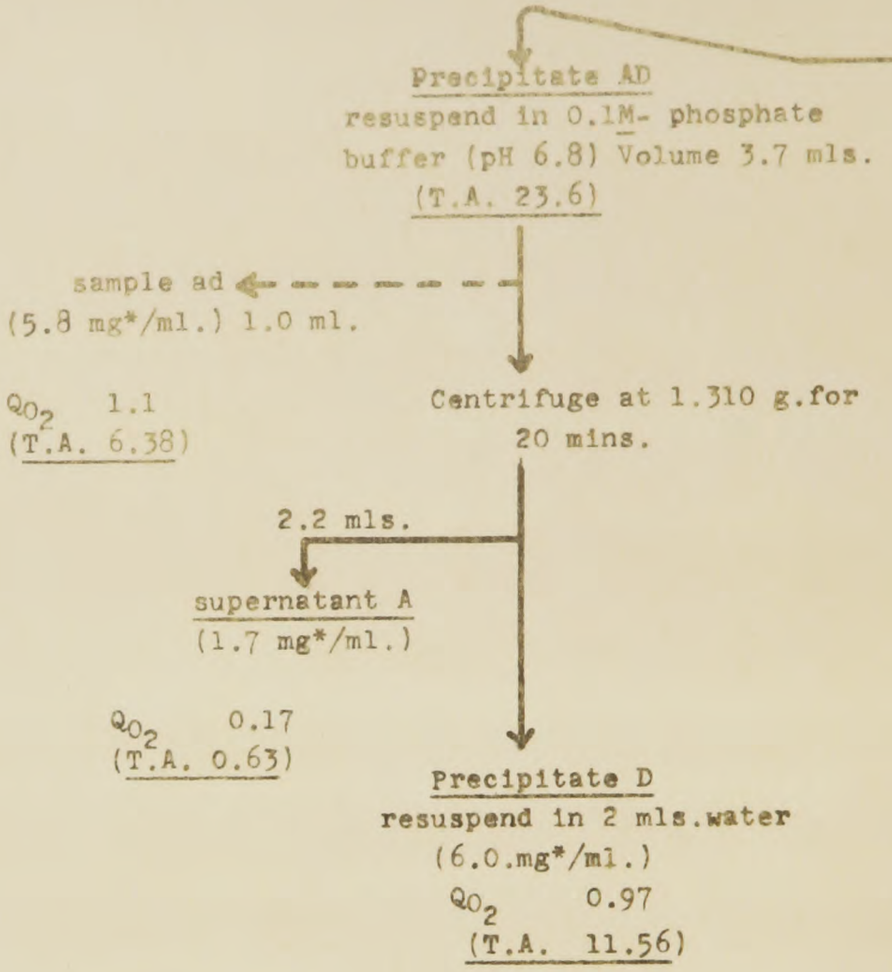
The fractionation shown in figure 20 was designed to investigate the activity of sodium phosphate and sodium chloride fractions with respect to their copper content. Unlike the phosphate fractionation of supernatant 1 illustrated in figure 18 the supernatants (A and C), derived from the resuspended precipitates AD and CE (fig.26), both show lower specific and total activities than the corresponding sediments (D and E).

Precipitate E showed the highest oxidase activity per µg. of copper ($Q_{O_2}^{Cu}$). Since the relatively inactive supernatants A, B and C contained, respectively, 0.046, 0.17 and 0.44% copper (w/w) of dried material (110°C for 15 hours) no immediate correlation of the content of this metal with the activity of 'exudate' protein fractions was discernible (fig. 20).

Supernatant B (fig. 20) was found, by chromatography (Whatman No. 1; solvent BAW (4:1:1) of the ethanolic extracts of each of the fractions, to contain almost the entire amount of protocatechuic

Fig. 20

Left gland 'exudate'



VALUES	A	D	AD	ad
Q_{O_2}	0.17	0.97		1.1
Total Activity	0.62	11.56	23.6	6.4
			17.2	
µg Cu	1.7	9.4	11.1	
$Q_{O_2}^{Cu}$	0.36	1.23	1.41	
% Cu ($^{W}/_{W}$)	0.046	0.078		
			AD-ad	

FIG: 20 CORRELATION OF COPPER CONTENT AND NaCl PRECIPITATION, AND

* : T.C.A. precipitate

'exudate'

(11 mls)
(4.4 mg*/ml.)
Dialysed 36 hrs vs. distilled water at 2°C, centrifuge 8000 g. for 30 minutes at 2°C.

10.5 mls. (T.A. 86.6)
supernatant BCE → sample bce (2.4 mg*/ml.)
1 ml. Q_{O_2} 3.44
(T.A. 8.25)

NaCl added to 0.2M-
Centrifuge at 1.310 g. for 20.mins.

9.mls.
supernatant B
(0.4 mg*/ml.)
Q_{O_2} 2.02
(T.A. 6.48)

Precipitate CE
resuspend in 0.1M- phosphate buffer (pH 6.8) Volume 3.7 mls.

sample ce ← 1.0 ml.
(5.7 mg*/ml)
Q_{O_2} 3.55
(T.A. 20.3)

Centrifuge at 1 310 g. for 20 mins.

2.5 mls.
supernatant C
0.9 mg*/ml.
Q_{O_2} 1.88
(T. A. 4.23)

precipitate E
suspend in 2 mls. water
(7.2 mg*/ml.)
Q_{O_2} 2.94
(T. A. 42.38)

C	E	CE	ce	B	BCE	bce
1.88	2.94		3.55	2.02		3.44
4.23	42.38	74.5	20.3	6.48	86.7	9.2
		54.3			77.5	
9.9	7.1	17.0		6.1	29.4	
0.42	5.96	3.19		1.06	2.64	
0.44	0.049			0.17		
		CE-ce			BCE-bce	

ACTIVITY OF FRACTIONS OBTAINED AFTER CENTRIFUGATION, SUSPENSION IN PHOSPHATE BUFFER (pH 6.8).

T.A. Total activity.

acid glucoside present in 'exudate'.

This fact substantiates the earlier observation that 0.1M- NaCl dialysis rids the 'exudate' of glucoside, and possibly other phenolic substances, much more effectively than dialysis against distilled water (see fig. 14).

(iv) Use of acetone (in collaboration with Dr. P. C. J. Brunet)

Homogenizatinn of left glands in 0.1M- phosphate buffer (pH 6.8) followed by centrifugation yields a precipitate (5) with high total activity (fig. 21). The resulting supernatant can be further fractionated by precipitation of heavy white flocks on the addition of an equal volume of cold acetone, but this sediment (precipitate 6) has very low initial activity (method of Hess 1958)

An acetone powder of homogenized left glands (method of Karlson and Schmid (1955)) is found to contain oxidase activity which apparently is not readily solubilized in phosphate buffer (pH 8.0) (see precipitates 3 and 4) for the $(NH_4)_2 SO_4$ precipitate (8) of the phosphate extract has low initial activity. (Fig. 22).

However, this precipitate 8, and precipitate 6 (fig. 21) described earlier, both promote an extraordinarily high oxidation of protocatechuic acid (15 µmoles) after 21 hours of incubation in phosphate buffer (0.1M, pH 6.8) at 37°C, (see fig. 23). Indeed

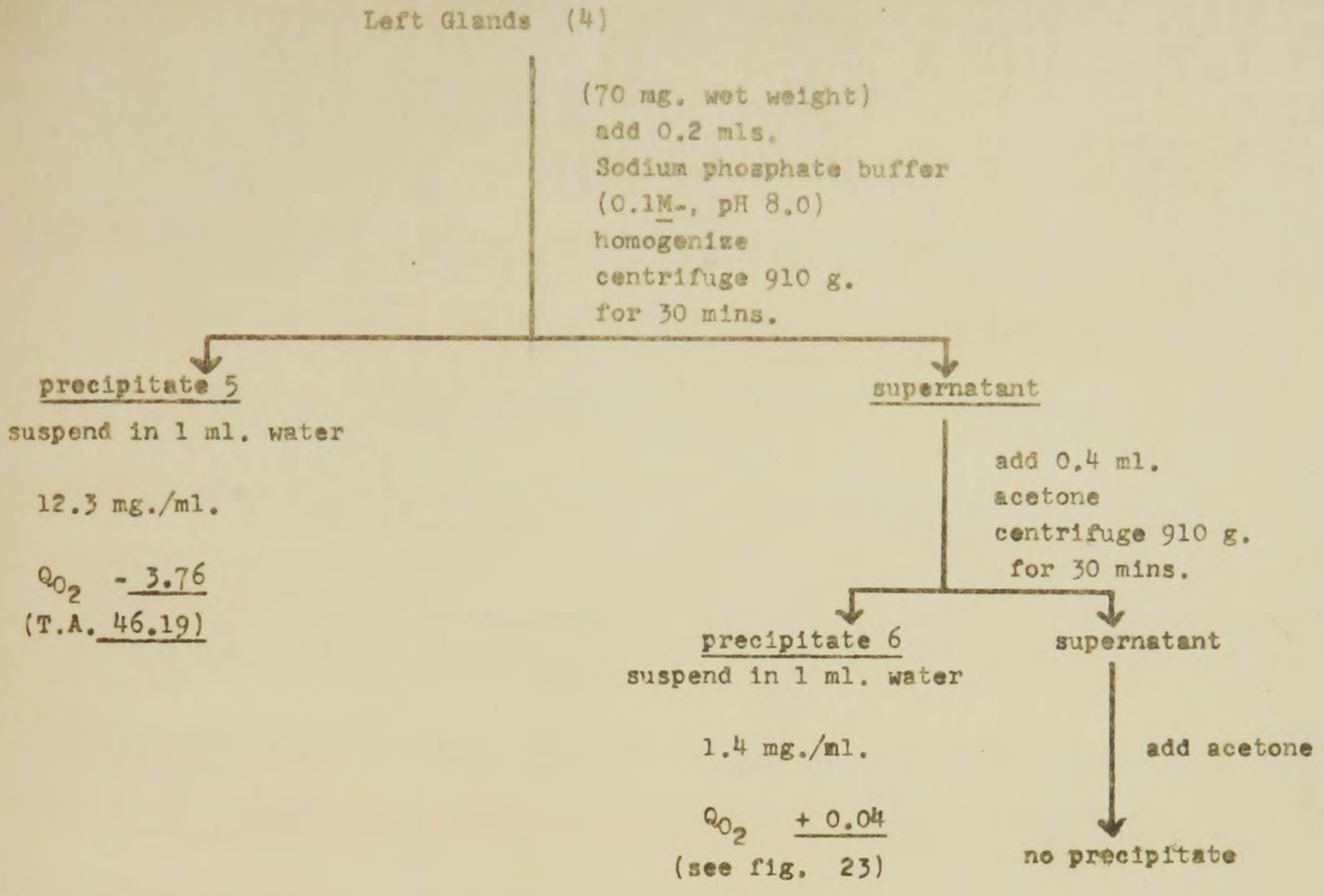
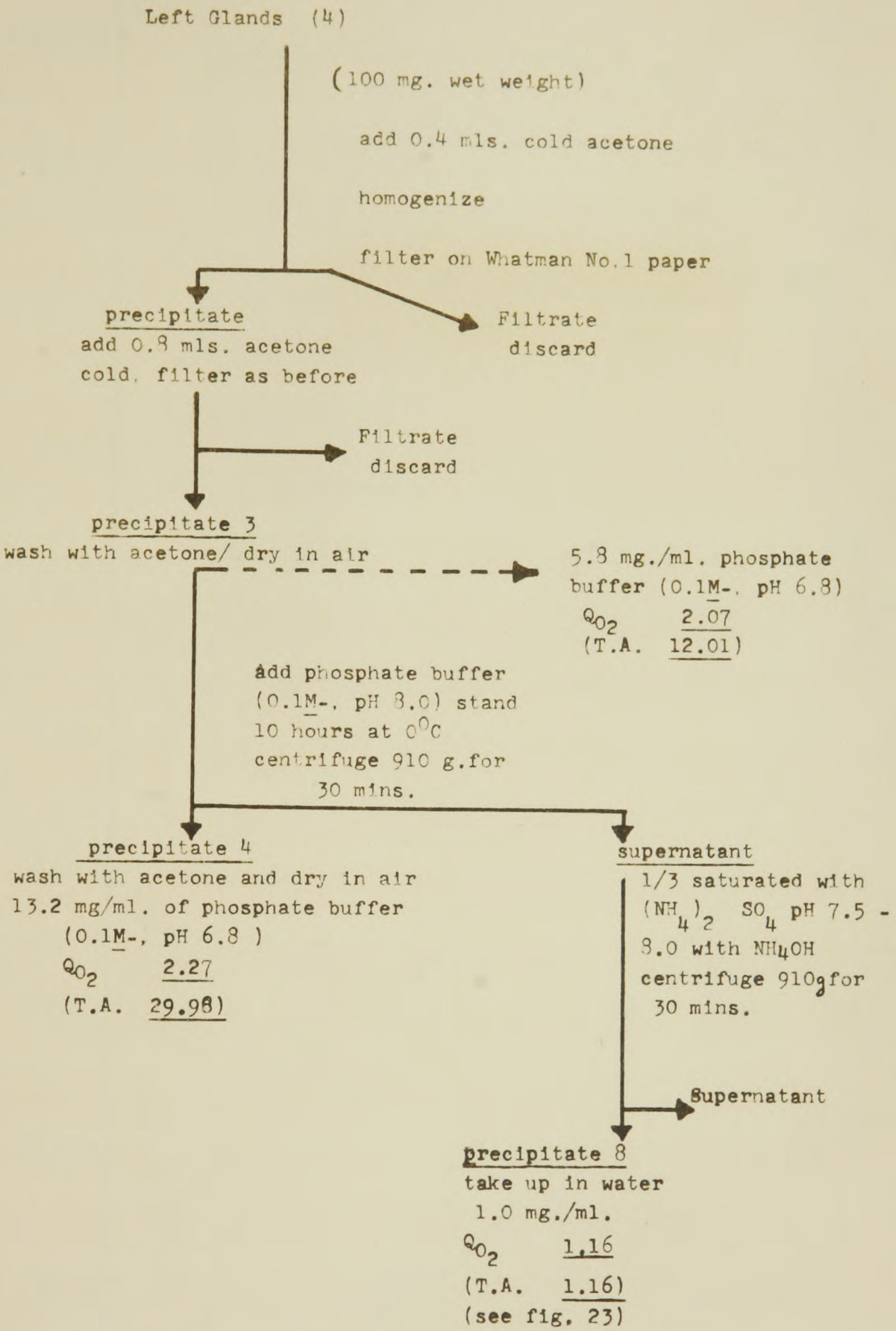


FIG: 21 USE OF ACETONE TO FRACTIONATE THE SUPERNATANT OF PHOSPHATE TREATED GLAND HOMOGENATE (METHOD OF HESS (1958))

T.A. Total activity.



FIG; 22 FRACTIONATION OF ACETONE POWDER OF GLAND HOMOGENATE IN SODIUM PHOSPHATE AND AMMONIUM SULPHATE BOTH AT pH 8.0. (METHOD OF KARLSON AND SCHMIDT (1955))

T.A. Total activity.

Fig: 23 Rate of oxidation of protocatechuic acid (15 μmoles) by homogenate fractions (fig. 21).

P/S: Penicillin and streptomycin (0.1%)

atmospheric pressure 751 mm. Hg.

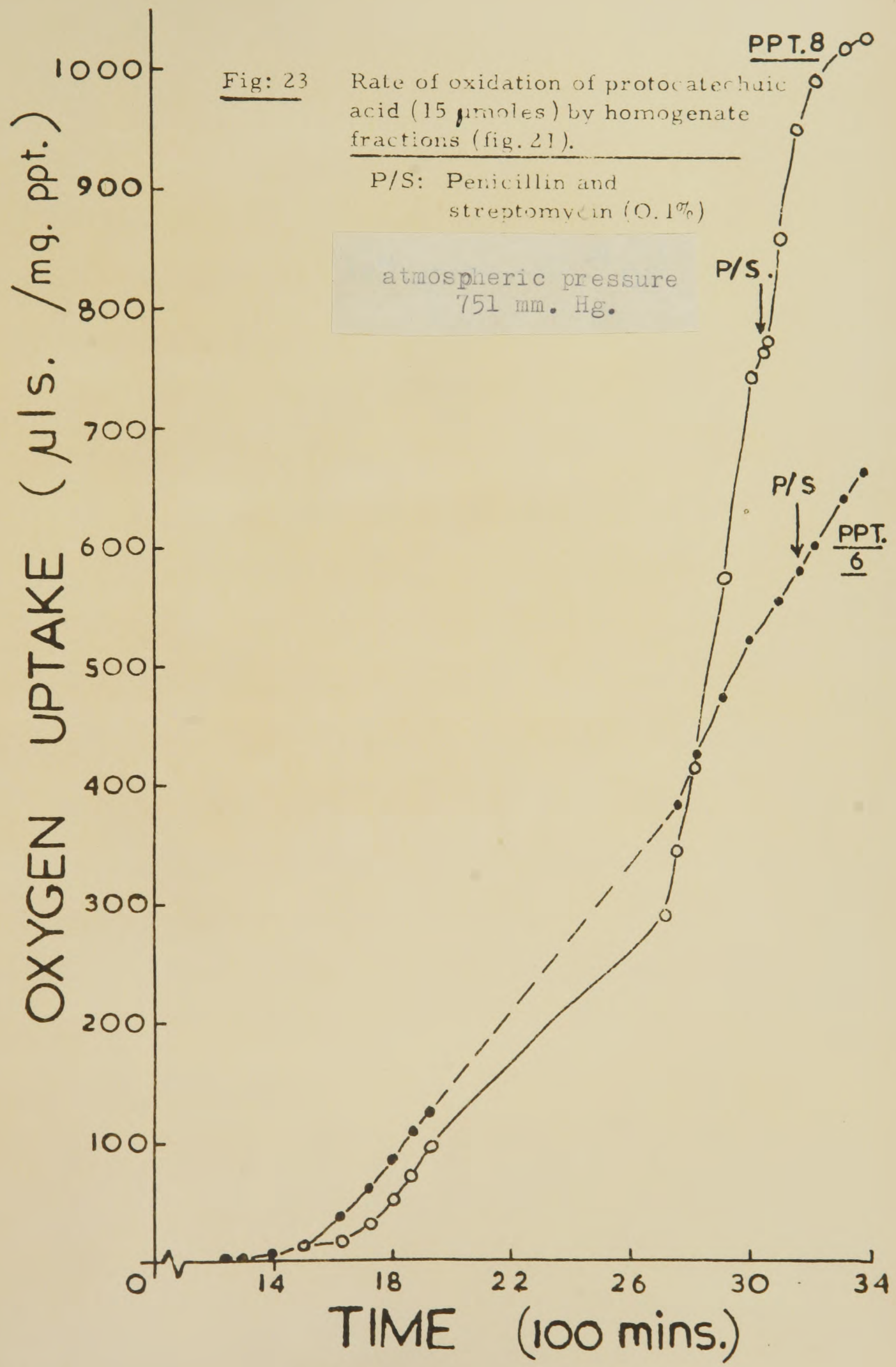
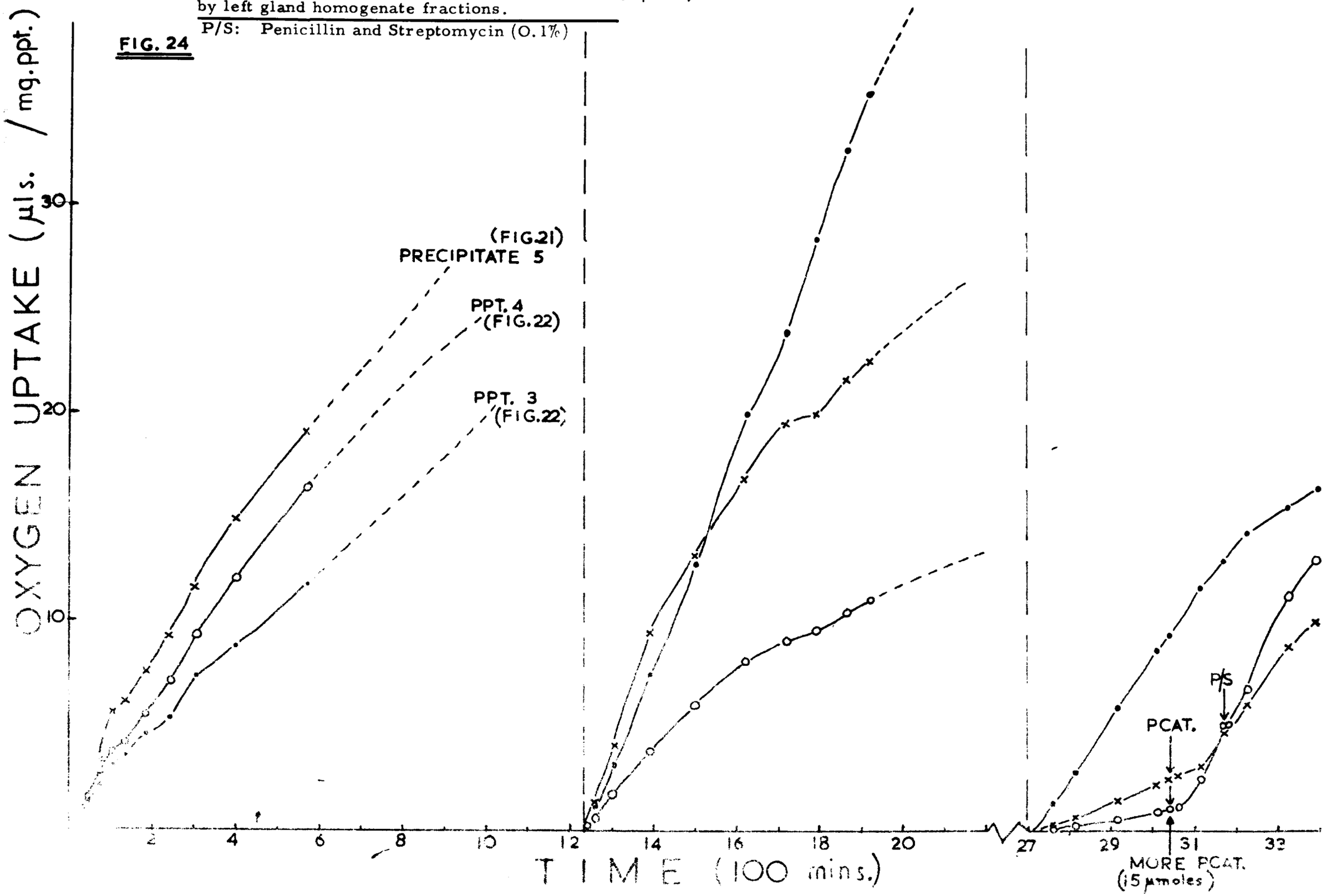


FIG. 24

Rate of oxidation of protocatechuic acid (Pcat.) (15 μmoles) by left gland homogenate fractions.

P/S: Penicillin and Streptomycin (O.1%)



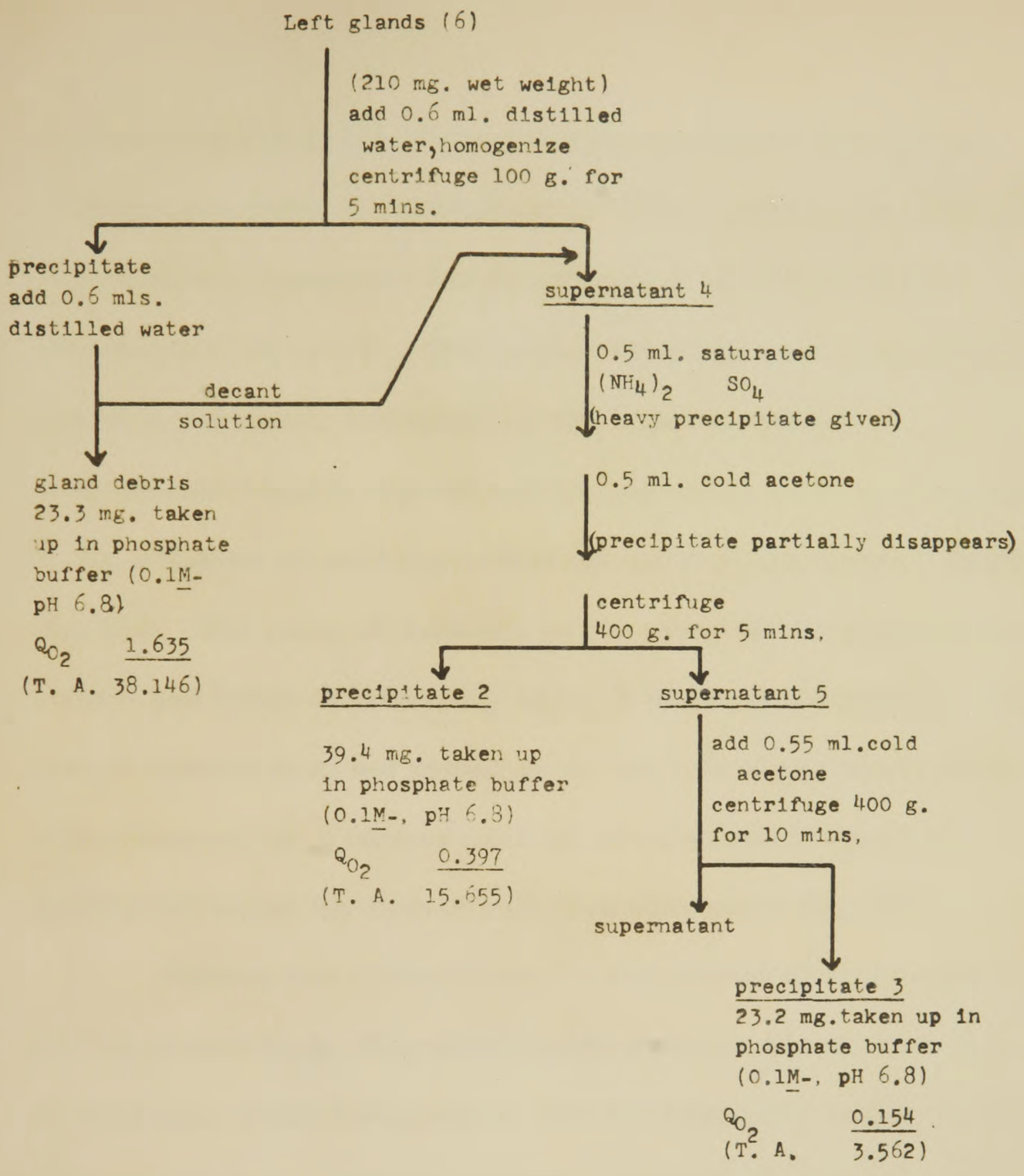


FIG: 25 SALTING INTO ACETONE-WITH SATURATED AMMONIUM SULPHATE-OF 'EXUDATE' PROTEINS. (METHOD OF BROWN AND WARD (1951))

T.A. Total activity.

precipitate 8 (fig. 22) is responsible for the uptake of 3 atoms of oxygen per mole of protocatechuic acid in spite of the introduction of a mixture of penicillin and streptomycin (0.1% (w/v)) final concentration of each). The reason for the 21 hour-long induction period is not clear, especially as the acetone powders (precipitates 3 and 4, fig. 22) and the phosphate sediment (precipitate 5, fig. 21) show no such lag before oxidation of the diphenol begins (fig. 24). The rates of oxidation of the substrate by precipitates 4 and 5 had fallen substantially after 50 hours of incubation. This loss of activity does not appear to be due entirely to inactivation of the enzyme, as a further addition of substrate (15 µmoles) greatly increases the rate of oxidation once more (fig. 24).

Salting into cold acetone (by the method of Brown and Ward (1951)) of the $(NH_4)_2 SO_4$-precipitated proteins of supernate 4, derived from gland homogenate, was not apparently very successful although the initial heavy precipitate did partially disappear upon the addition of acetone (fig. 25). These fractions were incubated, and their rates of oxidation of protocatechuic acid measured, over a period of 24 hours.

(v) Paper electrophoresis

All attempts to fractionate the proteins in 'exudate' by

paper electrophoresis failed. The proteins appeared to be completely insoluble in most buffers including sodium phosphate (0.1M-), barbital (0.05M) and tris (0.05M) at pH 8.0, for there was no movement from the origin (in 2 hours, 178 volts) on paper, of a bromophenolblue-staining component (indeed as there was with bovine serum albumin as a standard).

(vi) Ultracentrifugation (in collaboration with Dr. A.G. Ogston)

11 mls. of fresh 'exudate' were freeze-dried and resuspended in 2 mls. of distilled water. 1 ml. of this was centrifuged at 900 revs/sec (182,000 x g) in the Svedberg analytical ultracentrifuge (27.5°C). The opaque particulate material (A) began to sediment at 6 mins. (320 revs/sec) and had nearly all disappeared after an hour (360 revs./sec) (fig. 26). Small amounts of (perhaps) three protein components in solution diffused from the meniscus (B, C and D) as the rotor was accelerated to 900 revs/sec.

Centrifugation of another resuspended 'exudate' sample (concentrated by precipitation with 0.5M- NaCl) showed rapid (10 mins) sedimentation of the opaque material and lack of any other components.



Figure 26. Ultracentrifugation of concentrated (freeze-dried) 'exudate' (11 mls.) resuspended in 2 mls. distilled water opaque material left meniscus at 6 mins. (320 revs/sec). Photographs taken at

mins	52,	57,	78,	84,	88,	98,	148,	158,	168
revs/sec	345	360	920	900	900	900	900	900	900

while temperature of head rose from 20.4° to 27.5°C.

show A, pink, opaque, perhaps quinone-tanned material

B, C, } minute peaks rapidly diffusing

D, perhaps lipoprotein.

After centrifugation of the same concentrated 'exudate' suspension for 125 minutes at 31,200 g. (0°C), the specific activity(protocatechuic acid) of the supernatant (1 ml.) which contained approximately 0.1 mg. of TCA precipitate, was found to be about 40. The suspension was buffered as usual and the activity was measured in the normal way.

CHAPTER FIVE

DISCUSSION

The existence of a phenoloxidase in insect cuticle has been recognised for nearly 4 decades. However, little information exists about the nature, specificity and biological functioning of this enzyme. For such investigations, the left colleterial gland of Periplaneta presents a convenient and concentrated source of an insect phenoloxidase.

The nature and specificity of phenoloxidase of Periplaneta left colleterial gland.

The 'enzyme' present in the secretion from the gland exhibits a marked specificity for o-dihydroxyphenolic compounds with the notable exception of 3,4-dihydroxyphenylalanine (dopa) and its derivatives (i.e. dopamine and noradrenaline). It also appears to catalyse the oxidation of some p-diphenols including homogentisic acid lactone and hydroquinone and the related compound, p-phenylene-diamine. Neither the ortho- or para- isomers of L-tyrosine nor any

of the other monophenols tested are oxidized by the "enzyme" system (Table 1 and fig. 27).

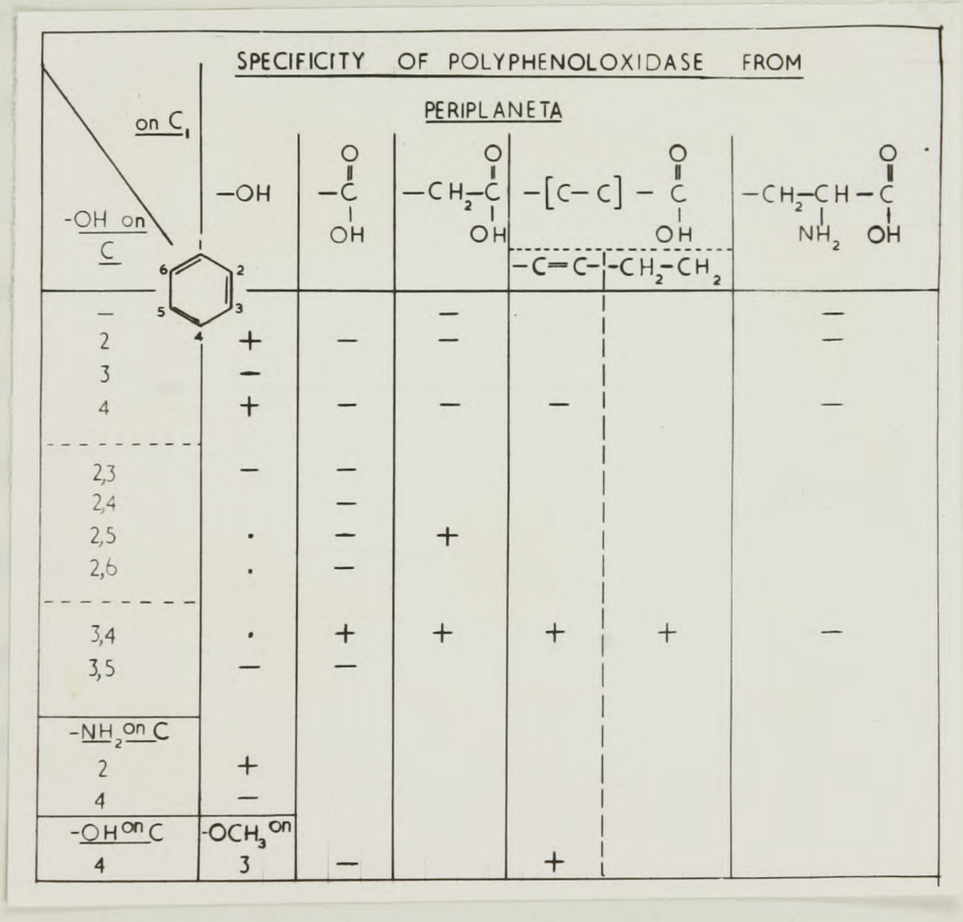
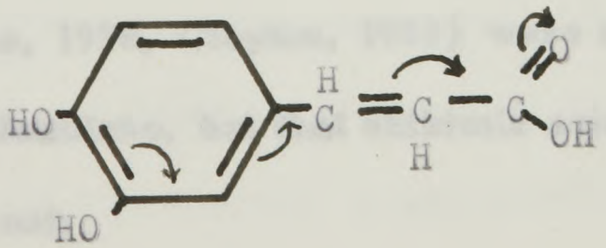


Figure 27: Specificity of phenoloxidase from left colleterial gland of Periplaneta.

These results clearly indicate that this phenoloxidase does not fall into the category of a tyrosinase (polyphenoloxidase) of either plant or animal origin, (Whitehead, Brunet and Kent 1959) the properties of which have been discussed in chapter three (page 49) and summarised in table 8.

The substrate specificity and relative activity of the insect phenoloxidase appears to be governed to a marked extent by the nature of the side chain of the substituted diphenolic substrates (table 1). For example, protocatechuic acid is more readily oxidized than the corresponding phenyl-acetic and propionic acids perhaps because the length of the side chain has reduced the electron withdrawing effect of the carboxylate group on the aromatic ring. Also caffeic acid is found to be more readily oxidized than homoprotocatechuic acid. This is consistent with the view that the double bond in the conjugated side chain of the former, appears to encourage electron shift (which facilitates oxidation) thus:-



caffeic acid

Of the 3-methyl ethers investigated, vanillic acid is not oxidized by the enzyme system, in contrast to ferulic acid (methyl ether of caffeic acid). This suggests that the unsaturated side chain of ferulic acid is involved in the formation of an enzyme-substrate complex, or that de-etherification occurs. However, the presence of the side chain double bond in itself does not appear to influence substrate specificity since 4-hydroxycinnamic acid is inactive. Presence of an amino group in the side chain (not for liver-fluke phenoloxidase (Mansour 1958)) seems to be a contributory factor in preventing the enzymic oxidation of catecholamines and dopa. However, if these substances attach themselves to the active sites of the protein-complex they may prove to be competitive inhibitors. This is in agreement with the observation that dopa but not tyrosine, was able to retard the rate of enzymic oxidation of protocatechuic acid (table 4).

It is of interest that the 3,4-dihydroxyphthalic acid and the polyphenol, chlorogenic acid (which is a plant phenoloxidase substrate, (Sisler and Evans, 1958, Clayton, 1959) were also oxidised by the cockroach phenoloxidase, but that shikimic acid and 2,3-dihydroxy-naphthalene are not.

The phenolase, like the cytochrome C (horse heart)-cytochrome oxidase (pig heart or rat kidney cortex) system can oxidise an o-aminophenol, and in this respect differs from tyrosinase, (Nagasawa, Morgan and Gutmann 1958, Nagasawa, Gutmann and Morgan 1959). The cockroach oxidase is incapable of oxidizing phloroglucinol whereas the "peroxidase-polyphenoloxidase system" of cabbage can readily do so (Rubin and Ivanova 1959). But Schneider and Schmidt (1959) report that p-cresol activity of potato polyphenoloxidase is inhibited by phloroglucinol, resorcinol and orcinol.

Catechol, hydroquinone and homoprotocatechuic acid are still oxidized by gland 'exudate' preparations which have been boiled (98°C for 10 minutes), (Table 3). This suggests that in this case the system is capable of a non-enzymic oxidation. Significantly perhaps the above three substances are quite rapidly autoxidized in solution at pH 6.8 (tables 1 and 3). This may be of some importance since their rate of oxidation in buffer alone could be increased catalytically by the mere presence of copper ions (Gillette, Watland and Kalnitsky 1954, 1955; Ingraham 1959) or of protein-bound copper (McCarthy, Green and King 1937). The oxidation of diphenols by tyrosinase can be distinguished though, from that caused by metals in solution (Gillette et al 1954)

However, the activity of gland secretion cannot be completely non-enzymic because the oxidation of protocatechuic acid and its aldehyde, which are only slowly autoxidised at pH 6.8 is heat sensitive.

Hydroquinone is initially oxidized more rapidly than catechol but this may only be of experimental significance for there is little evidence that the p-diphenol occurs in the left gland secretion or in oothecal fluid (see page 159). Dennell (1958b) alone claims to have demonstrated the presence of hydroquinone in insect cuticle, but some of his chromatographic evidence is disputed (Hackman 1958).

Catechol in solution buffered at pH 6.8 is autoxidised more slowly than hydroquinone (table 1) which is to be expected since their relative redox potentials (E_O) are + 0.792 v and + 0.696v respectively (Ball and Chen 1933). Protocatechuic acid (E_O+ 0.883v) is oxidized very much more slowly than catechol in the absence of "enzyme".

Hydroquinone is not oxidized by tyrosinases (Dawson and Tarpley 1951, Bonner 1957). But tyrosinase of Harding-Passey mouse melanoma oxidizes it - in the presence of catalytic amounts of dopa - more rapidly than catechol (Lerner, Fitzpatrick, Calkins and Summerson 1951) though for other tyrosinases this oxidation is

less rapid. Trace amounts of dopa did not increase, but rather inhibited the oxidation of protocatechuic acid by 'exudate' (table 4).

Many of the properties of Periplaneta phenoloxidase indicate that this enzyme system resembles a laccase more nearly than any other type of copper oxidase. The principal differences, it seems, (see Chapter three) lie in:-

1. the inability of the colleterial gland "enzyme" to oxidize pyrogallol and ascorbic acid
2. that it is not associated with a polysaccharide (i.e. Molisch test negative)
3. that it possesses no blue colour or absorption band at 615 mμ.
4. that the entyme appears to be particulate in nature and readily soluble
5. that it is stable to heating (for protocatechuic acid) at 60°C for 10 minutes (rather like plant peroxidases, which, however, generally contain iron - table 8)
6. the fact that 5% or 10% T.C.A. appears to coagulate most of the protein in 'exudate' preparations.

TABLE 8: SUMMARY OF SOME GENERAL PROPERTIES OF PEROXIDASE AND COPPER OXIDASES FOR COMPARISON WITH PHENOLASE OF PERIPLANETA AMERICANA

PROPERTIES	LACCASE[a]	INSECT PHENOLASE	TYROSINASE[a] (POLYPHENOLOXIDASE)	ASCORBIC ACID[a,c]	PEROXIDASE[b]
Source (Convenient)	Rhus latex	Left colleterial gland of cockroach	Mushroom	squash plant	Horse radish
Colour	blue	white	yellow	-	-
Associated with	copper polysaccharide	possibly copper	copper	copper	iron
Substrates Monophenols:	No	No	Yes - with lag (no lag in presence of diphenol)	No	Yes - in the presence of H_2O_2
Diphenols:	Yes - ortho and para	Yes - ortho and hydroquinone but not dopa and catechol-amines	Yes - ortho; not para in absence of ortho	No	Yes - in presence of ortho and hydroquinone
Others:	Ascorbic acid pyrogallol p-phenylenediamine	p-phenylenediamine	pyrogallol phenylalanine ascorbic acid	l-ascorbic acid l-glucoascorbate l-galactoascorbate d-araboascorbate	diamines monoamines pyrogallol resorcinol salicylic acid lenco dyes tryptophane ascorbic acid cytochrome C
	NOT resorcinol cytochrome C	NOT resorcinol ascorbic acid pyrogallol salicylic acid phenylalanine	NOT p-phenylene diamine	NOT polyhydric phenols i.e. resorcinol pyrogallol p-phenylenediamine	
Availability	soluble	Insoluble particulate Aggregated by ions	Partially assoc. with particles. Soluble with treatment.	Soluble	Soluble

Inhibitors Inhibited by:	Chelators:- KCN, H_2S, NaN_3 DDC	KCN NaCl- inorganic ions? borate dopa? NaN_3?	Chelators:- KCN, DDC, BAL, Fluoro-, amino-, and acetyl-tyrosine thiourea, thiouracil p-ABA, K-ethyl xanthate, p-nitrophenol cysteine glutathione oxidation products, inorganic ions (NaCl).	KCN, DDC, NaS K-ethylxanthate 8-hydroxyquinoline by the products of substrate oxidation	Catalase CC-light sensitive Excess H_2O_2 Chelators: HCN, H_2S, NaN_3, NO, H_2NOH, NaF, Na dithionate
not especially inhibited by:	carbon monoxide BAL (relative to tyrosinase)	Chelators: DDC, EDTA, phenylthiourea 4-chlororsorcinol o- and p-fluorophenol products of substrate oxidation	p-chloromercuri-benzoate iodoacetamide trivalent arsenicals. NaN_3	H_2S, carbon monoxide	
pH	2-9.5	?	5.5 - 7.0	4.0 - 7.0	-
Stability heat	not stable at 60°	stable at 60°- 70°	inactivated at 60°	-	stable at 60°

(a) Dawson and Tarpley 1951, Bonner 1957 (b) Theorell 1957 (c) Dodds 1948

Apart from the obvious (specificity) similarities between laccase and the phenolase of Periplaneta, it is known that heating (100°C for 2 minutes) does not precipitate the protein in laccase preparations (Nakamura 1958a). Boiling 'exudate' preparations does not appear to alter the state of dispersion of the protein particles, although phosphate-precipitated, boiled 'exudate' (Le*) is more sticky and gel-like than 'exudate' (Le) which has been similarly treated with buffer.

Presence of 'exudate' actually inhibits the autoxidation of pyrogallol, ascorbic acid, dopa, o-tyrosine and resorcinol (table 2). This observation is significant only as a check on the enzyme and the solutions used, for it is known that free and complexed cupric ions in solution can catalyse the rapid oxidation of ascorbic acid (Dawson and Tarpley 1951, Scaife 1959) and some diphenols (Gillette et al 1954, 1955; Isaka and Ishida 1953). Ascorbic acid is usually not autoxidisable within the physiological range of pH (Mapson 1958), but if caused (catalysts) autoxidaion can often be inhibited by the presence of protein.

Ascorbic acid, especially in small amounts, is capable of decreasing the lag period and of substantially increasing the rate of oxidation (by 'exudate') of protocatechuic acid (fig. 15, table 4).

This effect may be due to the ability of ascorbic acid to reduce the o-quinone molecules back to the parent diphenol, thus maintaining the substrate concentration by a coupled reaction. (Dawson and Tarpley 1951, Krueger 1950).

The decrease in the induction period and increase in the oxidation rate might be caused (Mason et al 1955, Krueger 1958) by the reduction of the metal cofactor to the active cuprous state - see page 63, equation 2). This latter explanation would account for the sudden stimulation of 'exudate' diphenolase activity observed, although it is not thought to explain satisfactorily the removal by ascorbate of the induction period for monophenolases (Krueger 1958).

Similarly traces of catechol (Bordner and Nelson 1939) or dopa (Lerner et al 1949, 1951) reduce the monophenol induction period of plant and animal tyrosinases, whereas presence of oxidising agents lengthen this lag. Added catechol and dopa served only to reduce the rate of oxygen uptake of protocatechuic acid and 'exudate' (table 4, fig. 15).

That L-p-tyrosine in small amounts increased protocatechuic acid oxidation (table 4, fig. 15) may possibly be explained by the fact that the amino acid itself could be oxidatively deaminated - i. e. by reaction with a diphenol according to the cycle for glycine

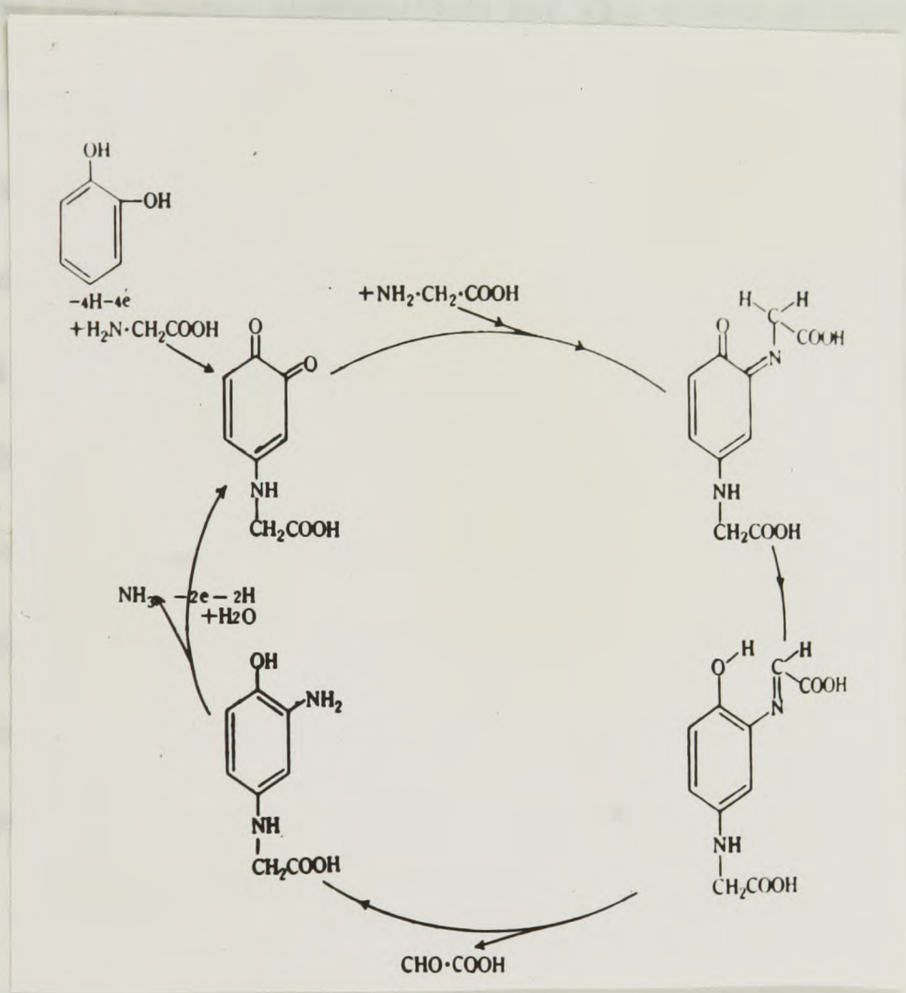


Figure 28: Scheme for the continuous oxidation of amino acid by mono substituted o-quinone (Mason 1955, Trautner and Roberts 1951)

(fig. 28 (proposed by Trautner and Roberts 1951)). It is not known yet whether glycine would increase the oxidation rate of protocatechuic acid by gland phenolase. Catechol oxidation by plant phenoloxidase (Beevers and James 1948, Mason 1955) is stimulated by glycine.

The less likely explanation for the effect of tyrosine on protocatechuic acid oxidation would be that the enzymically formed o-quinone is capable of hydroxylating the amino acid to form dopa. That o-benzoquinone formed from catechol could itself be responsible for the monophenolase action of tyrosinase is strongly contested (Bordner and Nelson, 1939, Dawson and Tarpley 1951).

Homogenized right colleterial gland greatly stimulated the left gland diphenolase (fig. 15) possibly, in small part, because of substrate liberation by the β-glucosidase activity upon the small amount of masked diphenol still present in 'exudate'. The presence in the right gland of some reducing agent or oxidase co-factor must be suspected from this result, while it is almost certain - from the present author's investigation and from that of Brunet and Kent (1955) - that a diphenolic substrate is not produced in any amount by this organ.

Concentrated NaCl dialysate of 'exudate' affected oxidation of protocatechuic acid so slightly (table 4) that the presence of a

dialysable co-factor in left gland secretion is very unlikely. The adduct was not desalted for the final concentration of NaCl in the incubation mixture was small (20 umoles in 1.5 mls.)

Inactivation of the phenoloxidase and utilization of substrate

The "enzyme" in 'exudate' is remarkably less subject to irreversible inactivation by the products of the oxidation of its substrates or by the actual functioning of the oxidase itself than any other phenolase reported (Dawson and Tarpley 1951, Bonner 1957).

Furthermore, this phenoloxidase retains its activity under conditions (temperature, pH change (fig. 13, table 7)) that would destroy most enzymes. (Dixon and Webb 1958).

Saline (0.01M) dialysed 'exudate' stored at 2°C retained most of its activity for several months. Preparations which had not been dialysed lost 52% of their original activity after standing for 2 days at 2°C. This inactivation appeared to be related to the fact that the white protein was tanned first pink, red then brown by the quinones formed from the diphenol released by hydrolysis of the comparatively large amount of endogenous glucoside present in gland secretion (see Fig.44).

(a) Quantitative aspects

Utilization of substrate by the oxidase, and thus subsequent inactivation (whether caused by product or not) was only brought about slowly compared to insect blood tyrosinase, (Pryor 1955, Onishi 1954a, b, 1953) and other phenoloxidases (Dawson and Tarpley 1951). Calculations made, even on the basis of one molecule of substrate requiring one atom of oxygen, showed that only the most active and concentrated 'exudate' preparations when buffered had been able to catalyse the oxidation of 3 μmoles of protocatechuic acid in about 400 - 600 minutes, of incubation at 37°C.

In the most active preparation (0.1M- NaCl precipitate of 'exudate' - precipitate 1, fig. 19) the uptake of 74.2 μls of oxygen was achieved in about 200 minutes. In the cases where extended incubation was carried out (e.g. fig. 24) the rate of oxygen uptake was usually linear even beyond the point, where one atom of oxygen per molecule of protocatechuic acid had been consumed. The rate of oxidation, as in the instance (fig. 24) of an acetone powder (precipitate 3, fig. 22) of gland homogenate, sometimes increased, unaccountably, long after the incubation had been initiated. After an extraordinarily lengthy lag period, precipitate 8, (fig. 22) eventually

catalysed the uptake of about 3 atoms of oxygen per molecule of substrate (i.e. (atmos. 751 mm. Hg) 1025 µls - see also precipitate 6, figure 23 (± 926 µls.)) after which the activity appeared to be about to cease abruptly.

After 51 hours incubation with protocatechuic acid (15 µmoles) the other gland homogenate fractions (i.e. precipitates 3, 4 and 5 - fig. 22 and 21) were still active (fig. 24). For precipitates 4 (acetone powder) and 5, the oxidation rate of the initial 15 µmoles of substrate had fallen considerably, although by this time (3100 minutes) less than 2 atoms of oxygen per molecule of protocatechuic acid (620 µls) had been taken up by the acetone powder (precipitate 4) and less than 3 per molecule by the (latter) phosphate precipitate 5 (850 µls). At 3390 minutes the acetone powder (precipitate 3 - fig. 22) from which precipitate 4 had been prepared, had also catalysed the uptake of less than 2 atoms per molecule of substrate (617 µls). This stoichiometric difference in "enzyme" affinity between the still (at this time) active acetone powders and other homogenate fractions cannot be readily explained. These concentrated preparations would be able to oxidize a great deal of substrate before they were finally inactivated (by auto-tanning) whereas the saline-dialysed 'exudate'

preparation, for example, the result of whose incubation is shown in figure 16, was much less sensitive, after 1160 minutes incubation, to further addition of protocatechuic acid. The sample that had been incubated without substrate until this juncture oxidized this diphenolic acid (in presence of "Tween" 85) at the normal rate only for about 100 minutes before the rate fell off considerably (fig. 16). The response (at 1250 minutes) by the 'exudate' preparation to the addition of "Tween" and a further 15 μmoles of catechol (at 1160 minutes) was larger than for protocatechuic acid but the disparity in the autoxidation rates for these substrates (and therefore between the role which the "enzyme" itself had played, until then, in the two cases) must be taken into account. This response to "Tween" and further addition of catechol is not manifested in the boiled control. Stoichiometrically there appeared to be no marked difference between the final result of the enzymic oxidation of hydroquinone, catechol and protocatechuic acid if allowance is made for the autoxidation (not boiled control-oxidation) of the former two substances. For 3 atoms of oxygen to be consumed per molecule of diphenol, oxidation followed by disubstitution (in reaction with amines) on the aromatic ring could have taken place. Alternatively, after monosubstitution, polymerization could have occurred.

(b) Induction of the "enzyme"

In the majority of experiments, especially those carried out in the preliminary survey, an induction period of between 30 and 60 minutes (sometimes longer - e.g. fig. 15) was observed for the enzymic oxidation of substrates. This characteristic (common to some other phenolases, especially to monophenolase activity in the absence of diphenols, (Dawson and Tarpley 1951, Bonner 1957)) appeared to be overcome here (see e.g. figs. 16 and 24) by the prior dialysis of the gland preparation against saline (0.01M) - not water - and, as already mentioned in this chapter, by ascorbic acid and to some extent by presence of right gland homogenate (fig. 15). This temporary lack of activity could be explained by the presence in 'exudate' of the phenolic glucoside or some other inactive phenolic substance (e.g. dopa) which could compete with the substrate for the active sites of the "enzyme". The existence of some equally dialysable (NaCl) component with (temporary) substrate-protecting property (e.g. a "quinone reductase" (Heller and Szarkowska 1958) or dehydrogenase (Dennell 1957) system cannot be ruled out yet.

The stimulation of the phenoloxidase activity of left gland 'exudate' by right gland homogenate may be caused, besides by an indirect functioning of the β-glucosidase but perhaps by a steroid,

(Dennell 1958a) lipid or lipoprotein (c.f. cytochrome oxidase (Hatefi 1958)). If such a component were already present in the secretion of the larger gland, then washing with acetone (see fig. 22) would be expected to partially inactivate the enzyme. The long induction period (fig. 23) shown by precipitates 6 and 8 (figs. 21 and 22) cannot therefore be explained on this basis, as the acetone powders (precipitates 3 and 4, fig. 22) and fractions 2 and 3 (fig. 25) did not show this characteristic.

The role of copper in the phenoloxidase

Oven-dried 'exudates' and T.C.A. precipitates (from 'exudate') contained on analysis between 0.1 and 0.2% (w/w) copper. 2, 2'-Diquinolyl gave no reaction with suspensions of 'exudate' or resuspended dried 'exudate' until the material had been ashed and a reducing agent added. This suggests either that the copper is very firmly bound and enclosed by the protein, or that it is present in the oxidized form (for the reagent is specific for cuprous ions). Kertesz (1957) claims that the native mushroom polyphenoloxidase contains cuprous ions, but there is some possibility that during preparation the enzyme protein itself might have (in this case) caused the reduction (Frieden 1958). Ingraham

(1959) argues that cupric-proteins would, however, be blue and at the pH values where most phenolases are active the potential of the catechol-o-quinone system would be so low that the cupric ion would not oxidise catechol. For laccase, which is blue, Nakamura (1958a) finds that redox potential of the enzyme at 25°C and pH 7.0 was + 415 mV, and he considers that the copper of the native enzyme is in the oxidized form even in the absence of oxygen.

That the white phenolase of cockroach resembles laccase with respect to the state of the bound copper is partially suggested by the observation (fig. 15) that ascorbic acid reduces the lag period for the oxidation of protocatechuic acid. On the other hand the vitamin is reported to depress the polyphenoloxidase activity of ripening tobacco leaves (Andrejcheva and Bajlov 1959) - which would be in keeping with Ingraham's (1959) arguments - but it has the opposite effect on most phenolases (Dawson and Tarpley 1951, Mason 1955, Krueger 1950, 1958).

In spite of the indication that copper is present in 'exudate' phenolase there appears to be no close correlation between the copper content of fractions of the "enzyme" and the ability of these fractions to catalyse the oxidation of protocatechuic acid (fig. 20).

However, as it will be pointed out later in this chapter, the extent of dispersion of the "enzyme" protein in these fractions should, under these circumstances, be taken into account when assessing their activity.

Together with this unexpected but perhaps superficial lack of activity - copper correlation, two other facts emerged from the preliminary investigation of this problem. The supernatants (B and C) of fractionated 'exudate' fractions (BC E and CE) (fig 20) contained a comparatively large amount of copper per mg. of protein. This suggests either that the metallic ion is being removed by the phosphate and saline treatments or that another non-enzymic copper protein is secreted by the left gland.

The second untoward fact to emerge was that metal chelating agents (James 1953) other than cyanide (table 6) and perhaps azide (fig.37, chapter 7) were without significant effect on the gland oxidase activity (towards protocatechuic acid). In view of the fact that other copper oxidases are sensitive to chelating agents such as EDTA, DDC, phenylthiourea etc. (Dawson and Tarpley, 1951 Bonner 1957) - see Table 8 - this anamolous situation may, possibly, only be explained by the particulate nature and relative insolubility of the 'enzyme' under investigation. Since

the chelating agents were always tested on 'exudate', already precipitated or aggregated by phosphate buffer, perhaps molecules or ions larger than cyanide were slow to reach the bound copper atoms. Perhaps, alternatively the chelated copper itself could oxidize diphenols, as in the case of the tyrosinase studied by Isaka and Ishida (1953). This is a likely explanation, which will be tested, in view of the fact that part of the copper associated with 'exudate' may be removed by fractionation with phosphate (fig. 20). It is known, though, that combined serum copper (Zuckerkandl 1959a) does not react readily with dithiocarbamates.

Despite the lack of evidence of a copper-activity interrelationship, the activity of the phenoloxidase, deactivated by long treatment with cyanide and dithiocarbamate (followed by dialysis) appears, nevertheless, to be partially restored on addition of curpric sulphate (table 7). That this effect could be caused by the mere presence of added cupric ions in solution is possible (Gillette et al 1954, 1955) but there was no significant change in activity of the dialysed 1M- K_2CO_3- treated control when copper sulphate was added. In addition to the inactivation of oxidase brought about by any removal of copper present, the

high alkalinity of the cyanide used (pH 10) by Lerner et al (1950) in their experiment on tyrosinase, might have drastic effect on the enzyme itself, judging by the inactivity of the sediment from the K_2CO_3 (pH 10) control included in our investigation (table 7) (the supernatant was less affected however). The milder dithiocarbamate treatment appeared to be more effective than that of cyanide, but subsequent addition of the 5 µg. of cupric ions stimulated oxygen uptake to the same extent (i.e. by 0.4 µls./mg. protein/hour - table 7) in both cases.

The dialysed supernatant of the dithiocarbamate-treated sample had no activity as expected, but the similar fraction resulting from the cyanide-reacted 'exudate' is unexpectedly active (Q_{O_2} 5.5, table 7). This cannot be readily explained. The non-sedimented fraction (125,000 g. for 1 hour) from K_2CO_3-treated 'exudate' has not suffered inactivation too greatly (QO_2 9.5) from contact with the alkaline carbonate (table 7). Removal of the cyanide (and cyanide complex) from the supernatant fraction by dialysis has accounted for nearly half of that activity registered in the carbonate supernatant. It would seem, then, that copper does play some role in the gland phenolase.

In conclusion, some slight evidence of the fact that molecular oxygen is involved in the oxidation of the diphenol by the

copper oxidase (see Mason et al 1950, Ingraham 1959) was indicated by the fact that "enzymic" oxidation of protocatechuic acid in air was 34% less rapid than the control in oxygen.

The specificity requirement to be met by the diphenolic substrate is emphasised by the effect of borate (which forms cyclic derivatives with o-dihydroxyphenols and not p-dihydroxyphenols) (Mallette 1950) on the oxidation of protocatechuic acid (table 6). The oxidation of hydroquinone was not altered by borate, as expected.

In character with caeruloplasmin (Curzon 1960) and many phenolases (Dennell 1943, Lerner and Fitzpatrick 1950, Onishi 1954c) sodium chloride temporarily inhibits the activity of the left gland "enzyme" (fig. 19).

Studies on the fractionation of left gland 'exudate' and homogenate

The results of attempted fractionation of 'exudate' gave little indication that prosclerotin or structural protein could be distinguished from the phenoloxidase by these methods. That these proteins may be one and the same (Whitehead, Brunet and Kent 1959) remains an attractive proposition, but it has been noticed (fig. 18) that successive water washings of low-speed "exudate" precipitate

show a diminishing activity per ml.

There was some suggestion (in the fractionation shown in fig. 20) that a non-enzymic copper protein might be present in "exudate" because there was no apparent correlation between phenoloxidase activity and the protein or copper content of the fractions obtained. Inactivation of the 'enzyme' would not account for the range of percentage copper values found for the T. C. A. precipitates (fig. 20) (even within the approximate accuracy of that method).

The results of the activity tests on various fractions obtained showed that, in general, an increase in the total activity of the preparations was recorded (viz. NaCl fractionation of supernatant 1 - fig. 18). In most cases, however, the opposite effect was noticed (e. g. fractionation of precipitate AD, fig. 20).

The tentative explanation was that the sediment from centrifuged previously unfractionated 'exudate' might have a greater total activity if resuspended in a certain volume of water than if resuspended in much less. Concrete proof of this protein-dispersion effect has not been obtained but some evidence for the hypothesis will be lead shortly.

The situation is complicated because the phosphate buffer, in which all activity measurements were made, brought about

considerable precipitation or aggregation of the protein, with the result that the values obtained may not represent the true activities of some fractions. It is notable that the total activity of the fractions yielded after phosphate fractionation show that a decrease in the potential activity (in phosphate) has taken place (fig. 18 and fig. 20). The search for a more suitable buffer, though extensive has been unsuccessful so far.

Addition of sodium phosphate (pH 6.8) to 'exudate' preparations resulted, after centrifugation, in supernatant and precipitate fractions both having enzyme activity. If the buffer is added to the sediment of 'exudate' obtained centrifugally, little effective solubilization or redispersal of the active protein was achieved (e.g. precipitates AD and CE, fig. 20) whereas, if phosphate fractionation of the supernatant of this 'exudate' is attempted, the precipitate then obtained seldom contains 50% of the original activity of the fraction before phosphate addition (e.g. see fig. 17).

Protein dispersion and particle size.

An attempt was made to deduce a relationship between oxidase activity and dispersion (or particle size) of 'exudate' protein in various fractions, by statistical correlation of the T.C.A.

precipitate (mg.) values /ml. with respective figures for hourly oxygen uptake (µls) /ml. of "enzyme" solution or /mg. (i.e. specific activity) of T.C.A. protein found.

Figures 28[a] and 29 show the scatter diagrams of these two sets of activity values plotted against the T.C.A. protein content of each fraction investigated. It is of interest that, as for a soluble enzyme system, the protein concentration (>1.0 mg/ml.) has little effect on the specific activities of these fractions (fig. 29) and in fact the scatter of all values lies on a rather sharp hyperbole.

The correlation coefficient (r) of these Q_{O_2} and protein values is calculated to be -0.469 (31 degrees of freedom (D.F.)) which is significant (Student t-test) at the 1% level, (Appendix 1b). The correlation coefficient for the two sets of values plotted in the scatter diagram shown in figure 28[a] is found to be +0.459 (39 D.F.) which is also significant at the 1% level (Appendix 1a). However, the values found for the supernatant fractions (marked ⊗x) only (fig. 28[a]) have a correlation coefficient of +0.471 (11 D.F.) which is not significant even at the 10% level (Appendix 1a).

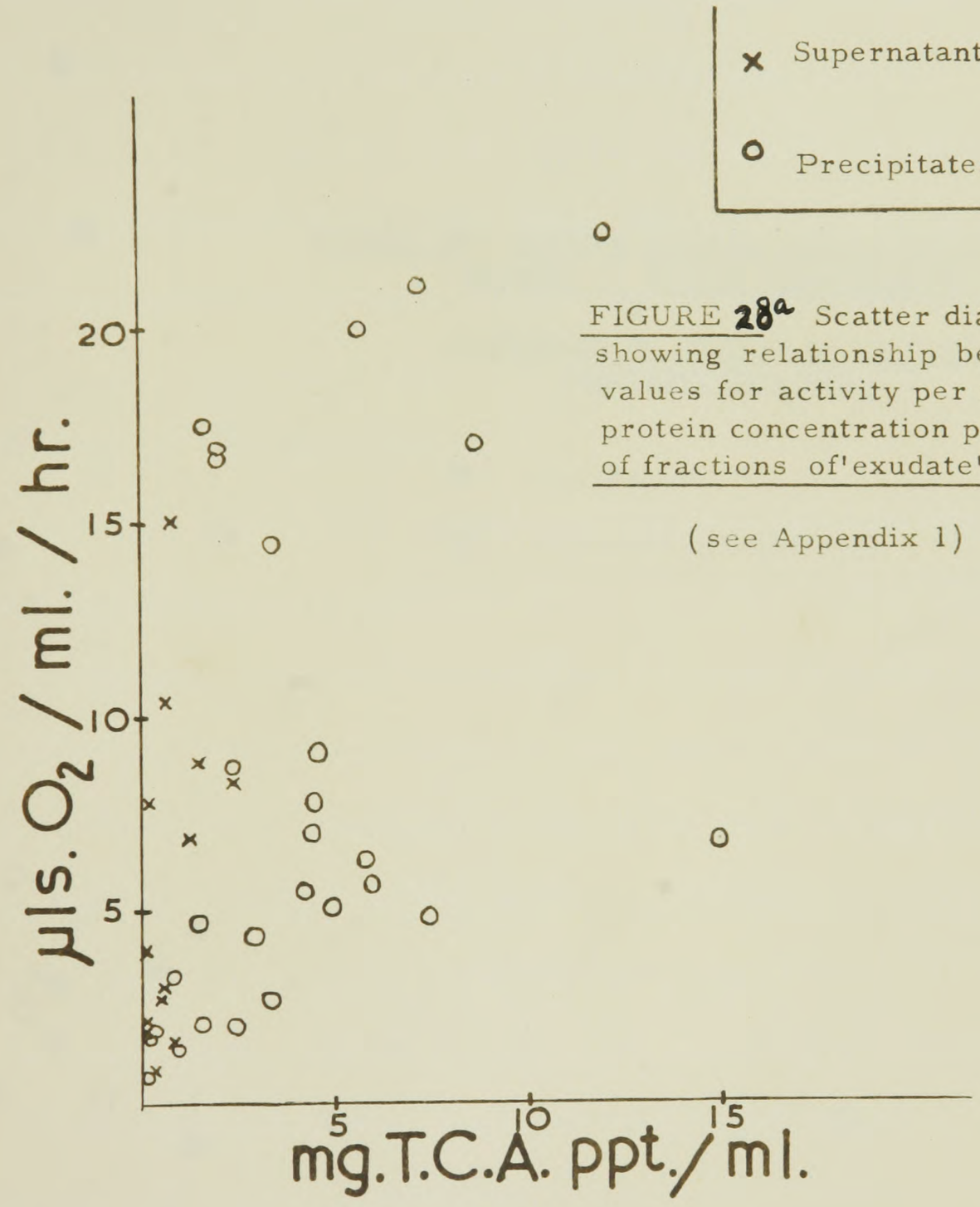


FIGURE 28a Scatter diagram showing relationship between values for activity per ml. and protein concentration per ml. of fractions of 'exudate' obtained

(see Appendix 1)

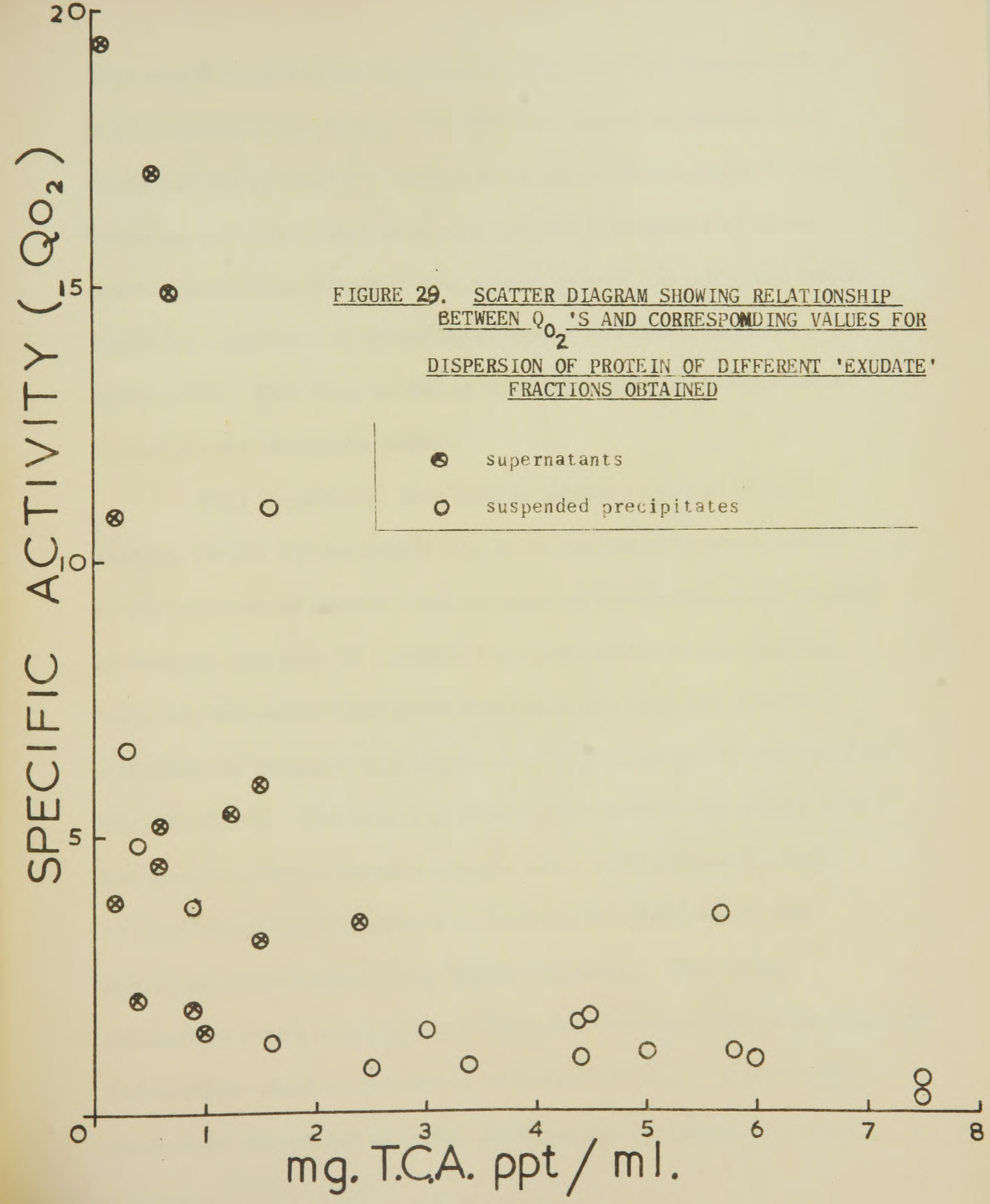


FIGURE 29. SCATTER DIAGRAM SHOWING RELATIONSHIP BETWEEN Q_{O_2}'S AND CORRESPONDING VALUES FOR DISPERSION OF PROTEIN OF DIFFERENT 'EXUDATE' FRACTIONS OBTAINED

This would suggest that the state of the protein (in supernatants) is of importance here, i.e. the more dispersed or diluted the "enzyme" the greater its affinity to catalyse protocatechuic acid oxidation relative to that of a resuspended precipitate or more concentrated supernatant. The effect, though, is not great enough under the conditions of these experiments to show a negative correlation. This may be due to the aggregation of the particles caused by the phosphate buffer.

This hypothesis, that size or surface area of protein particle (which can be considered to be reflected to some extent in the measure of protein concentration of supernatants) is related to enzymic activity, is supported to some extent by the fact that ionic and non-ionic detergents increased the total and specific activities of 'exudate' and fractions of it (viz. pages 81-6 Section B(ii) Chapter four). The action of detergents could be due partly to the decrease in surface tension brought about at the liquid-particle or liquid-aggregate interface or because the particles in the aggregate may become more highly dispersed. The latter alternative would mean that a greater surface area and more "enzyme" active sites would be presented to the substrates. Under these conditions, the action of metal chelating agents should be more efficient.

When detergent was added to an incubation mixture some time after the initiation of the reaction at 37°C, the effect on activity was not very noticeable (table 5 and fig. 16) possibly for the reason that the phosphate-aggregated particles had by this time firmly adhered to the walls of the flask (which were oscillating). The effect of added "Tween" in the experiment whose result is shown in figure 16, may have been minimized because the preparations had by this time been partially inactivated by quinone auto-tanning of the 'enzyme'. Sodium hexametaphosphate, a colloid surface active agent, was unable to disperse the protein aggregates once formed (in the presence of sodium phosphate buffer).

The phenoloxidase may actually be solubilized or depolymerized by the non-ionic ("Tween") detergent, as is cytochrome oxidase (Yonetani, Takemori, Sekuzu and Okunuki 1958, Ambe and Venkataraman 1959 by 1% 'Emasol 4130' (polyoxyethylene sorbitan mono-oleate)). Nevertheless, addition of phosphate buffer still produced some precipitate in the preparations treated with "Tween" and deoxycholate. "Tween" is reported to increase the induction period of tyrosinase (Lerner 1953) but if anything the effect was the opposite in this investigation. Sodium deoxycholate and other ionic detergents are known to

inactivate (temporarily) cytochrome oxidase (Yonetani et al 1958, Yonetani 1959) but deoxycholate (and acetone) activated plant chloroplast-phenolase (Mayer and Friend 1960) and the cockroach "enzyme" (viz. page 84 Section B(ii) Chapter four). Anionic, but not non-ionic wetting agents were found to activate the 'latent' phenolase of bean leaf (Kenten 1958).

Conclusion

The results suggest that protein aggregation or particle dispersion appears to influence the measureable activity of phenoloxidase preparations. The activities recorded for variously fractionated "enzyme" preparations must be viewed with some caution. The findings of the preliminary survey of the specificity, although not suitably corrected in all experiments are perhaps more reliable and comparable since the treatment of the separate non-dialysed 'exudates' used was as uniform as possible. In spite of this the variation in the results obtained for protocatechuic acid (3 µmoles) oxidation (Q_{O_2} 3.93 ± 1.15 (10)) was large. However, the possibility must not be overlooked that some seasonal variation in the left gland secretions might occur. An effort was made by careful selection of the cockroaches to minimize the effects of age,

disease and of the oothecal-laying cycle upon the experiments carried out during the course of these investigations.

The time lag or induction period found for some 'exudate' fractions especially for homogenate precipitates 6 and 8 (fig. 23 and see figs. 21 and 22 respectively) is quite unexpectedly and abnormally long for any enzyme. These lags may be caused either by presence of oxidizing agents or a quinone reductase (see Farkas and Ledingham 1959, Heller and Szarkowska 1958) or possibly by the absence of some co-factor or even by the temporary or partial denaturation (i.e. inactivation) of the 'enzyme' during the experimental manipulations.

The fact that this 'enzyme' behaves unusually raises the question (again) whether the oxidations which it catalyses, although remarkably specific, are truly enzymic. That some prepantions of gland 'exudate' and homogenate are inactive for an extraordinary length of time, may mean that all fractions should be incubated for as long as 50 to 60 hours before judgment on their catalytic ability is passed.

It is unlikely that bacteria could be responsible for the oxidations since high concentrations of antibiotics did not reduce "enzyme" activity (fig 23) and microscopic investigation of 'exudate' (Gram –

(Mackie and McCartney, 1956)

stained and under phase contrast) before and after incubation revealed very few micro-organisms.

The "enzyme" under investigation is to be understood, as a catalytic system designed to function in the insect vestibulum, over a period of hours, in the form of apparently insoluble particles contained in a substrate-rich, highly viscous secretion. (Perhaps, then, it was no accident that the "enzyme" failed to be solubilized.) Any attempt to dissolve these particles or to alter the natural state of protein aggregation may, besides, result in the modification of the in vivo character of the system.

In conclusion, as expected, the phenoloxidase of the cockroach left colleterial gland apparently resembles closely the cuticle enzyme, described by Onishi (1954a) and Wigglesworth (1957). Before any successful direct application to cuticle of the findings of these investigations, the experiments already undertaken should be extended and augmented to reveal more clearly the characteristics of the insoluble copper-containing protein from the left gland.

SECTION II

THE PHENOLIC INTERMEDIATES IN LEFT COLLETERIAL GLAND

CHAPTER SIX - INTRODUCTION

The rise and fall in the tyrosine content of insect blood at pupation (Dennell 1947, Fraenkel and Rudall 1947, Onishi 1954b, Hackman 1956) suggests that the diphenolic substrate for cuticle phenoloxidase, and hence the tanning-quinone precursor, arises from blood tyrosine (Mason 1955, Hackman 1958). Dennell (1958a) has questioned this theory on the grounds that tyrosine in the blood serves only to form melanin in the cuticle. Pryor (1940b) thought that 3,4-dihydroxyphenylalanine (dopa) formed by the action of blood tyrosinase on tyrosine at pupation - and found in cuticle (Schmalfuss and Barthmeyer 1929, Levenbook, 1950) - underwent deamination in the cuticle before sclerotization was to occur.

This suggestion is supported by Mason (1955) because:-

(1) tyrosine and dopa themselves are unable to tan cuticle (Pryor 1940b, Fraenkel and Rudall 1947),

(2) the nitrogen content of cuticle is inversely proportional to the degree of hardening and darkening caused by tanning (Evans 1938) for the increase in its weight (equivalent to blood tyrosine lost), which follows sclerotization, is not accompanied by an increase in its nitrogen content (Frankel and Rudall 1947), and

(3) possible derivatives (fig. 30) of dopa have been isolated from cuticle; namely 3, 4-dihydroxyphenyl-lactic (Pryor, Russell and Todd 1947), 3, 4-dihydroxyphenylacetic (Schmalfuss and Bussman 1935, Pryor et al 1946, Hackman, Pryor and Todd 1948) and protocatechuic acids (Pryor et al 1946, Kawase 1958).

Richards (1951) indicates that 3, 4-dihydroxyphenyl propionic acid has also been identified, but this more recently is questioned (Hackman 1958) although Malek (1960) claims its presence (on seemingly little chromatographic evidence) in Schistocerca gregaria soft cuticle.

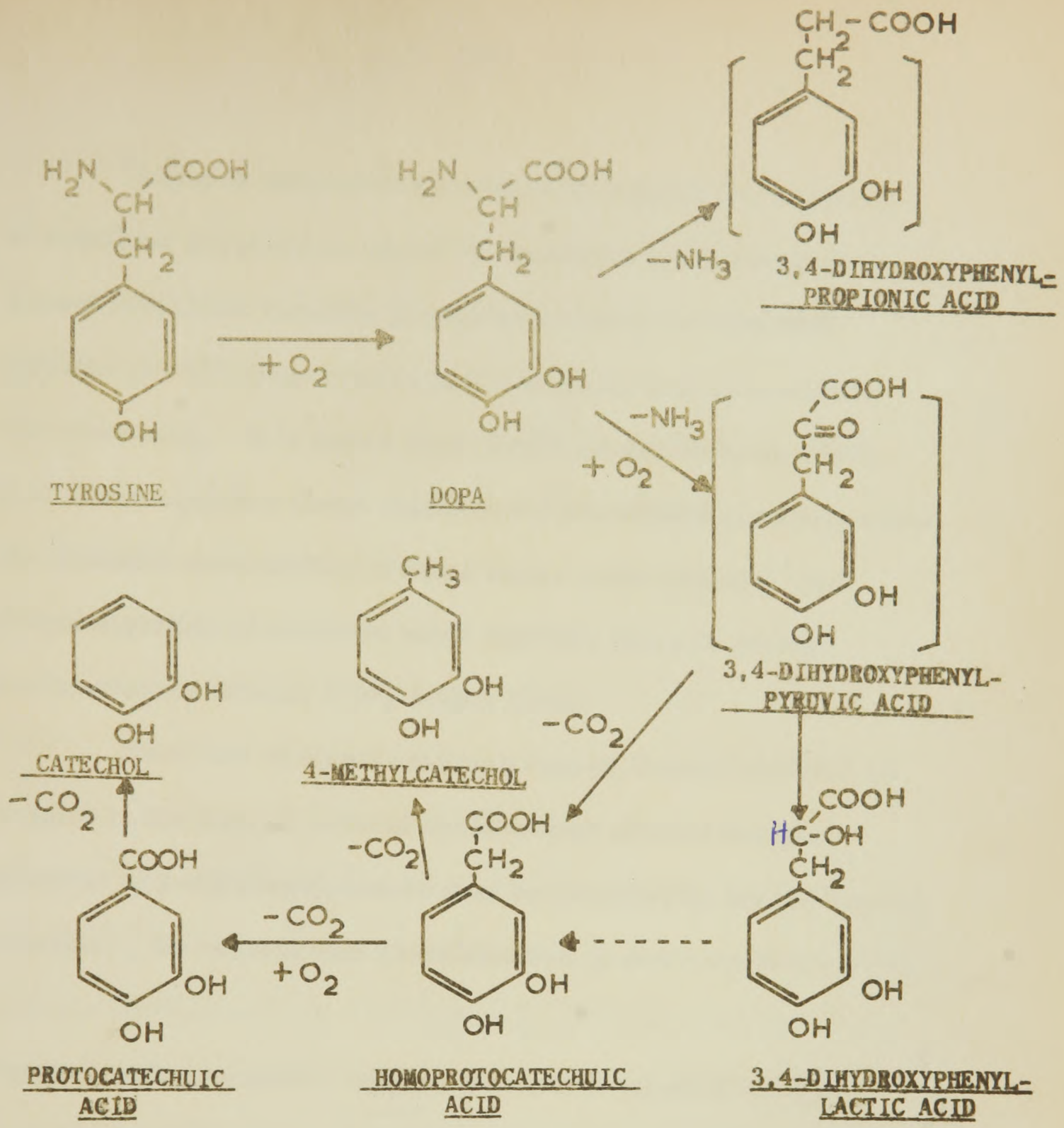


FIGURE 30. STRUCTURAL FORMULAE OF TYROSINE, DOPA AND DERIVATIVES CLAIMED TO BE FOUND IN ARTHROPOD CUTICLE (Hackman 1958, Richards 1951)

There is little direct evidence to support the sequence of reactions proposed in figure 30 (Hackman 1958) except that Brunet (1961) has recently presented evidence to show that tyrosine-G-C^{14} gives rise to protocatechuic acid glucoside in the cockroach. It is known also (Trautner and Roberts 1950) that o-benzoquinone (from catechol and phenolase) can bring about the oxidative deamination of some amino acids (fig. 28), and decarboxylation of aromatic acids can take place in certain circumstances (Mason 1955, Malek 1960).

This lack of direct evidence caused Dennell (1958b) to suggest in the light of several observations (1958a) that the quinones of p-dihydroxyphenols may be responsible for the tanning reaction. He reports that phenylalanine, p-and o-tyrosine (1956) together with pyrogallol, hydroquinone, and catechol (1956) have been identified by him in soft white cuticle of Calliphora vomitoria larvae and suggests that only a system that brings about non-specific hydroxylation of aromatic amino acids can explain their occurrence in vivo and their synthesis in vitro. o-tyrosine has also been reported in cuticle of Periplaneta americana (Kennaugh 1958). Hackman (1958) disputes the claims of Dennell mentioned above and

considers the evidence (chromatographic) given insufficient to support the hypothesis of non-specific hydroxylation.

Nevertheless, protocatechuic acid is still found in exuviae of silkworm pupal (Kawase 1958), oothecal fluid (Pryor et al 1946, Brunet and Kent 1955) and Calliphora puparia and in larval cuticle (Dennell 1958a) after sclerotization is complete. Dennell (1958a, b) and Kennaugh (1958) claimed that this acidic diphenol played no part in cuticle formation for it was unable to tan soft cuticle in vitro, but there is reason to believe that their incubations were carried out in the absence of any buffer (Brunet 1958). Protocatechuic acid appeared to tan 'dewaxed' cuticle of albino locusts Schistocerca gregaria (Jones and Sinclair 1958).

Dennell (1958b) supposes that resorcinol in the epicuticle is responsible for retardation of tanning at the site of quinone formation. He has also suggested (1958c) that epicuticle is not immediately tanned after commencement of sclerotization because the steroid-or lipid-protein complex, which may be identified with the phenoloxidase, may itself migrate inwards to the diphenol-rich protocuticle instead of the substrate having to diffuse outwards to the epicuticle.

It is of interest that Pryor (1955b) suspected that the tryptophane derivatives kynurenine and 3-oxykynurenine demonstrated by him in mature cuticle of Calliphora and Lucilia could be involved in selerotization, as well as in pigment (ommochrome) formation (Butenandt 1957).

Pryor et al (1946) identified protocatechuic acid as the diphenol present in Blatta odhecal fluid. The role of this diphenol in the formation of the sclerotin walls of cockroach ootheca was confirmed by Brunet and Kent (1955) who found that it was produced by the left colleterial gland as the 4-0-β-D-glucoside (Kent and Brunet 1959). Silk worm larval integument and the exuviae of pupae also contain this substance and since it appears rather suddenly in the integument before pupation, Kawase (1958) considered that, as in the cockroach, it might have been previously masked or alternatively, that it is formed in situ from an inactive precursor.

1-Benzoyl-β-D-glucose (periplanetin) has been isolated from the latero-cervical organ of Periplaneta and Blatta (Quilico, Piozzi, Pavan and Mantica 1959), but the role of the substance is not clear.

m-Aminophenol and hydroquinone when injected into locusts are excreted as their glucosides (Myers and Smith 1954) which are

non-toxic. In this mode of phenol detoxication, insects differ from higher animals, in which, as a rule, the glucuronides are formed (Smith 1955).

Candy and Kilby (1960) have demonstrated that trehalose biosynthesis in locusts is achieved by a uridine diphosphate glucose (UDPG)-enzyme system (i.e. a glucosetransferase). Dutton and Duncan (1960) showed that the source of glucose for detoxication of o-aminophenol in homogenized hepatic caecae of Periplaneta also involved UDPG, which could be replaced by ATP +UTP + glucose-1-phosphate, but not by UDP-glucuronic acid. UDPG + protocatechuic acid incubated in presence of left colleterial gland homogenate failed, in first attempt, to give rise to the 4-O-β-D-glucoside of the diphenol (Kent 1958). Brunet (1961) has shown that tyrosine-G-C^{14} injected into the haemocoel of a ligatured abdomen of a female cockroach (P. americana) is incorporated between 4 - 24 hours into the protocatechuic acid glucoside found in the left colleterial gland.

Recently, Malek (1960) has demonstrated the presence after acidic hydrolysis (or, under reducing conditions, after alkaline hydrolysis) of 4-methylcatechol in mature, but not in soft cuticle of Schistocerca gregaria. This substance and a

similar unidentified compound were also found in soft cuticle that had been artifically tanned with homoprotocatechuic acid and 3,4-dihydroxyphenylpropionic acids respectively. As the latter phenolic acid itself gave rise to homoprotocatechuic acid upon incubation with cuticle (and 4-methylcatechol is formed from homoprotocatechuic acid under the same conditions) the existence of a mechanism in cuticle which can bring about decarboxylation was suspected (see fig. 30). Hackman and Todd (1953) previously had demonstrated that catechol arose from the action of a plant phenolase on protocatechuic acid, or rather that the carboxyl group of the acid, is readily displaced by amines. They studied the oxidation of catechol and related substances in the presence of aniline. Whether the decarboxylation reported by Malek (1960) is enzymic or not is not clear. Aerts, Vercauteren, and Decleir (1960) demonstrated that decarboxylation of p-hydroxyphenyl-pyruvic acid takes place in presence of crude extracts of homogenized Calliphora erythrocephala larvae (to which diethyldithiocarbamate had been added to inhibit the phenolase).

Malek (1960) has tentatively proposed that the exocuticle of insects would be amber if tanned by p-quinones, but darker if o-quinones were responsible. This is of interest for it suggests that insects could be classified by the metabolism of their

phenolic compounds (i. e. by the nature of the parent, diphenol formed in the cuticle). Definite evidence of the participation of p-diphenols does not appear to have been established (Hackman 1958) judging by the lack of detail (of results) attached to the claims of Dennell (1956, 1958a) and Malek (1960).

It has been already established with regard to melanization (Wigglesworth 1953, p. 402) that it "seems to be the localization of the chromogen" and not of the phenolase, "which determines the distribution of pigment patterns" in cuticle. Changes in the surface layer of the cuticle, which are probably governed hormonally and through the nervous system, are dictated by the epidermal cells immediately below the area in question (Wigglesworth 1953). Although it is difficult to distinguish between the darkening of insect cuticle caused by melanization and by that due to phenolic tanning, Malek (1957) has proposed that the two processes (in the desert locust) may be governed by sparate phenoloxidases. This suggestion would appear to be supported by Dennell's (1958a) observation that melanin formation and not tanning, in the puparium of Protophormia terrae-novae was inhibited by phenylthiourea injection.

The active substances in some insect poisons especially of Coleoptera and Blattoidea are most probably synthesised from p-benzoquinone (Pavan 1959). This is of great interest since venoms are secreted in stinging insects by the modified sexual accessory glands (Wigglesworth 1953). For instance Triboleum castaneum produces ethyl-p-quinone. It is known, though, that the quinones contained in venoms are not derived directly from the diet (Pavan 1959), as is often the case for the phenolic substances secreted by odoriferous glands (Wigglesworth 1953). The characteristic smell of some insects may be attributed to alkylbenzoquinones secreted by these glands (Thomson 1957, Hackman, Pryor and Todd 1948, Estable, Ardao, Brasil and Fieser 1955).

It is also known that some ants and caterpillars secrete formic acid and some Mycetophilid larvae oxalic acid (Wigglesworth 1953). This is of interest for the left colleterial glands of Periplaneta exude calcium oxalate in their secretion (Brunet 1952) but its function is not understood.

The metabolic pathways by which formate and oxalate are produced in different insects are not known. It is possible that those pathways involved in quinone (and diphenol) synthesis in various insects stem from a common precursor especially where

these diphenols or quinones are secreted by the sexual accessory glands. This precursor would appear to be the tyrosine found in the amino acid-rich haemolymph (Brunet 1961), as has been suspected (Pryor 1940a, b) for some time. Since catechol and protocatechuic, homoprotocatechuic, 3,4-dihydroxyphenyllactic and possibly even 3,4-dihydroxyphenylpropionic acids have been demonstrated in insects, the hypothetical pathway (Hackman 1958), shown in figure 30 would suggest itself. In the next Chapter some evidence will be presented which suggests that biosynthesis of protocatechuic acid from tyrosine does not follow the pathway shown in figure 30.

CHAPTER SEVEN

RESULTS

A. SURVEY FOR PHENOLIC COMPOUNDS IN THE TISSUES OF PERIPLANETA AMERICANA.

In preliminary experiments the findings of Brunet and Kent (1955) (see page 17) were confirmed. The presence of protocatechuic acid glucoside in (50% aqueous ethanolic) homogenates of left colleterial gland was demonstrated. The chromatographically separated subetance (Rf% 53-56; BAW, 4:1:5), when aluted with water, exhibited u. v. spectral properties (λmax 245 and 288 mμ; λmin 227 and 258 - see page 75) characteristic of the synthetically prepared subetance (Brunet and Kent 1955, Kent and Brunet 1959). The glucoside was hydrolysed by the action of -glucosidase of emulsin or right gland homogenate to give glucose (Rf% 13) and protocatechuic acid (Rf% 81-82). The latter component showed spectral properties identical with the authentic compound (λmax. 253, 289 mμ; λmin 233, 274 mμ), when eluted from the paper (Whatman No. 1) with 50% aqueous ethanol.

Other organs of the female cockroach were homogenised in aqueous ethanol and their extracts examined chromatographically (BAW, 4:1:5). The components detected with the ferric chloride/ferricyanide spray (Fe^2) and by u.v. contact printing (253 mμ. emission) are listed in Table 9 below.

In this solvent (BAW, 4:1:5) the Rf% values of known phenolic compounds are:-

Compound	Rf%
dopa	20-22
p-tyrosine	34-37
protocatechuic acid glucoside	53-56
protocatechuic acid	80-83

Other possible sources of protocatechuic acid glucoside in the cockroach, besides the left colleterial gland, are the fat body, malpighian tubes and the mid-gut, (column 5, Table 9). Only the component found in the gut tissue showed both phenolic properties and absorption near 253 mu. However, upon elution (water) of this component, incubation for 5 hours at 37° C with emulsin, and after chromatographic separation, no evidence could be found for the presence of a phenolic compound resembling protocatechuic acid.

Table 9. Components detected in aqueous ethanolic

Organ or tissue	Method of detection				Rf%
fat body	u. v.	7.2	19.3	23.6	29.9
	Fe^2	11.1	20.5	24.5	
malpighian tubes	u. v.				30.2
	Fe^2	9.4	25.2		
mid-gut	u. v.	6.9		23.1	28.7
	Fe^2	9.2	19.2	23.4	
hind-gut (colon)	u. v.	7.8	24.8		
	Fe^2	11.3	24.1		
ovaries	u. v.	8.9	25.6		
	Fe^2	14	25.9		
blood	u. v., Fe^2	—— streak to ——			
contents of gut	Fe^2				
nerve cord and ganglia	u. v.				
	Fe^2				
left colleterial gland	u. v.				
	Fe^2	7.5	22.9		
oothecal fluid	u. v.		15.8		
	Fe^2	8.1	12.1	20.2	

extracts of cockroach tissues by descending chromatography (organic solvent layer, BAW, 4:1:5).

35.5		44		
	40.8		51.8	91.7
35.5		45	54.4	68.5
35.2	41.2			
36		44.8	53	65.2
	40		51	77.2
	41			87.7
		43.9		93.7
→		44		
				70.6
	41.3			
	41.5			
34.4	41.6	45	53.1	
34.2	40	46	52.9	
33.7			54	81.5
	38.1		54.2	81.8

B. EXAMINATION OF PHENOLIC COMPOUNDS IN LEFT COLLETERIAL GLAND.

(i) In 'exudate' dialysates

Preparation of the phenoloxidase from left colleterial glands (see page 28) involved dialysing the glandular secretion against 0.01 M - NaCl to remove endogenous substrates. The combined dialysates were evaporated to dryness at 40° to 50°C under reduced pressure and stored at -15°C, final traces of water being removed by freeze-drying.

The glucoside and other constituents were extracted from the residue (of inorganic salt) in the manner shown in figure 31.

Samples (0.5 ml.) of the ether extract (F1) and aqueous layer (F2) were examined chromatographically in BAW, 4:1:1, 2% acetic acid and in n-butanol:ethanol:borate (BEB)-4:1:1 on borated paper. The results are shown in Table 10.

The glucoside was almost entirely confined to the aqueous layer (F2). After resolution of this fraction in BAW, the four components indicated in Table 10 were excised and eluted separately with 50% aqueous ethanol (4 ml.). Corresponding areas of

Figure 31

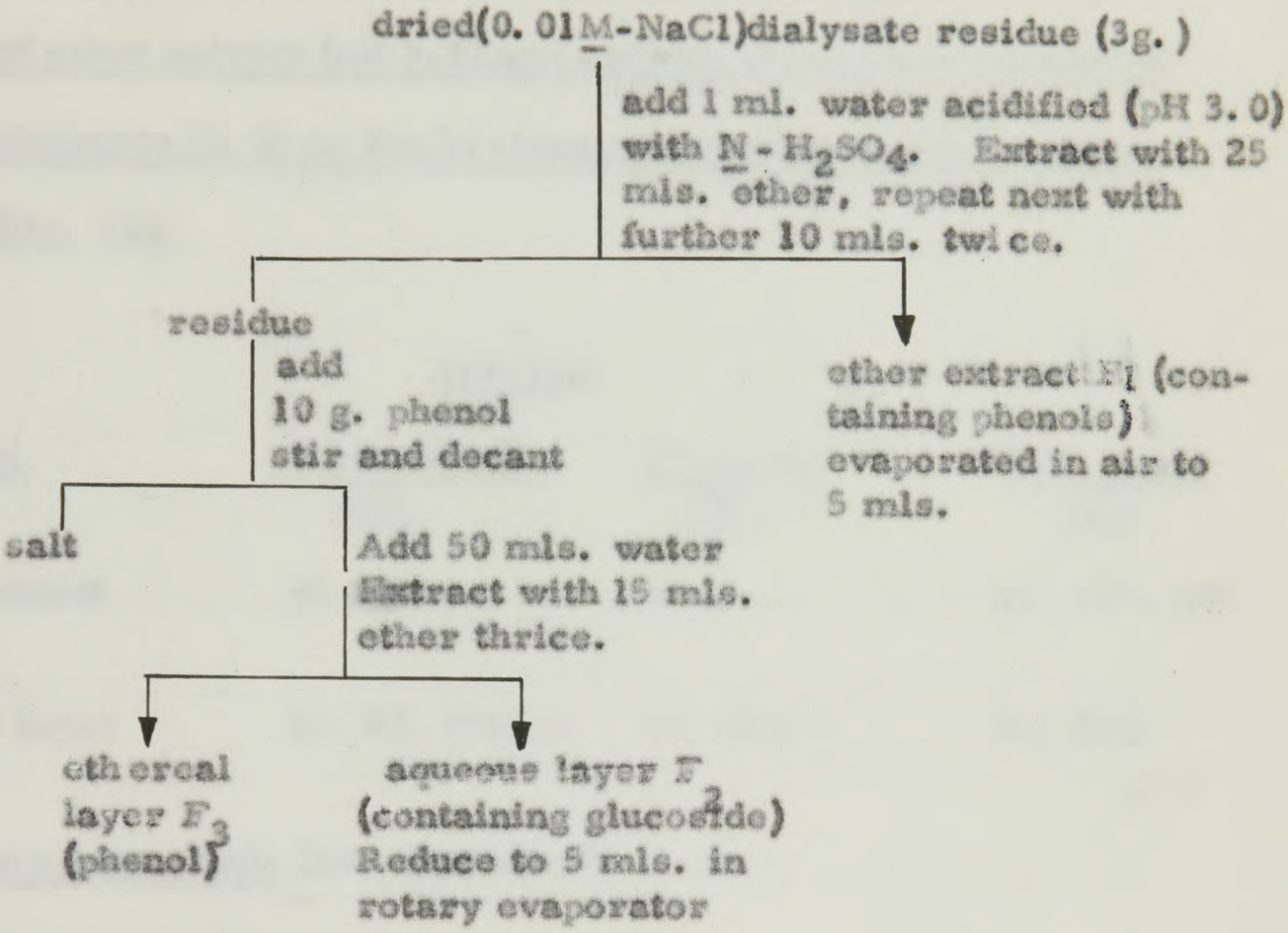


Fig. 31. Extraction of phenolic compounds from dried dialysates (0. 01 M-NaCl) obtained from left gland 'exudate'

Table 10. Chromatographic (descending) examination[1] of ether extract (pH 3.0) and aqueous layer from residue of dialysate (0.01 M-NaCl) obtained from left gland 'exudate' (fig. 31).

Fraction	Solvents: BAW, 4:1:1 Rf%	2% acetic Rf%	BEB, 1:1:1 Rf%
ether extract (F_1)	48, 90	60	25, 79*, 90*
aqueous layer (F_2)	17, 22, (36), 47	68, (81)	26, (54)
Reference compounds (see appendix 3)			
Protocatechuic acid glucoside	46-48	67-69	26
Protocatechuic acid	79-81	46-48	24
tyrosine	34-36	84-86	45-47
dopa	17-19	68-71	17-19
catechol	90-92	71-73	63-65

1 Components were detected with ferricyanide (Fe^2) spray and by u. v. contact-photography.

* Not sensitive to ferricyanide
( ) No absorption at 253 mμ. } on paper

blank portions of the chromatogram were excised, and eluted for purposes of control. The spectral properties of the faster-moving (Rf% 47) substance indicate that it was protocatechuic acid glucoside, whereas the Rf% 36-component had λmax 277 mμ and λmin. 248 mμ. The two slower-moving constituents showed no appreciable absorption at this dilution.

The two fast-moving components in F_1, separated by BEB, 1:1:1, were not ferricyanide positive but they absorbed strongly at 253 mμ on paper. They could not be identified due to lack of further material. Similar components in incubated gland homogenates were noticed very near the solvent front (BAW, 4:1:1), as for example in figure 32.

(ii) In left gland homogenates.

(a) Evidence for catechol (cat)

(1) Descending paper chromatography. Evidence for the formation of catechol during the incubation (37°C) of homogenate of left gland (L) and of left with right (LR) colleterial glands came to light as the result of a chromatographic investigation of the ethanolic extracts (± 0.2 mls.) of these incubation mixtures.

About 8 to 10 left colleterial glands (L) and a similar number which had been dissected out together with the right glands (L+R) were homogenized in water (± 5 mls.) separately. 10 right glands (R) were also homogenized in water (± 2 mls.). Samples (± 0.2 mls.) were taken from each of the homogenates, i.e. at 0, 1, 3, 8 and 26 hours after initiation of incubation at 37°C. At the time of sampling an equal volume of 96% ethanol was added to each sample and after slow speed centrifugation in narrow test tubes the supernatants were applied to Whatman No. 1 paper.

The results of descending chromatography of these samples in BAW, 4:1:1 - using the ferric chloride/ferricyanide (Fe^2) spray and ultraviolet (253 mμ) contact photography for detection of diphenols - are shown in the photographs below (fig. 32b, 33).

The extract of the homogenate of left and right gland which had been incubated for eight hours (LR_8) contained a component with Rf% 80 (fig. 32).

This phenolic substance which did not absorb strongly at 253 mμ (fig. 32) had also been formed in homogenate of left gland after 26 hours (L_{26}) incubation at 37°C (fig. 33). In LR_{26} this component was most evident (figs. 33, 34) for the protocatechuic acid

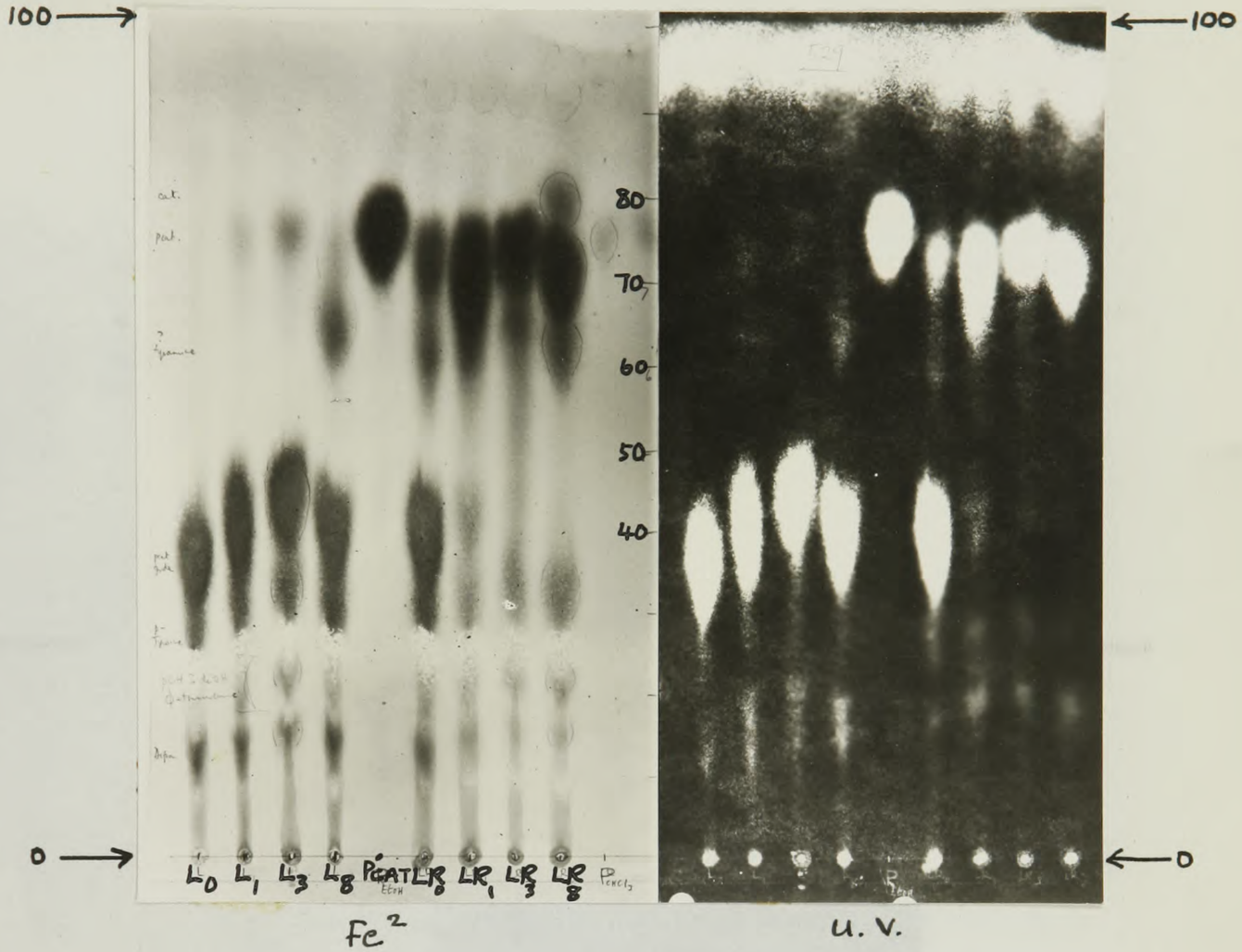


Figure 32: u/v contact print of and paper chromatogram (BAW, 4:1:1) showing separation of phenolic compounds present in ethanolic extracts of left (L) and left with right (LR) gland homogenates after incubation (37°C) at 0, 1, 3 and 8 hours. Reference compound - protocatechuic acid (Pcat).

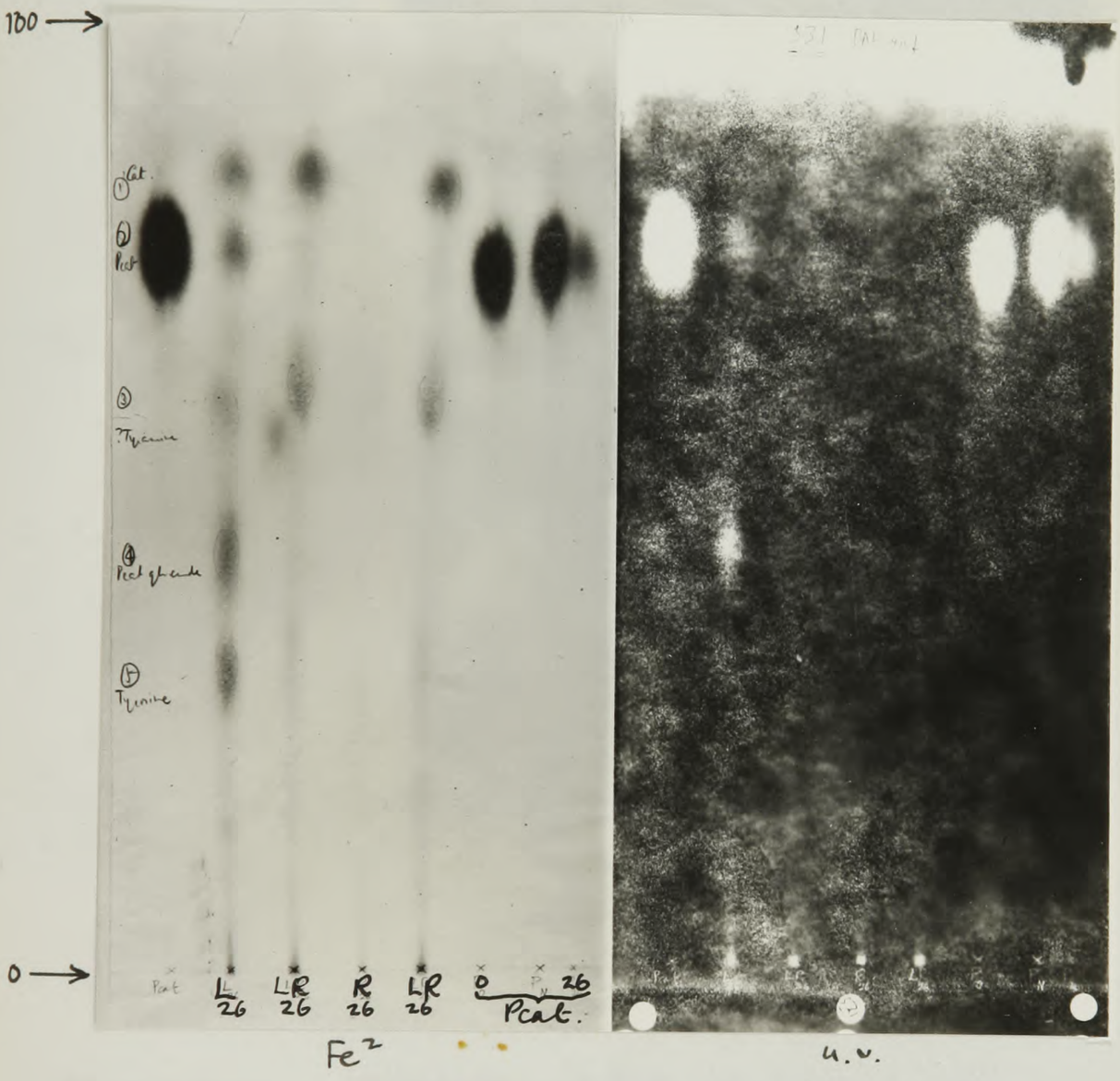


Figure 33: Separation of homogenate extracts (as described in figure 32) after 26 hours incubation (37°C). Right gland (R); reference compound protocatechuic acid (Pcat).

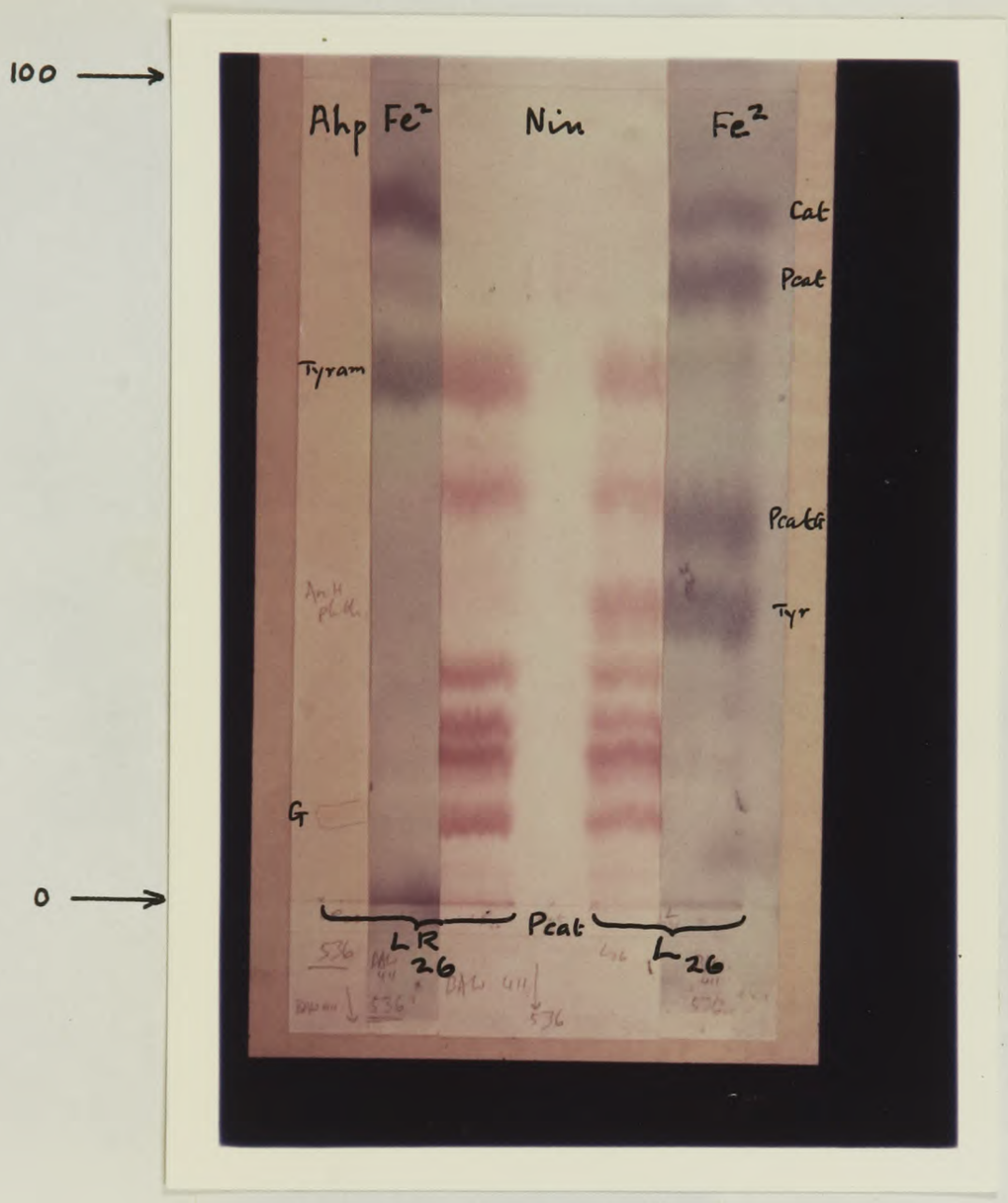


Figure 34: Separation (BAW, 4:1:1) of phenolic and amino compounds in ethanolic extract of left (L) and left with right (LR) gland homogenate incubated (37°C) for 26 hours. Phenolic compounds (blue) detected with ferricyanide spray (Fe^2). Amino compounds (red) with ninhydrin (Nin), and glucose with aniline hydrogen phthalate spray (Ahp).

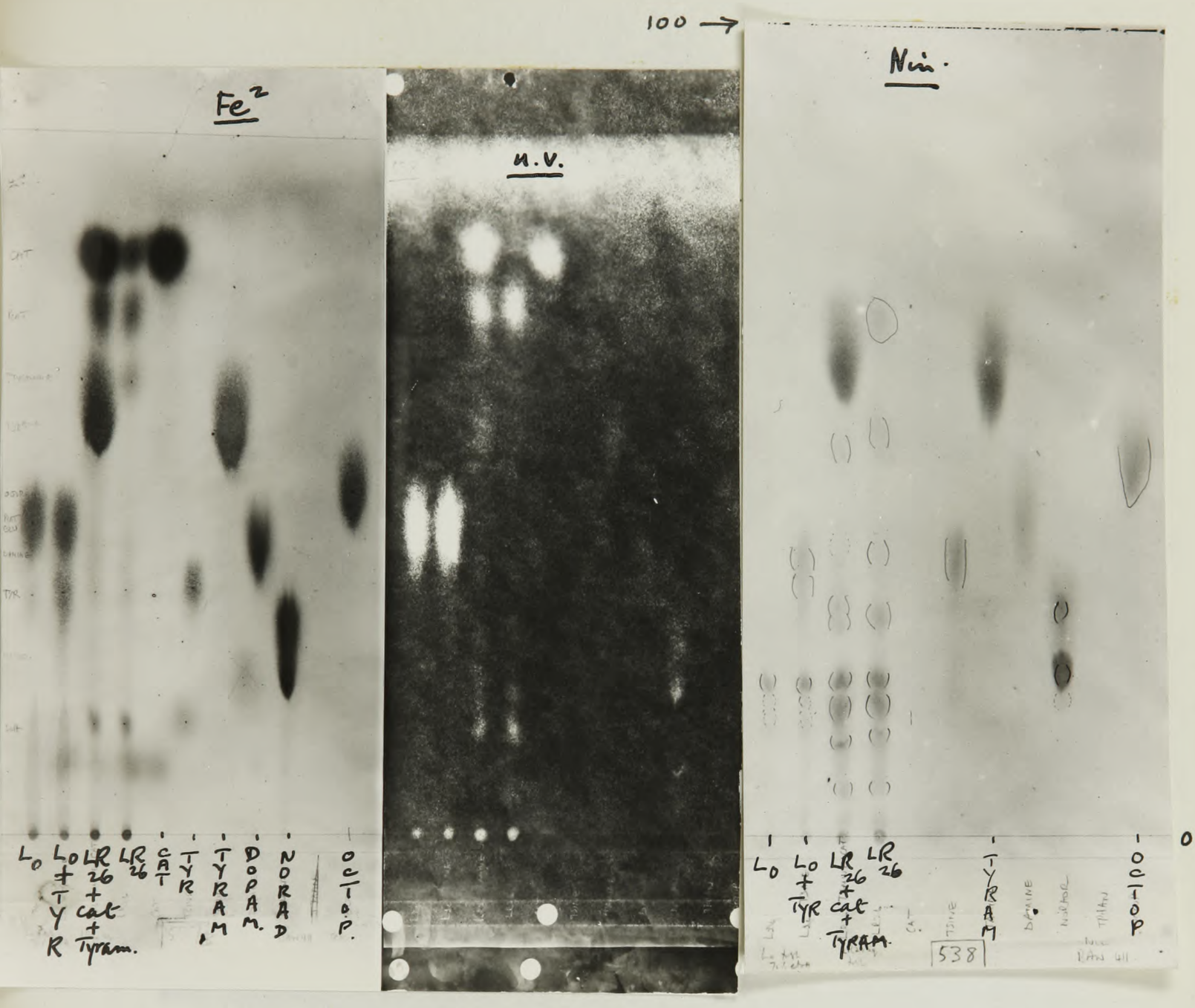


Figure 35: Chromatographic separation (BAW, 4:1:1) of phenolic compounds contained in ethanolic extract of left and right (LR) gland homogenate incubated for 26 hrs at 37°C, together with authentic reference phenolic compounds shown. Detection - ferricyanide (Fe^2) and ninhydrin (Nin) sprays and u/v contact printing.

(Rf% 71-74) moving behind it (formed by the hydrolysis of the glucoside (Rf% 41-44, fig. 32)) had almost entirely disappeared. This component was augmented by the addition of authentic catechol to LR_{26} (fig. 35) and it was found to separate in BEB, 1:1:1 on borated paper with an Rf% of 63 (see Table 10).

In a repeat experiment, the ethanolic extract of homogenated left glands that had been incubated for 22 hours were chromatographed (as 6 cm. bars) on paper in BAW, 4:1:1 and in 2% acetic acid. Authentic catechol was also run (as a similar bar) in both solvents. The appropriate regions were excised (after a u.v. contact print had been made for the purpose of locating the diphenol) and eluted with 50% aqueous ethanol (spectrographically pure). The spectral properties (λmax. 277-8, λmin. 243-6) of the components eluted from both chromatograms compared with that of the eluted synthetic compound.

In a further experiment, homogenates (1 ml.) of left and left with right glands were incubated (37°C) in the presence of (i.e. L+ or LR+) protocatechuic acid (Peat) and/or p-tyrosine (Tyr) (3 µmoles each) for 16 hours.



Figure 36: Chromatographic examination (BAW, 4:1:1 (solvent stood 24 hrs)) of ethanolic extract of homogenates of left (L) right (R) and both glands together (LR) which were incubated (37°C) with protocatechuic acid (Pcat) and/or p-tyrosine (Tyr).

Reference compounds: Pcat, catechol (cat) and p-tyramine (Tyram).

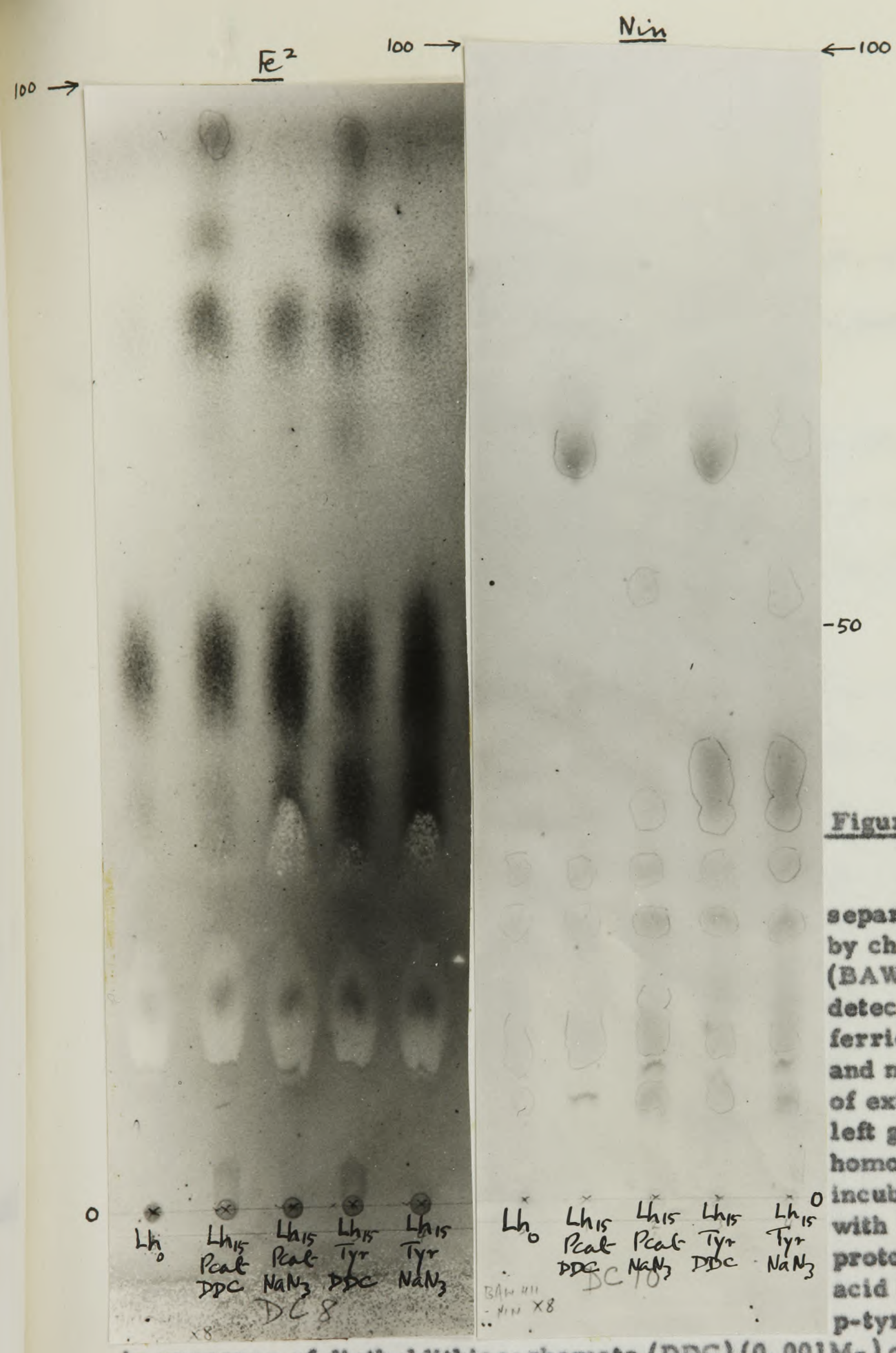


Figure 37: separation achieved by chromatography (BAW, 4:1:1 - detection with ferricyanide (Fe^2) and ninhydrin (Nin)) of extract of left left gland homogenate (Lh) incubated (37°C) with 3 µmoles protocatechuic acid (Pcat) or p-tyrosine (Tyr) in presence of diethyldithiocarbamate (DDC) (0.001M-) or sodium azide (NaN_3) (0.01M-) for 15 hours.

The result of the chromatographic (BAW, 4:1:1) examination of the ethanolic extracts (0.2 mls.) of these mixtures is shown in figure 36.

A ferricyanide-positive component with Rf% 82, similar to reference catechol, is evident in the extracts of L+Pcat and L+Tyr. It is less evident in LR + Pcat and not at all in LR + Pcat + Tyr, and R + Pcat.

This component was detected by the same method (fig. 37) in homogenate of left colleterial (15 hours, 37°C) with Pcat or Tyr. - both in the presence of 0.001 M - sodium diethyldithiocarbamate (DDC). When this copper-chelating agent or phenoloxidase inhibitor (James 1953) was replaced by 0.01 M- sodium azide, the component with Rf% 80-82 could not be detected with ferricyanide.



Figure 38. Separation of protocatechuic acid (Pcat) and catechol (cat) by paper electrophoresis (Whatman No. 3 220 v, 2 hours) in sodium phosphate buffer (0.1 M -, pH 6.0). Detection with ferricyanide.

(2) Paper electrophoresis.

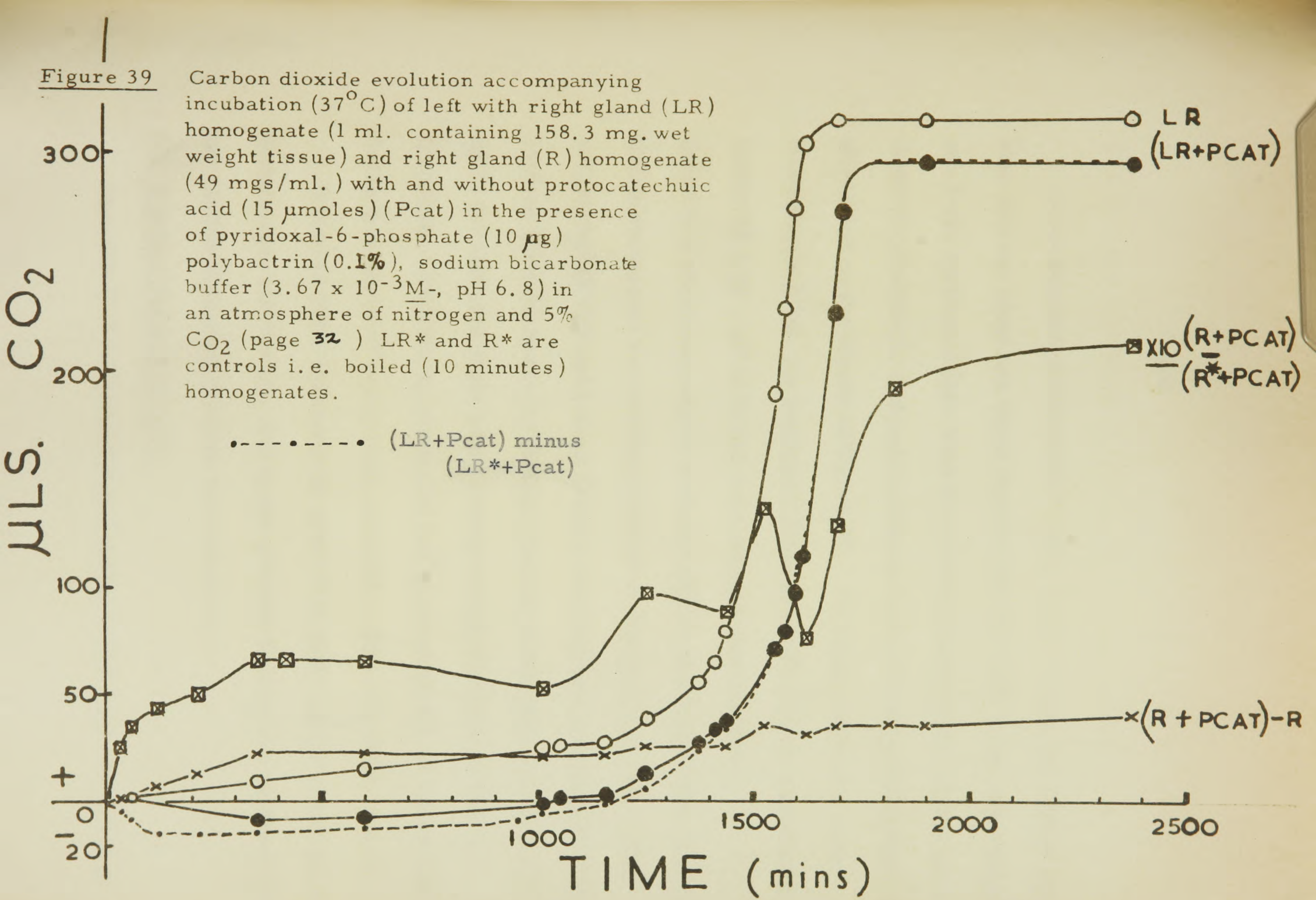
When in milligram quantity catechol could be detectably separated from protocatechuic acid by paper electrophoresis in sodium phosphate buffer (pH 6.0) (figure 38). This method was not suitable for separating the components contained in incubated homogenates, for the methods of detection (ferricyanide spray and u.v. contact-photography) were not sensitive enough under the conditions used. Separation of other diphenols was also limited by the quantities required for detection.

(3) Manometry.

There is some evidence that evolution of carbon dioxide accompanies anaerobic incubation (37°C) of right (R) gland homogenate (1 ml. containing 49 mg. wet weight of tissue) in the presence of (Pcat) protocatechuic acid (15 μmoles). In the absence of substrate, the homogenate (control) was responsible for half as much the gas evolution.

Although left and right glands (LR) when homogenated together also produce CO_2 upon prolonged incubation (fig. 39) and this system is heat labile, the volume evolved is not increased by the presence of protocatechuic acid.

Figure 39 Carbon dioxide evolution accompanying incubation (37°C) of left with right gland (LR) homogenate (1 ml. containing 158.3 mg. wet weight tissue) and right gland (R) homogenate (49 mgs/ml.) with and without protocatechuic acid (15 μmoles) (Pcat) in the presence of pyridoxal-6-phosphate (10 μg) polybactrin (0.1%), sodium bicarbonate buffer (3.67×10^{-3} M-, pH 6.8) in an atmosphere of nitrogen and 5% CO_2 (page 32) LR* and R* are controls i.e. boiled (10 minutes) homogenates.



In an experiment described later (page 173 and fig. 50) no gas was evolved when left gland homogenate (L) (39.6 mg. wet weight/ml.) was incubated with Pcat (15 μmoles) bicarbonate buffer, pyridoxal-6-phosphate, and polybactrin in an N_2-5% CO_2 atmospher as described in the legend of figure 39. However, when the same homogenate (1 ml.) is incubated alone and with Pcat in bicarbonate buffer (0.1 M , pH 6.8) and pure nitrogen for 15 hours ( ) there is a chromatographic evidence (BAW, 4:1:1 and BEB, 1:1:1) that a component resembling catechol is formed (fig. 51). With p-tyrosine (TYR) and L-dopa (both 15 μmoles) this component is also evident after 15 hours incubation, but not in the boiled control (L *) or in the left plus right gland homogenate (LR) alone or with p-tyrosine. Aerobic incubation of left gland alone for 15 hours produces a component resembling catechol. (fig. 51, BEB, 1:1:1)

There is little manometric proof then that - in the presence of bicarbonate and 5% CO_2 and in the absence of oxygen - protocatechuic acid is decarboxylated to give catechol.

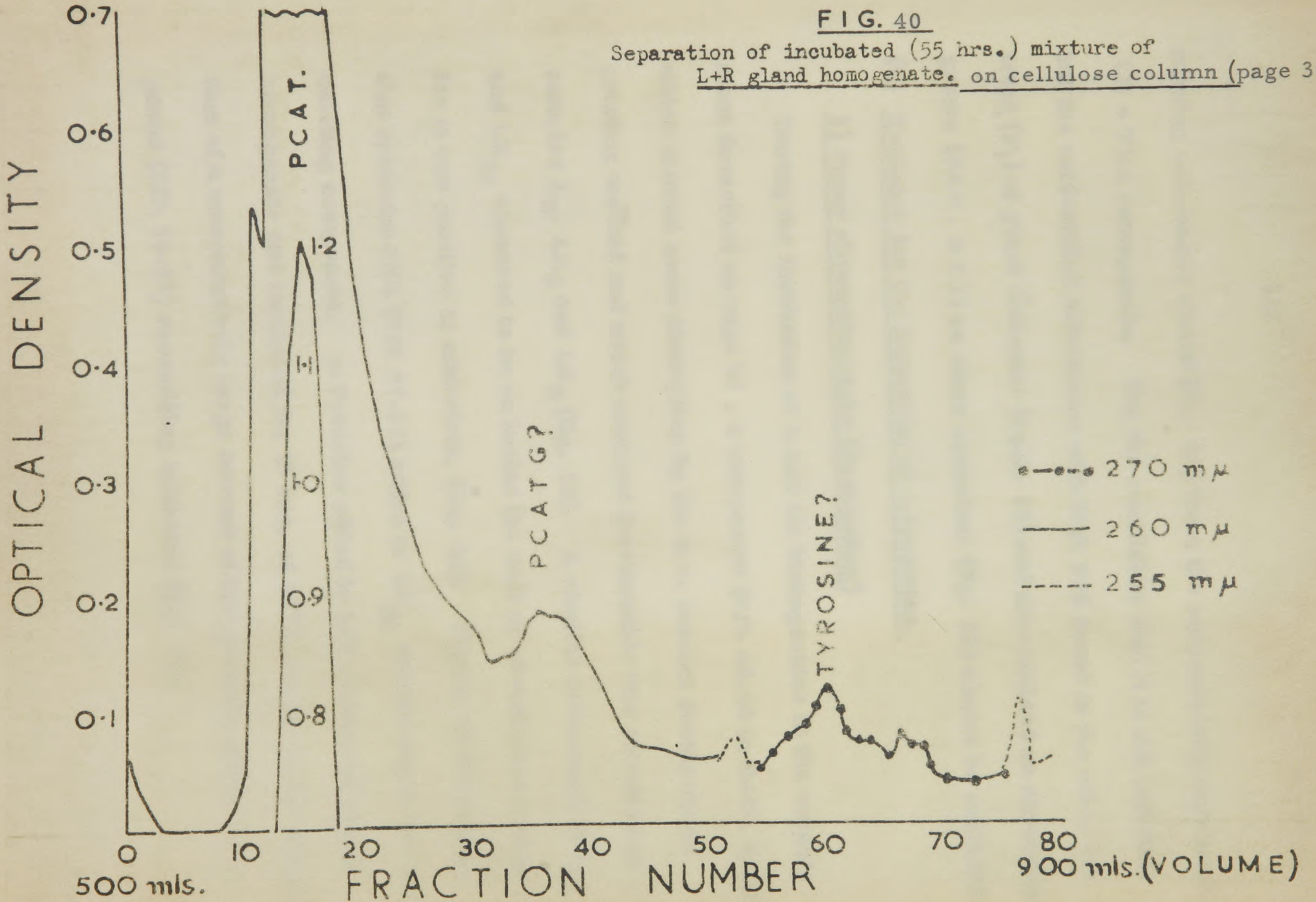
(4) Column chromatography

Samples (1.0 ml) of incubated mixtures (55 hours at 37°C) of

left and right gland homogenate (LR) with and without Pcat (15 μmoles) were chromatographed (BAW, 4:1:1) down a cellulose column (3 x 155 cms) as described on page 37-8. Figure 40 shows the result of the separation of LR alone, while figure 48 shows the result achieved for the same homogenate incubated with p-tyrosine. Figure 49 gives the elution pattern for known quantities of the phenolic compounds thought to be involved. In fresh BAW, 4:1:1 solvent catechol shows a λmax of 279-281 mμ. (see appendix 2). By the time the absorptions were measured for the standards (fig. 49) the max. λ was 276 mμ. Because of this, and because separation of catechol from protocatechuic acid by this method is not satisfactory (see fig. 49 and appendix 2), the likelihood of catechol being detected in the presence of about 0.70 mg. of protocatechuic acid in LR (fig. 40) is small. The elution peak of Pcat is not entirely regular, but catechol could not be demonstrated by subsequent chromatography of samples of tube 12 (fig. 40). Nevertheless a component with λmax. 280 mμ in a homogenate of left gland (L) incubated (55 hrs.) with tyramine in air is separated on this cellulose column (fig. 53). This component is obviously not identical with the fast-

FIG. 40

Separation of incubated (55 hrs.) mixture of L+R gland homogenate. on cellulose column (page 36-7)



(FIG.40)

running substances eluted (fig. 48) from the anaerobically incubated LR + TYA homogenate. But the possibility that it is not catechol but the unidentified substances with high Rf% found in the ether extract (E_1) of gland dialysate (Table 10) and also noticed on chromatograms (BAW, 4:1:1) on other occasions (fig. 32) cannot be excluded.

(b) Evidence for the formation of p-tyramine.

1) Paper chromatography (descending)

During the incubation of L and LR homogenates in the experiment described on page 161, a component (Rf% 62-64 in BAW, 4:1:1) which showed some absorption by the u. v. contact photographic printing method and which oxidised ferricyanide was revealed in samples L_8, LR_9 and LR_8 (fig. 32). A similar component in L_{26} and LR_{26} appeared to be an amino (as well as phenolic) compound for it was positive to ninhydrin, (fig. 34). Figure 35 illustrates that tyramine-HCl (Rf% 57-63) added to LR_{26} extract augments the existing component. p-Tyrosine added to left colleterial gland homogenate and incubated for 15 hrs. at 37°C results in the formation of a comparatively large amount of the phenolic amino compound (Rf% 58-63) resembling tyramine (fig. 41).

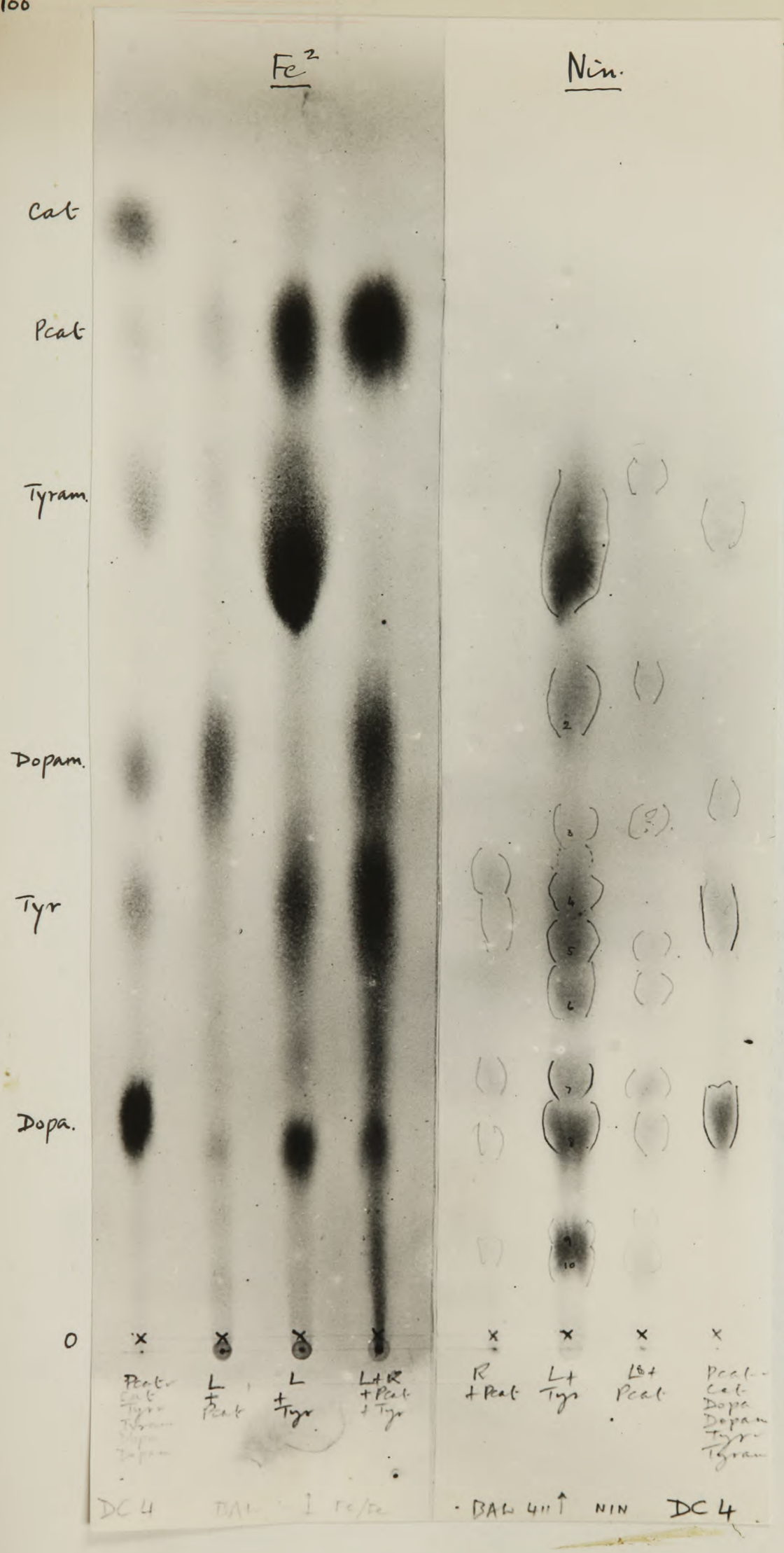


Figure 41

Result of incubation of left (L), right (R) and both (LR) gland homogenates (ml.) with Pcat and/or Tyr (3 μmoles each) The photograph shows the chromatographic separation (BAW, 4:1:1) of components detected with ferricyanide (Fe^2) and ninhydrin (Nin) sprays run against the six reference compounds shown.

Elution (0. ·01 N-HCl) of this Rf% 57-64 component separated (BAW, 4:1:1) by descending chromatography from the extract of left gland homogenate, incubated (37°C) alone for 22 hours, resulted in a solution having λmax. 225 and 278 mμ and λmin. 246 mμ with a slight shoulder at 281-2 mμ (Table 11). This absorption spectrum compared favourably with that of authentic tyramine-HCl which had been chromatographed and eluted in an identical manner.

The eluate contained a phenolic constituent which separated with Rf% 71-72 in amyl alcohol pyridine and water (2 : 2 : 1) and 87-90 in 2% acetic acid. Among many other substances when chromatographed (ascending) in these solvents tyramine separates in these regions (see appendix 3).

Furthermore, like tyramine, when the eluate (4 ml.) was neutralised (by adding 1g. of the carbonate form of Dowex-1 resin) and made alkaline (pH 10. 0) with a drop of 1 N-NaoH, the absorption maxima of the subetance it contained showed a shift from 224 and 279 mμ. to 241 and 298 mμ. (see fig. 42).

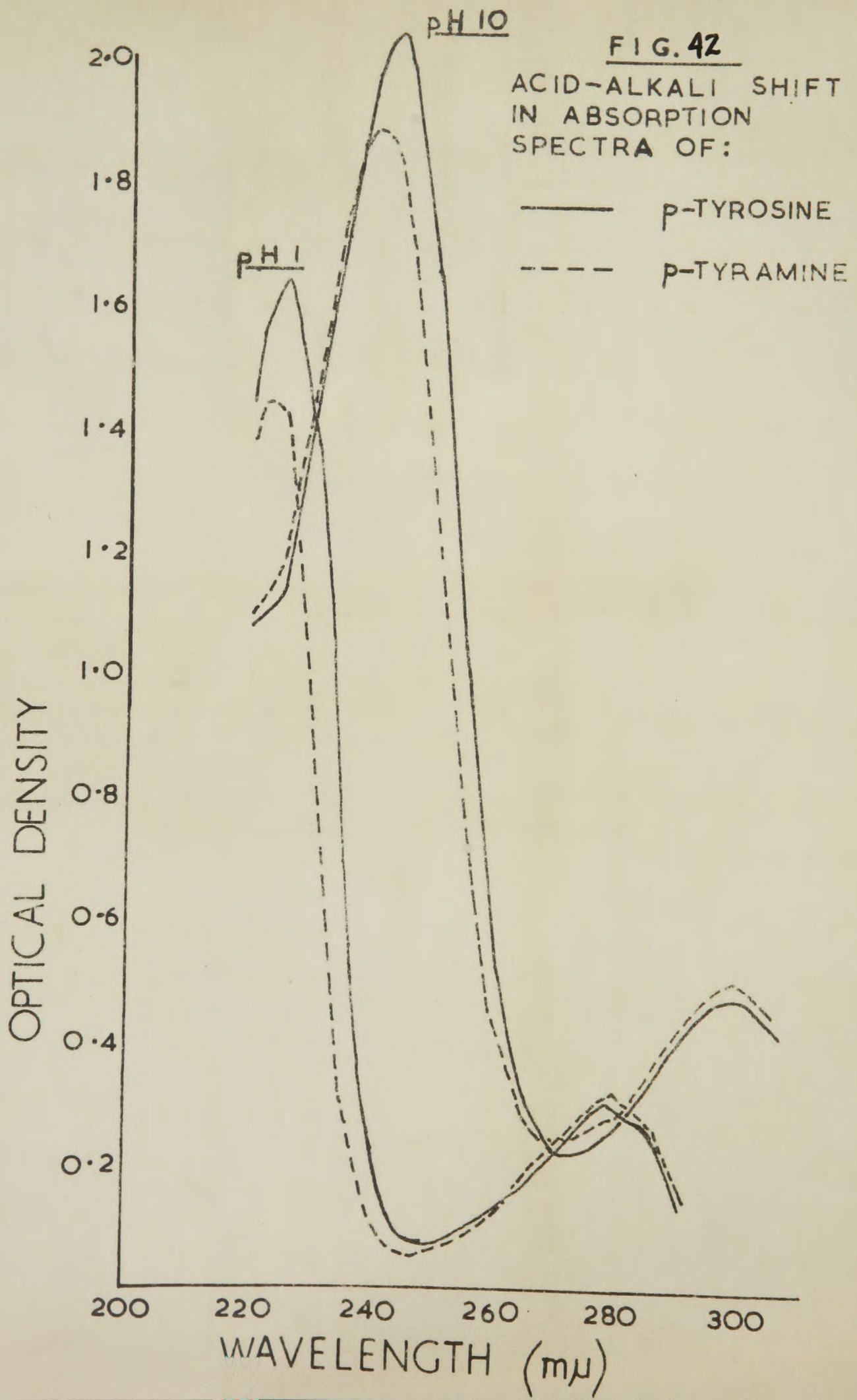


FIG. 42

ACID-ALKALI SHIFT IN ABSORPTION SPECTRA OF:

——— p-TYROSINE

- - - - p-TYRAMINE

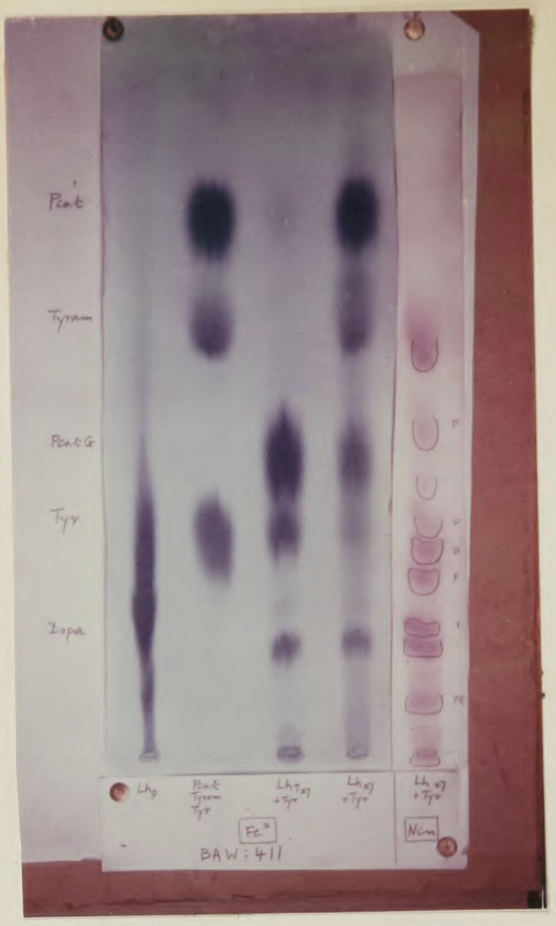
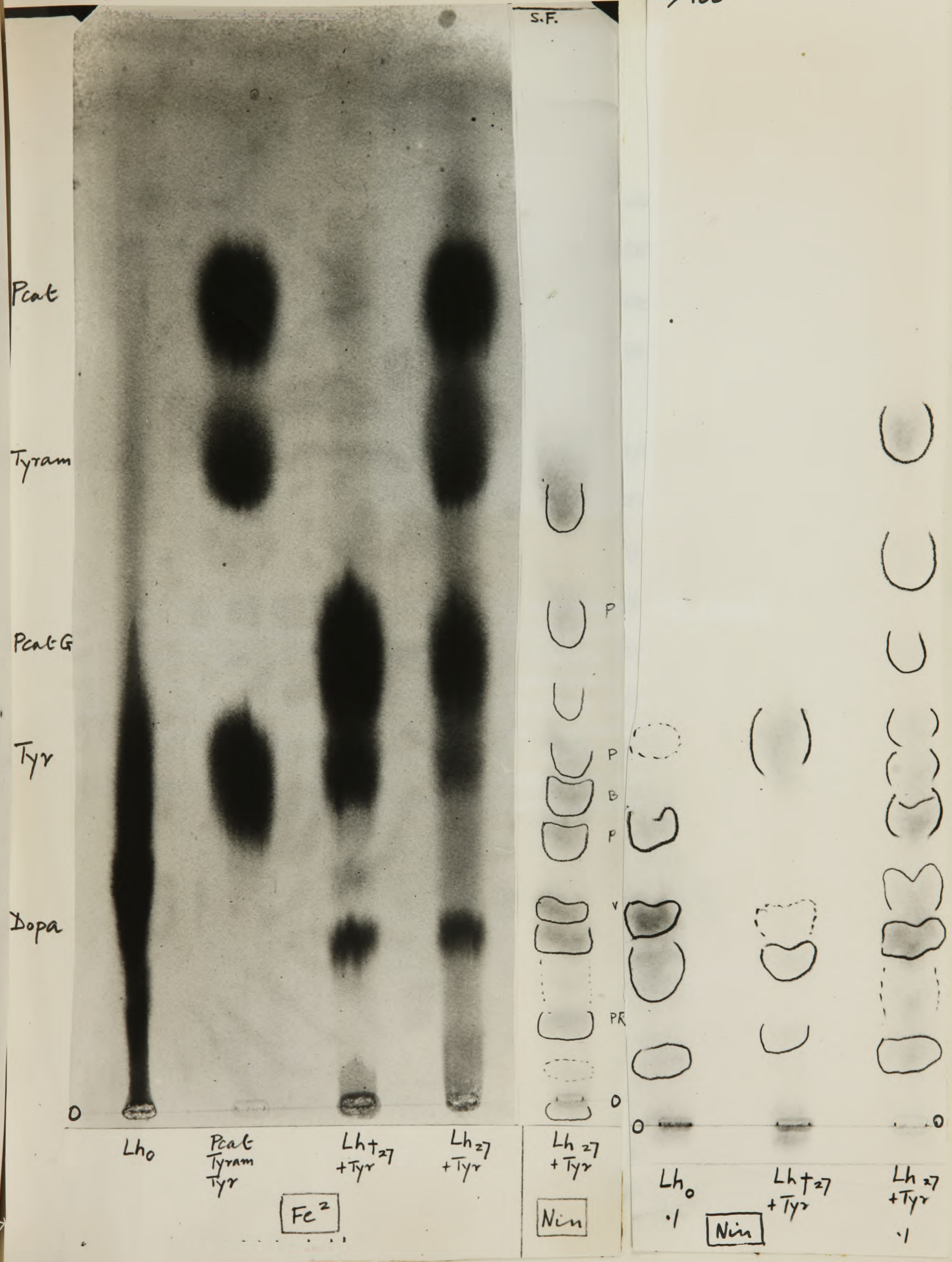


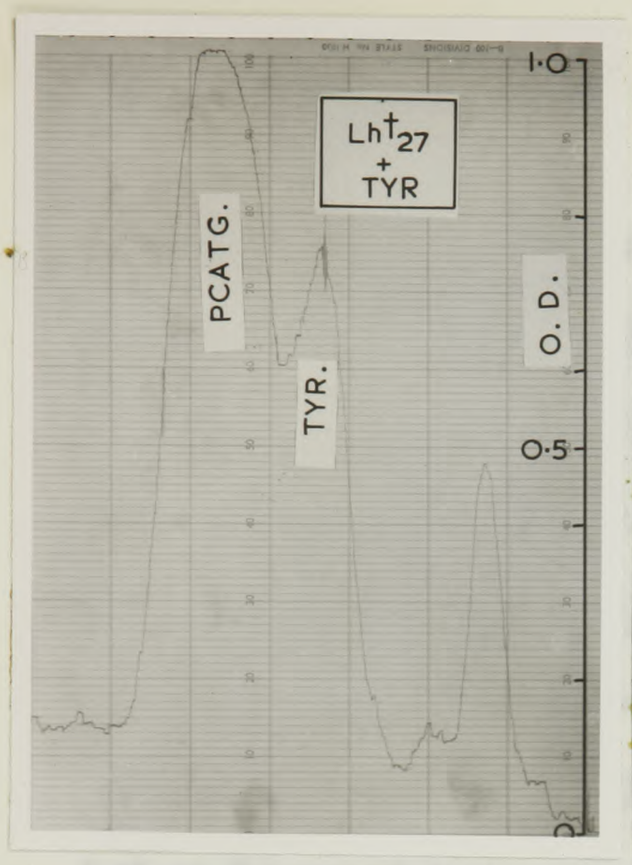
Figure 43: Separation (BAW, 4:1:1) of 0.1 ml. samples of homogenate (boiled (Lh*) and unboiled (Lh)) of left gland (1 ml.) incubated (37°C) with 1 µmole of p-tyrosine (0.25 ml.) for 27 hours. Detected with ferricyanide (Fe^2) and ninhydrin (Nin) sprays. Reference compounds (0.02 ml): protocatechuic acid (Pcat - 34 µg), p-tyramine (Tyram - 12 µg) and p-tyrosine (Tyr - 14.4 µg). Lh_O-homogenate without tyrosine before incubation

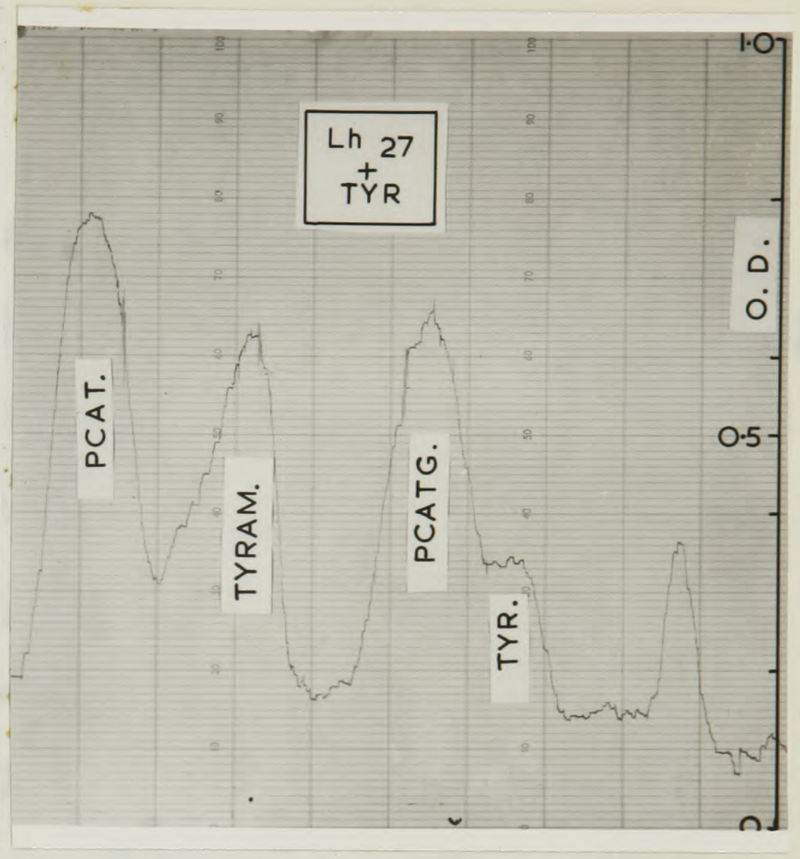

S.F.
→ 100
Pcat
Tyram
Pcat-G
Tyr
Dopa
O
P
P
B
P
V
PR
O
O
O
Lh0
Pcat Tyram Tyr
Lh+27 +Tyr
Lh 27 +Tyr
Fe2
Lh 27 +Tyr
Nim
Lh0 ·1
Nim
Lh+27 +Tyr
Lh 27 +Tyr ·1

When left gland homogenate is incubated in the presence of protocatechuic acid or p-tyrosine and DDC (fig. 37) a ninhydrin-positive, Rf% 60 (BAW, 4:1:1) component can be separated by chromatography. However, the substance does not appear to be diphenolic.

Two samples (1 ml.) of left colleterial gland (L) homogenized in water (0.1 g. wet weight/ml.) were incubated (37°C) for 27 hours with 1.0 μmoles (0.2%) of p-tyrosine (Tyr). One (L_h*) had previously been immersed in boiling water for 10 minutes. Before incubation the pH of the unbuffered homogenate was 7.4 - afterwards that of the boiled control (L_h*) was 7.0 and of the untreated homogenate was 6.0. Chromatographic examination (BAWm 4:1:1) of extracts of these incubated mixtures reveals that, as opposed to the unboiled ($L_{h_{27}}$ + Tyr), in the boiled sample (L_h *$_{27}$ + Tyr) none of the phenolic-amino component resembling tyramine has been formed (fig. 43).

Figure 44 shows on a quantitative basis the difference between the ferricyanide-positive components contained in both homogenates. These measurements were made with a recording densitometer (red filter) as described on page 36-7. For the total homogenate ($L_{h_{27}}$ + Tyr), 85.6 μg. (0.62 μmoles) of tyramine have been formed





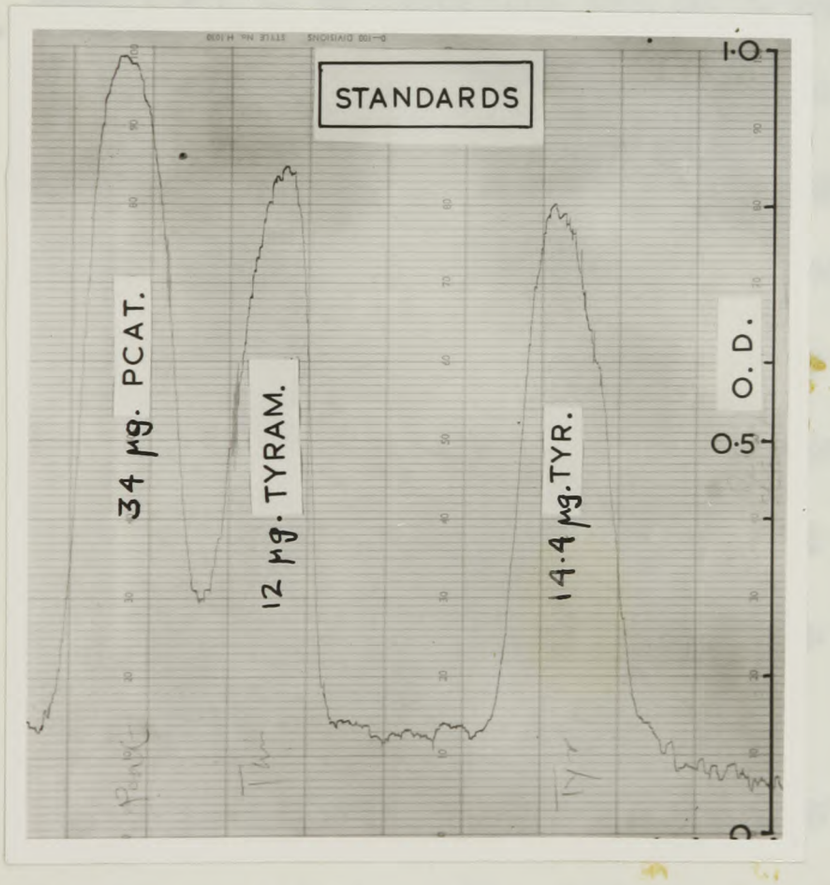


Figure 44

Optical densities of blue spots obtained by BAW chromatography of standards (.02 ml.) and of incubated homogenate (0.1 ml.) samples (fig. 43) followed by ferricyanide (Fe^2) spraying: measurements made with recording densitometer (page 36) along 3 cm. strips cut from chromatogram shown in figure 43 inset.

whereas, taking the amount (151 µg.) found after incubation in the boiled control (Lh^*_{27} +Tyr) as a standard, 108.1 µg. (0.60 µmoles) of p-tyrosine have been utilized (although 181 µg. of it were added originally at the initiation of incubation).

(2) Ion-exchange and cellulose column chromatography.

The pH of the incubated mixture (1 ml.) containing the free phenolic amine (ca. 75 µg.) was adjusted (pH 7.5) with 1 N-NaOH, before 0.1 g. of Amberlite IRC (H^+), type 1, resin was added. After shaking for an hour, the resin and solution were poured on to a short column of the same resin (page 39). The water eluate from the column and that obtained by elution with 1 N-HCl were reduced in volume (to ± 1 ml.) in a rotary evaporator, and the latter was neutralised by adding Dowex-1 in $CO_3^=$ form.

This entire treatment was repeated with the boiled homogenate (Lh^*_{27} + Tyr) to which about 100 µg. of p-tyramine-HCl had been added.

Chromatographic examination (BAW, 4:1:1 - descending) of the four eluates obtained (fig. 45) showed that passage through the ion-exchange resin (AMB) removed tyramine from the control homog-

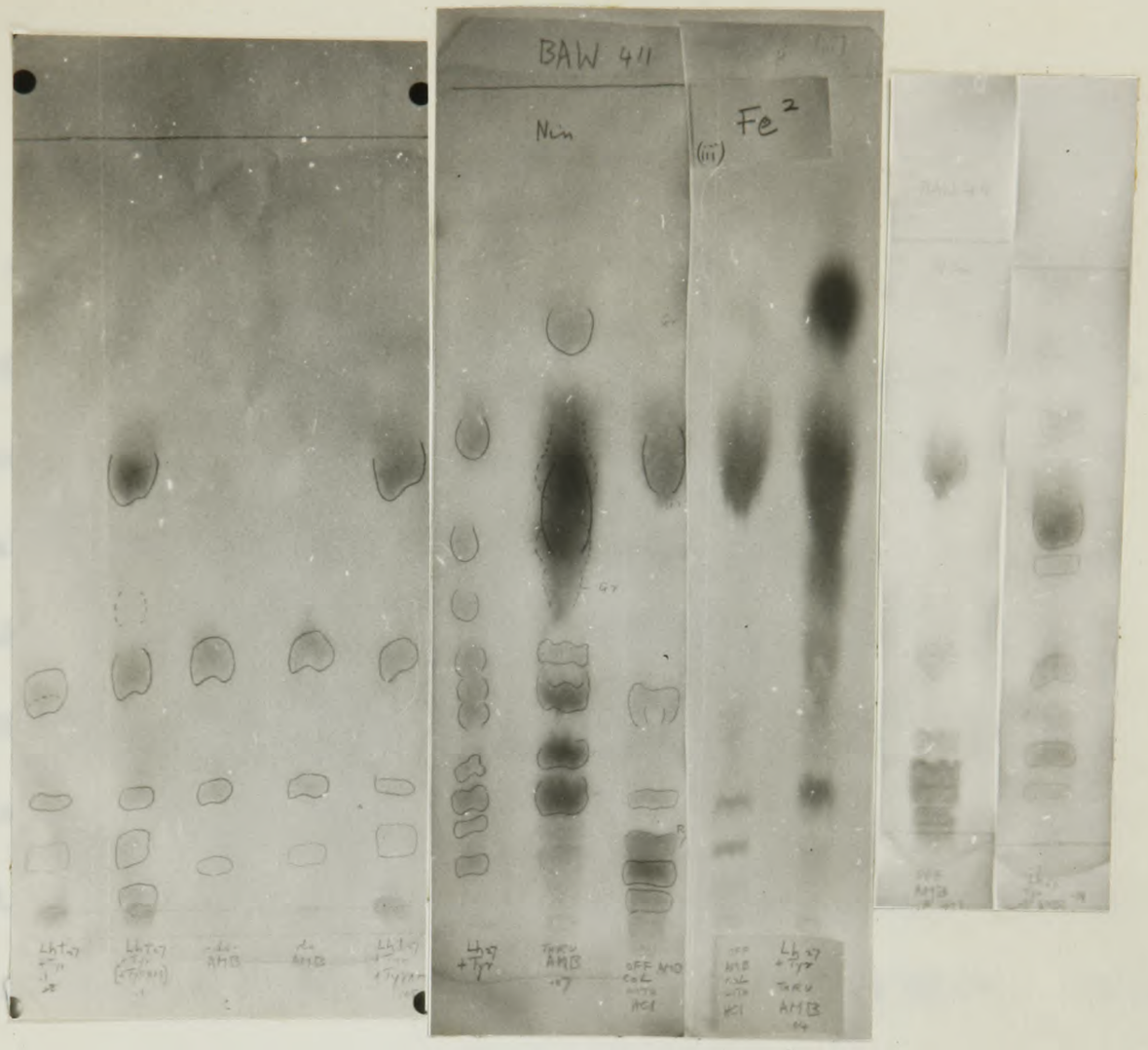


Figure 45: Separation by descending chromatography (BAW, 4:1:1) of boiled (Lh*) and unboiled (Lh) homogenates of left gland incubated (37°C) with p-tyrosine for 27 hours which have been eluted from an Amberlite IRC (H^+) column with water (thru' AMB) and HCl (OFF AMB HCl). To control, about 15 ug p-tyramine-HCl added after incubation (Lh^*_{27} Tyr + Tyram). Components were detected with ferricyanide (Fe^2) and ninhydrin (Nin) sprays.

enate. The acid eluate (OFF AMB - see fig. 45) of Lh_{27} + Tyr contained a component (Rf 58%) which was a phenolic amine. This eluate contained other ninhydrin positive components, but one in particular resembled tyramine (Rf% 73) when separated in phenol/water solvent mixture on Whatman No. 1 paper (fig. 46). The resolution in 2% acetic acid was as usual poor.

When separated in BAW, 4:1:1 and in phenol/water, the water eluate (0.07 ml.) from Lh_{27} + Tyr still contained a phenolic amine component with Rf% ranging from 50-65 and another at 74-76. They cannot be satisfactorily explained or identified in view of the fact that all the tyramine in the control (Lh^*_{27} + Tyr. + Tyram.) had been adsorbed on the resin (fig. 45). When less (0.01 ml.) of this eluate was chromatographed the slower component showed Rf% of 56 (Nin) which was consistent with tyramine. Perhaps an amino acid with similar Rf value (BAW, 4:1:1) is formed during incubation as well as tyramine.

Later, attempts were made in collaboration with Dr. P.C.J. Brunet to confirm the formation of tyramine in incubated homogenates of left glands using a column of Dowex-50 (Na^+). The method used is described on page 39 .

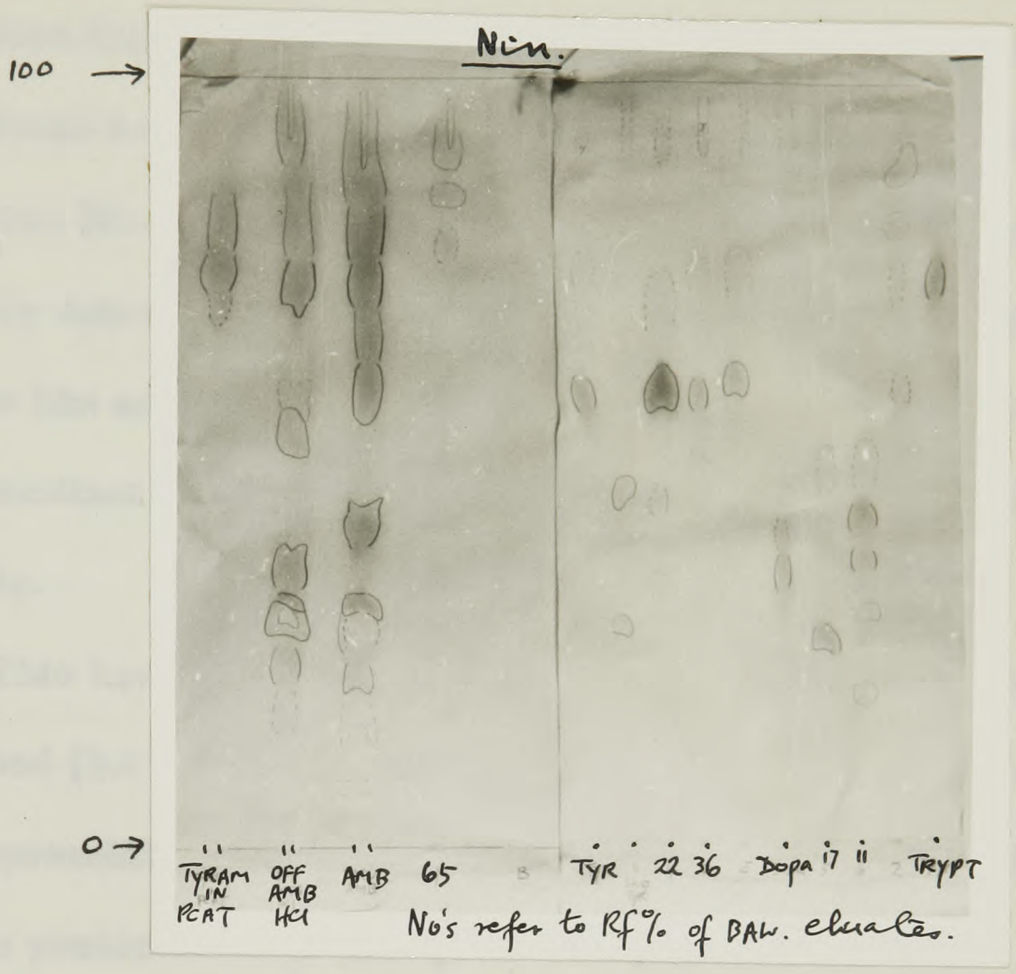


Figure 46: Paper chromatographic separation (phenol/water) of amines and amino acids contained in eluates of incubated (27 hrs) left gland homogenate samples from BAW; 4:1:1 chromatograms and from an Amberlite IRC ion exchange column with water (~~thru~~(AMB) and 1N -HCl (off AMB Hcl). References - tyramine (TYRAM) in presence of PCAT p-tyrosine (TYR) and tryptophane (TRYPT). Detected with ninhydrin spray.

The homogenate run down the ion-exchange resin (fig. 47) had been incubated anaerobically with p-tyrosine (15 µmoles) and pyridoxal-6-phosphate for 55 hrs. Although only 66.5% of the tyrosine (the elution peak for 0.91 m.g. of this amino acid previously determined) could be recovered after the incubation tyramine has not yet been conclusively identified by this method. Concomitant CO_2 production could not be demonstrated manometrically.

This homogenate of left and right gland was also chromatographed (BAW, 4:1:1) on a cellulose column described on page . Any tyramine produced would be eluted together with and obscured by the protocatechuic acid glucoside (figs. 48 and 40). Calculations based on the behaviour of the column when a known amount of p-tyrosine (2.17 mg.) is chromatographed (fig. 49) and see appendix 2) indicated that there had been no loss of tyrosine during incubation. As an analytical tool for this purpose the cellulose column is not very satisfactory owing to its low resolution and sensitivity in spite of its length. The spectral properties of the reference compounds chromatographed (fig. 47) are given in appendix 2, together with photographs showing a paper chromatographic (BAW, 4:1:1) check of the pattern for reference phenolic

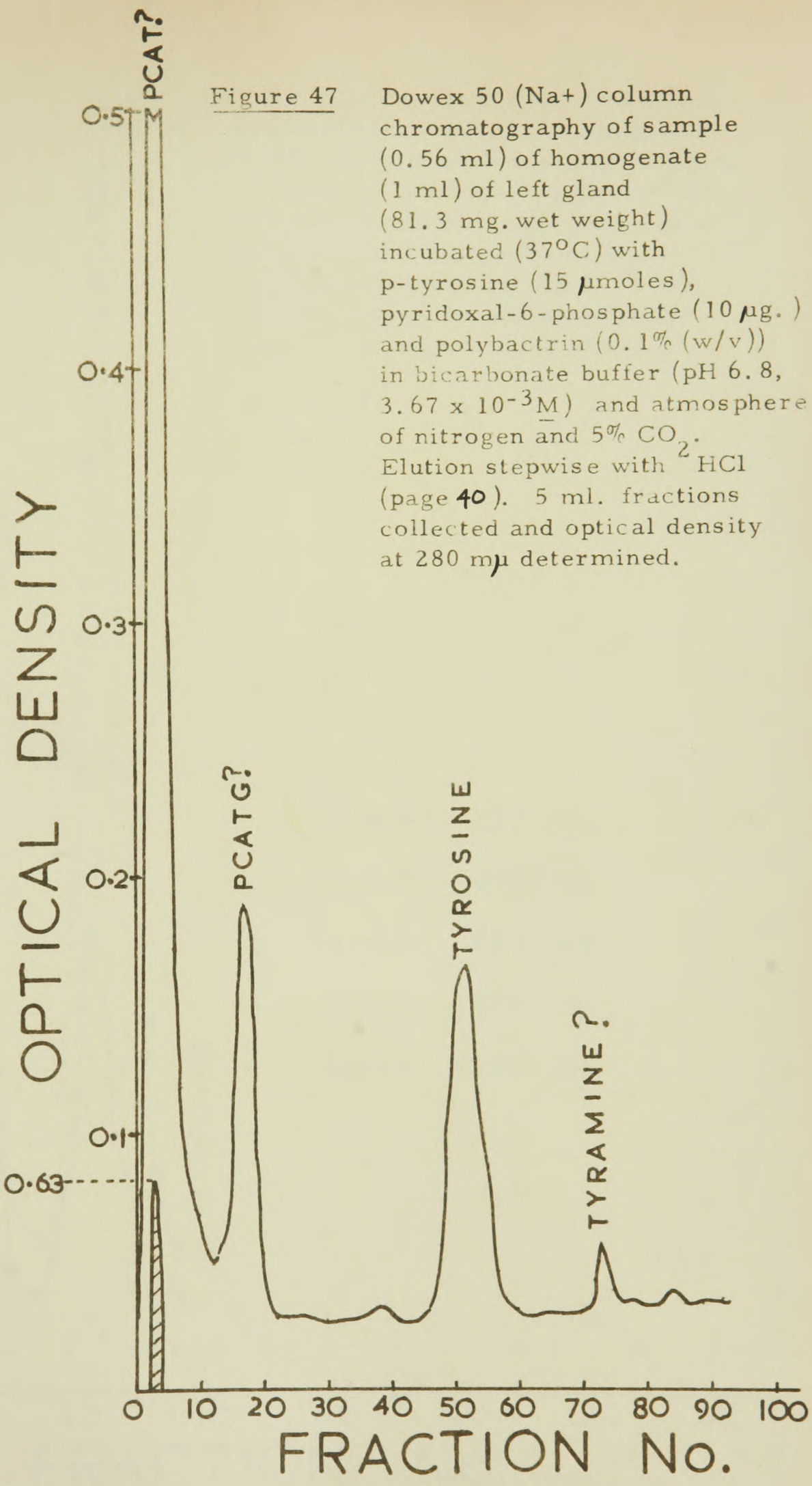
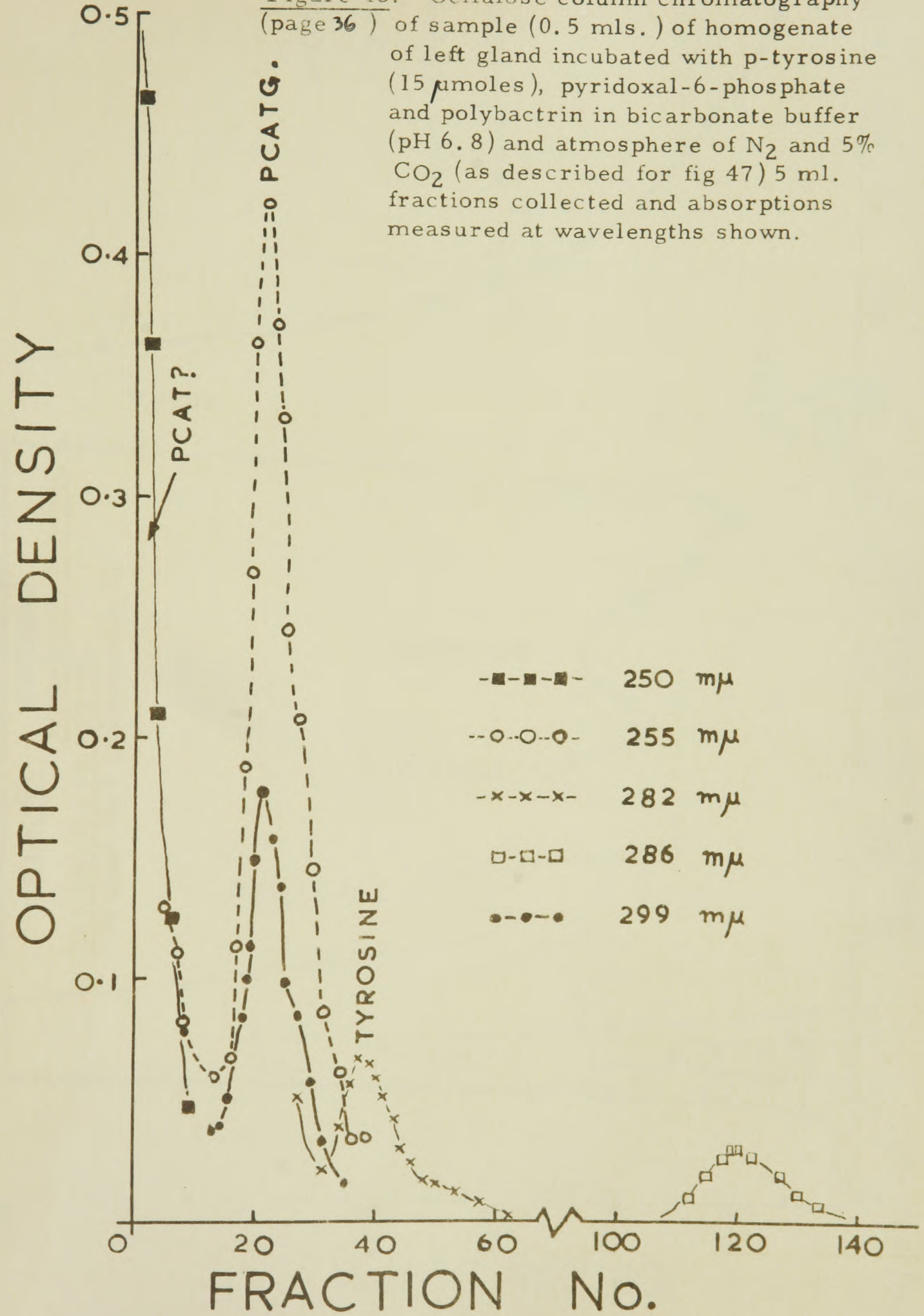
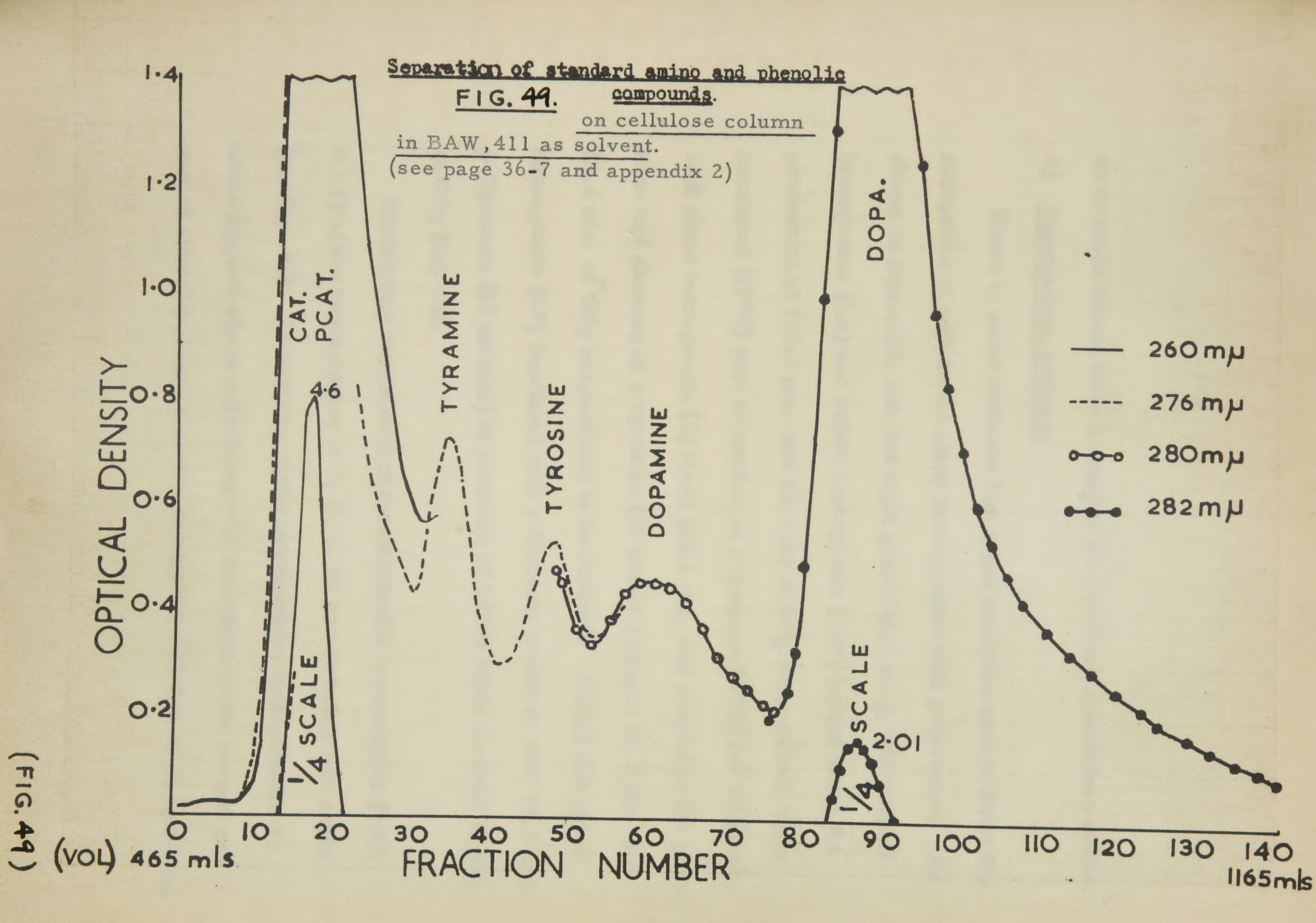


Figure 47 Dowex 50 (Na+) column chromatography of sample (0.56 ml) of homogenate (1 ml) of left gland (81.3 mg. wet weight) incubated (37°C) with p-tyrosine (15 µmoles), pyridoxal-6-phosphate (10 µg.) and polybactrin (0.1% (w/v)) in bicarbonate buffer (pH 6.8, 3.67×10^{-3}M) and atmosphere of nitrogen and 5% CO_2. Elution stepwise with HCl (page 40). 5 ml. fractions collected and optical density at 280 mµ determined.

Figure 48: (page 36) Cellulose column chromatography of sample (0.5 mls.) of homogenate of left gland incubated with p-tyrosine (15 μmoles), pyridoxal-6-phosphate and polybactrin in bicarbonate buffer (pH 6.8) and atmosphere of N_2 and 5% CO_2 (as described for fig 47) 5 ml. fractions collected and absorptions measured at wavelengths shown.




Separation of standard amino and phenolic compounds.
FIG. 49.
on cellulose column
in BAW, 411 as solvent.
(see page 36-7 and appendix 2)
OPTICAL DENSITY
1·4
1·2
1·0
0·8
0·6
0·4
0·2
CAT.
PCAT.
4·6
1/4 SCALE
TYRAMINE
TYROSINE
DOPAMINE
DOPA.
1/4 SCALE
2·01
260 mμ
276 mμ
280 mμ
282 mμ
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
(VOL) 465 mls
FRACTION NUMBER
1165mls
(FIG. 49)

compounds eluted from a smaller (2.5 x 44 cm.) cellulose colum.

3) Manometric evidence

There is some evidence that a gas evolution accompanies the incubation of colleterial gland homogenates with p-tyrosine. As shown in figure 50, left and right gland (44.7 m.g. wet weight) homogenate (LR) and boiled homogenate (LR*) brings about the production of 185.6 µls. and 12.3 µls of CO_2 respectively when incubated (37°C) with 15 µmoles of p-tyrosine (TYR) for 30 hours. Left gland homogenate (L) itself (39.6 mg. wet weight) in the presence and absence of p-tyrosine (15 umoles) causes 95.0 µls. and 30.6 µls. of CO_2 respectively to be evolved. Boiled left gland homogenate (L*) incubated with p-tyrosine produces only 21.3 µls. p-Tyrosine (15 µmoles) in presence of buffer alone evolved 14 µls. of CO_2 (fig. 50).

During this experiment chromatographic examination (BAW 4:1:1) of the homogenates at 0, 2, 6, 15 and 30 hours was made (see fig. 51). In samples of the homogenates incubated for 30 hours (apparently in agreement with the manometric results recorded above) a phenolic-amino component with Rf% of 56 had been

Figure 50: Evolution of carbon dioxide accompanying incubation (37°C of 1 ml) left (L) or left with right (LR) gland homogenate with or without p-tyrosine (Tyr) or protocatechuic acid (Pcat) (15 μmoles each) under conditions described on page 32. Boiled homogenate samples (L* and LR*) are used as controls. L contained 39.6 mg. wet weight of tissue/ml., and LR 44.7 mg/ml.

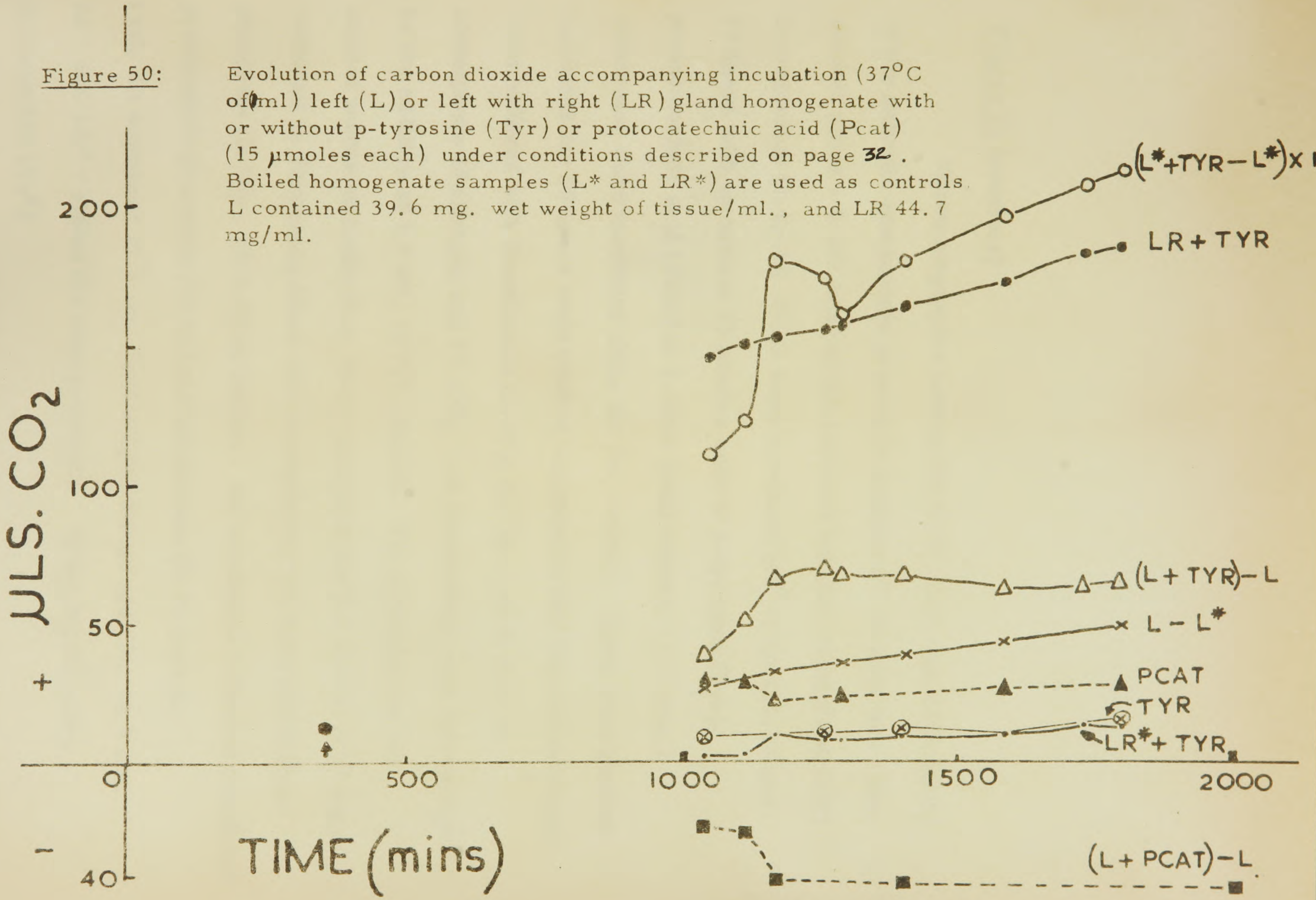
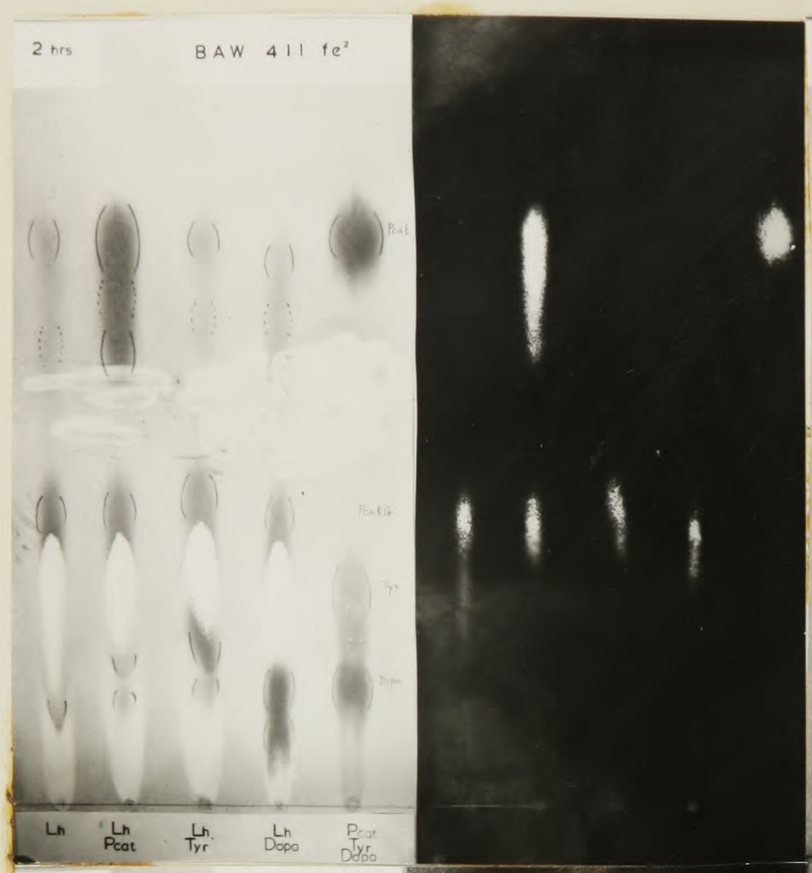
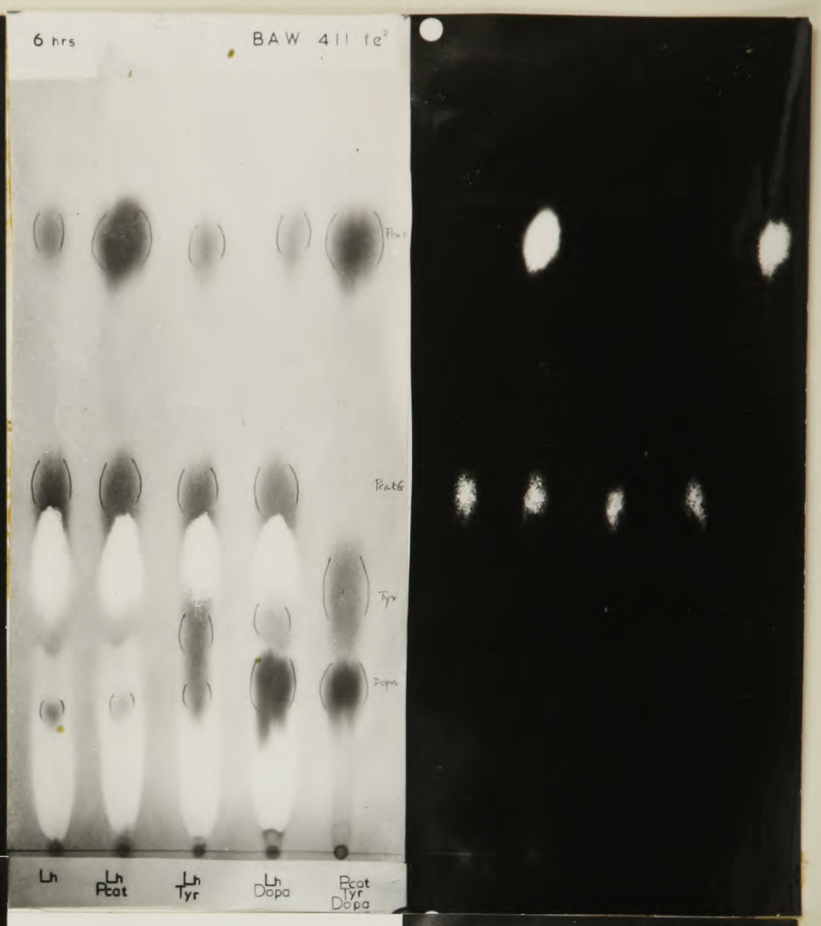


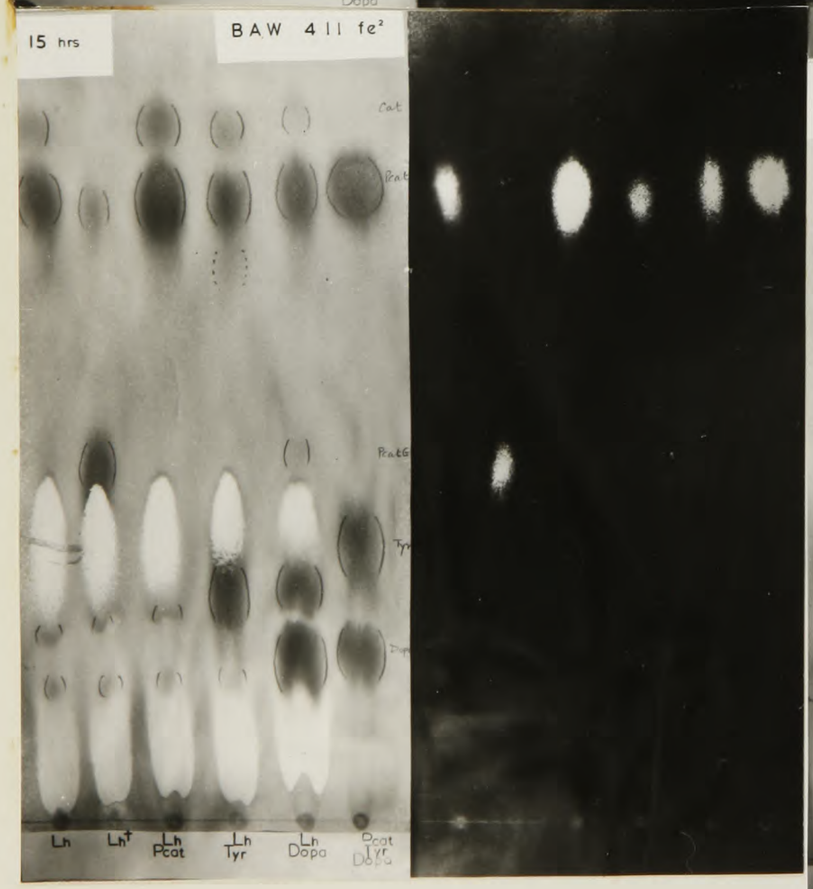
Figure 51 (overleaf)

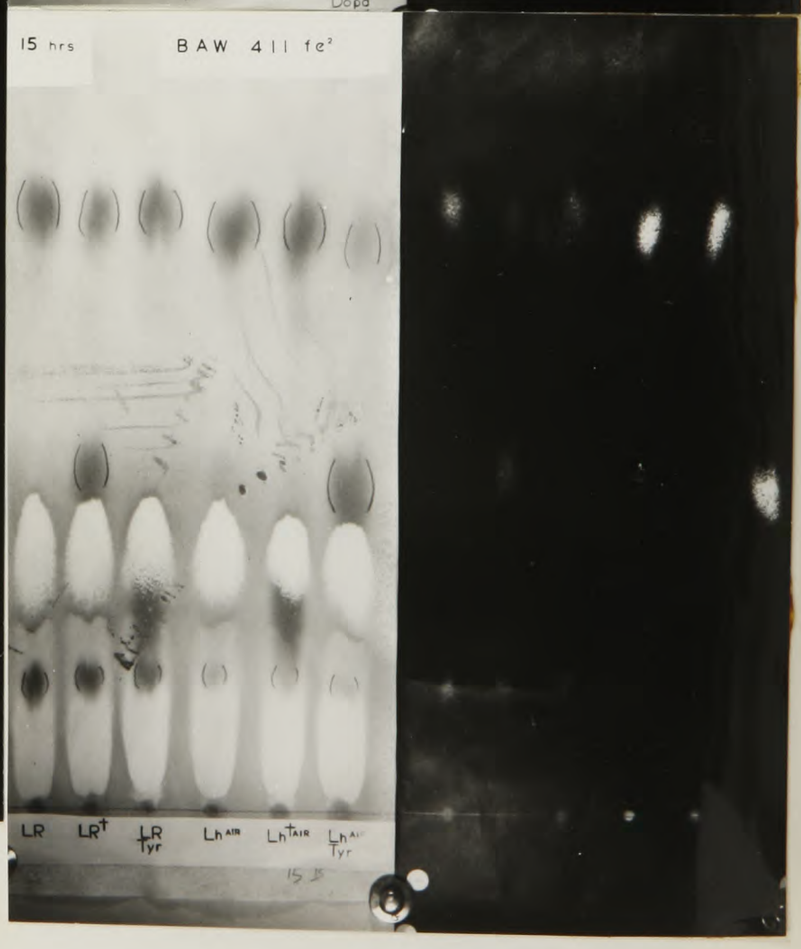
Chromatographic survey (in BAW, 4:1:1 and BEB, 1:1:1) of the phenolic components present in samples (0.05 mls. or - when ethanol added - 0.081 mls) of left (Lh) and left plus right gland (LR) homogenates (1.0 ml.), which were incubated (37°C) for 2, 6, 15 and 30 hours with or without 15 μmoles each of p-tyrosine (Tyr), protocatechuic acid (Pcat) or L-dopa (total volume = 1.5 mls.) Substrates were incubated alone too for control) Those homogenates incubated for 30 hours were studied manometrically (fig. 48) and hence were buffered with bicarbonate (3.67×10^{-3} M- , pH 6.8) in an atmosphere of nitrogen and 5% CO_2. All incubations were terminated by the addition of 1.0 ml. of 96% ethanol. The homogenates not studied manometrically (i.e. those incubated for 2, 6 and 15 hrs) were contained in Thurnberg tubes with atmosphere, nitrogen and sodium phosphate (0.1 M- , pH 6.8) as buffer. All incubation mixtures contained pyridoxal-6-phosphate (10 μg) and polybactrin (0.1% (w/v)).

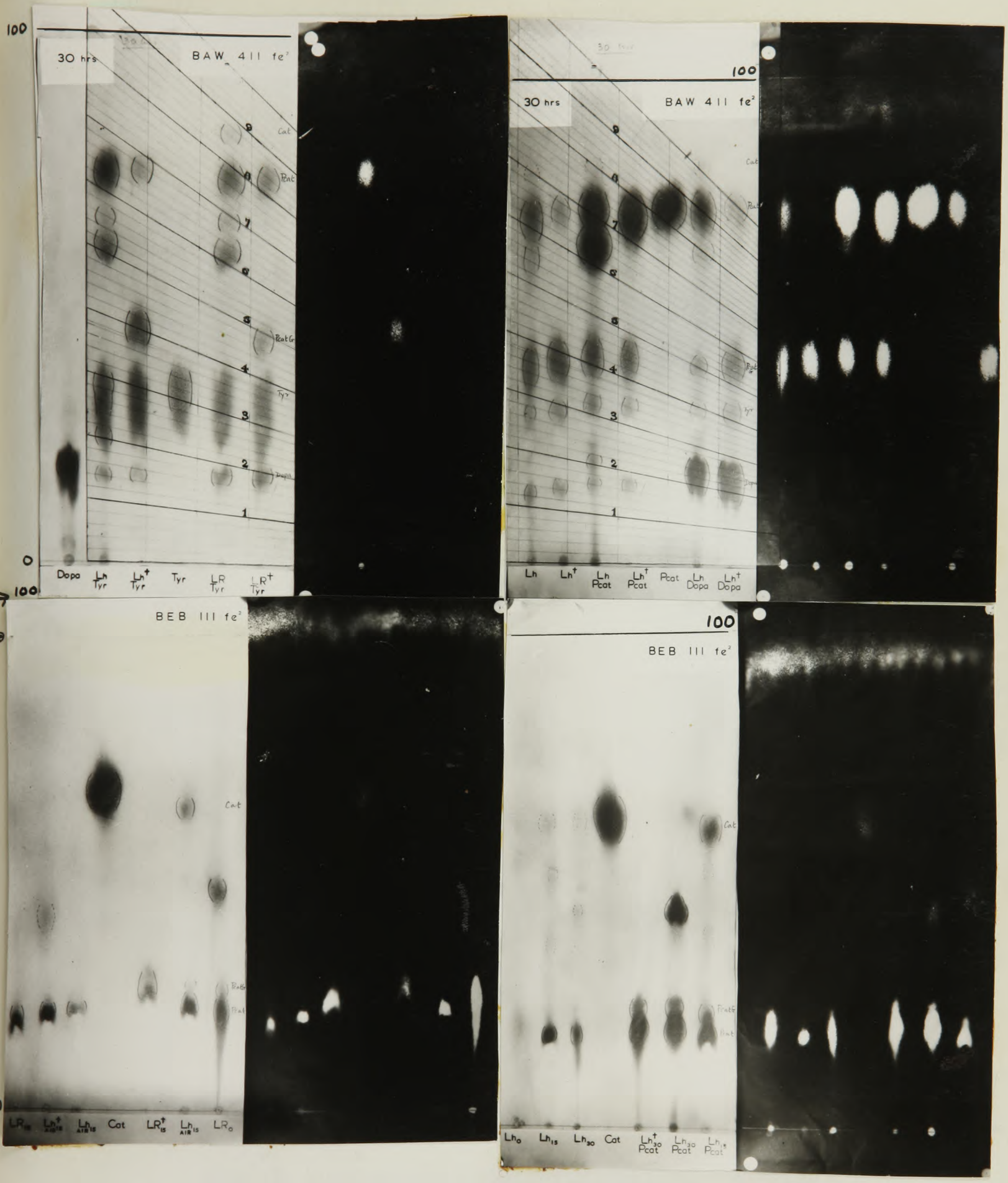
1 ml. Lh contained 39.6 mg. (wet weight) tissue and LR, 44.7 mg. Lh* and LR* - boiled (10 mins) controls. Spray for detection - ferricyanide (Fe^2).


2 hrs
BAW 411 fe²
Pcat
PcatG
Tyr
Dopa
Lh
Lh Pcat
Lh Tyr
Lh Dopa
Pcat Tyr Dopa


6 hrs
BAW 411 fe²
Pcat
PcatG
Tyr
Dopa
Lh
Lh Pcat
Lh Tyr
Lh Dopa
Pcat Tyr Dopa


15 hrs
BAW 411 fe²
Cat
Pcat
PcatG
Tyr
Dopa
Lh
Lh⁺
Lh Pcat
Lh Tyr
Lh Dopa
Pcat Tyr Dopa


15 hrs
BAW 411 fe²
LR
LR⁺
LR Tyr
Lh AIR
Lh⁺ AIR
Lh AIR Tyr


100
30 hrs
BAW 411 fe²
Cat
Pcat
Tyr
Dopa
Lh Tyr
Lh† Tyr
Tyr
LR Tyr
LR† Tyr
0
100
Lh
Lh†
Lh Pcat
Lh† Pcat
Pcat
Lh Dopa
Lh† Dopa
BEB III fe²
Cat
LR₀
Lh† LR
Lh AIR
Cat
LR† IS
Lh AIR IS
LR₀
100
BEB III fe²
Cat
Lh₀
Lh IS
Lh 30
Cat
Lh† Pcat 30
Lh 30 Pcat
Lh IS Pcat

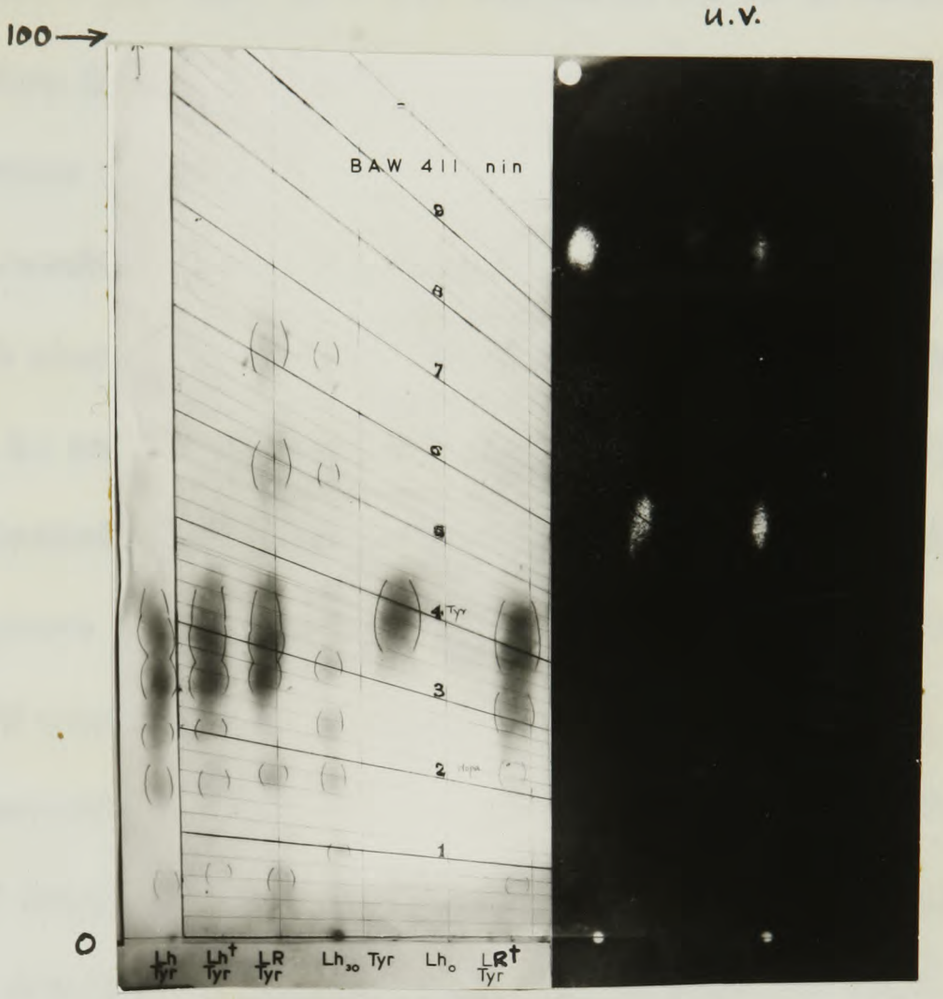


Figure 51a: Examination by paper chromatography (BAW, 4:1:1) of colleterial gland homogenates (Lh or LR) incubated (37°C) for 30 hours. Notation as shown in figure 51.
Spray - ninhydrin (Nin.)

formed in LR + Tyr and Lh + Tyr but not in either of these homogenates when they had been boiled for 10 minutes (fig. 51). That more tyramine was produced in LR + Tyr than Lh + Tyr is suggested by the result of the ninhydrin (Nin)-sprayed chromatogram (fig. 51a), which also shows some to be present in Lh alone after 30 hours - as would be expected (see fig. 34). That in the incubations which were terminated earlier (carried out in phosphate buffer (pH 6.8) and atmosphere of nitrogen) there is little sign of the formation of a Rf% 58-60 component even after 15 hours is surprising, in view of the manometric and chromatographic results described above for the 30 hour incubations. In the Lh + Heat incubation terminated at 2 hours, a ferricyanide-positive component (Rf% 66, BAW, 4:1:1) had been formed, as noticed - in figure 32 - for Lg, LR_0 and LR_1 but this appears to have disappeared again after 6 hours (fig. 51).

The manometric evidence that CO_2 is evolved when left or left with right gland homogenates is incubated (30 hours) with p-tyrosine is therefore supported by quantitative chromatographic evidence that tyramine is produced. Earlier aerobic experiments (figs. 41, 34) seem to suggest that tyramine is formed by left gland in greater amounts in the absence of buffers, and in the presence of air and/or

of right gland homogenate.

(c) Evidence for the presence of p-tyrosine.

1) Paper Chromatography

As already mentioned the component with Rf% 34-36 (BAW, 4:1:1) found in the aqueous layer (F_2) of extracted left gland dialysate (Table 10) was found, upon being excised and eluted (50% ethanol) from the paper, to have λmax. at 277 mμ and λmin. 248 mμ.

p-Tyrosine is known to separate in BAW, 4:1:1 in the region of this component. The aqueous layer referred to above also contained a phenolic substance or substances with Rf% 81 in 2% acetic acid, which is again similar to that shown by p-tyrosine.

Figures 33, 34 and 35 show that incubated left gland homogenate contains a ninhydrin-positive, phenolic component which resembles authentic p-tyrosine. When eluted from the paper with 0.01 N-HCl the absorption spectrum of this component (Table 11) resembles tyrosine, which had been similarly separated and eluted.

Table 11. U.V. spectral properties at pH 2-3 of reference compounds and components eluted (a) from paper after chromatography (BAW, 4:1:1) of left gland homogenate.

Component Rf%	λmax mμ.	λmin mμ.	λmax mμ.	shoulder mμ.
60-64	225	246	278	282
33	226	248	278	285
18-21	224	254	284	
References				
p-tyramine	223	248	279	284
p-tyrosine	225	248	278	284
Dopa	222	252	282	

(a) with 0.01 N-HCl (4 mls.)

The eluate was neutralised by adding 1 g. Dowex-1, $CO_3^{=}$ resin and the pH changed to 10 with drops of 1N-NaOH. The spectral properties of the solution showed λmax. about 243 and 299 mμ and λmin. at 272 mμ. (Carey), as does p-tyrosine and tyramine too, at pH 10 (fig. 42).

In section (b) of this chapter evidence has been presented that tyramine is formed during prolonged incubation of gland homogenates in the presence of p-tyrosine. The component with Rf% 33-36

begins to disappear when the homogenate of both glands (LR) is incubated in the absence of added tyrosine (figs. 32, 33, 34, 35). When eluted (aqueous ethanol) components with Rf% 22 and 36 in aqueous fraction (F_2) of dried dialysate (Table 10) both contain an amino constituent (resembling p-tyrosine - appendix 3) when separated chromatographically (Rf% 57-60) in phenol/water as solvent mixture (Fig. 46). However, resolution of both eluates in amyl alcohol pyridine, water (2:2:1) - ascending - produces ferricyanide-positive components at Rf% 67 and 48 (weak). p-Tyrosine separates at Rf% 44-49.

2) Column chromatography

The evidence obtained from chromatography of gland homogenates on cellulose and ion-exchange resin indicated that p-tyrosine might be present in cells of the left gland (figs. 40, 43 and 52).

d) Other constitutents.

1) The Amino compound resembling Dopa.

In Table 11 it can be seen that the eluate of the component with Rf% 20-22 (BAW) shows spectral properties which suggest those of dopa. However, the ratio of the extinctions at 227 and 282 mμ.

for synthetic DL-dopa is 4.3 to 1.5. This ratio for the cockroach eluate is 5.3 to 5.6, which indicates the presence of another aromatic substance(s) besides dopa or that dopa is not present.

More than one amino compound is present in incubated homogenate (L and LR) in this region of the BAW, 4:1:1 chromatogram (figs. 34 and 43), but the component migrating with Rf% 21-23 exhibits the same shade of violet which is characteristic of dopa when treated with ninhydrin (see spot no. 7, fig. 41). Nevertheless, in the 0.01 N-HCl eluates of LR homogenate separated and excised in the usual manner, it is the slower components which resemble dopa in spectral properties (Table 12).

Region 7 in figure 41 cannot contain dopa then, but among those substances which migrate more slowly dopa certainly might be present in small amounts. When rechromatographed in phenol/water the concentrated eluates examined above (Table 12) showed the following separation: (Fig. 46).

Eluate from region	Rf% (detected with ninhydrin)				
Rf% 22-28		(33)	. (43)	.	59
Rf% 14-18	27			(48)	
Rf% 10-14	(20)	. (29) .	36	. 42	(48)

( ) faint.

Table 12. Properties of components separated from homogenate of left and right glands (LR) by chromatography (BAW, 4:1:1)

Region excised (Rf%)	With Ninhydrin in Fig. 41		Spectral properties of eluates. (pH 2-3)			Ratio of extinctions
	Colour	No.	λ_{max} mμ.	λ_{min} mμ.	λ_{max} mμ	230 mμ/281mμ
22-26	Violet	7		233	264	
14-18	Magenta	8	232	254	281	2.0/3.4
10-14	Fawn	8-9	228	253	282	2.3/3.0
DL-dopa	Violet		227	252	282	4.3/1.5

The bracketed components are only weakly positive to ninhydrin. As dopa separates with Rf% 35 in phenol/water (appendix 3) only the region between spots 8 and 9 in figure 41 (Rf% 10-14) appears to c contain a similarly-migrating amino compound. With n-propanol / HCl (3:1) as solvent, the eluate from the excised region (of LR) with Rf% 15-20 (BAW, 4:1:1) reveals the presence of a phenolic subetance with Rf% 24 , (ascending) which cannot be dopa (Rf% 36) (Brunet, 1960).

2) Other amino compounds.

Figure 45 reveals that the two slowest-moving amino compounds may well be free amines for they disappear on passage through Amberlite IRC at pH 7.0. The components at Rf% 21 and 16 are not retained by passage through the resin (page 39) although the HCl eluate from the column, when neutralised and concentrated, contains traces of an aminophenol with Rf% of 16 (BAW). This eluate when chromatographed also reveals amines at Rf% 28, 7 and 3 together with a curious brilliant red (ninhydrin) component, which may be phenolic, at Rf% 10. (fig. 45).

The non-phenolic amino subetance (with Rf% 48-52) formed when colleterial glands are homogenated and incubated at 37°C

(fig. 34) 43 and 51a) resembles tryptophane in this solvent (BAW, 4:1:1) and, when excised and eluted with 0.01 N-HCl, in phenol/water too (Rf% 73) - when rechromatographed. It does not appear to be a free amine (fig. 45).

The separation of various excised and eluted components of incubated LR in phenol/water (e.g. fig. 46 and see page 178) suggest the presence of those amino acids such as alanine, valine, glutamic acid and glycine which are normally found in incubated tissues.

3) Other substances

Protocatechuic acid when oxidised catalytically (copper sulphate, pH 8-9) appeared to give rise to a phenolic component with Rf% 18-21 (BAW, 4:1:1). (So too did pyrogallol solution exposed to air). This artefact may be present then in homogenates incubated with protocatechuic acid for long periods.

When LR homogenate + Pcat (15 μmoles) was incubated at 37°C for 3 days (in the presence of polybactrin) the mixture smelt very strongly of the odour of cockroaches themselves, i.e. possibly 4-methyl quinone. Chromatographic examination of this homogenate reveals an intense ferricyanide-positive spot at Rf% 81 (perhaps

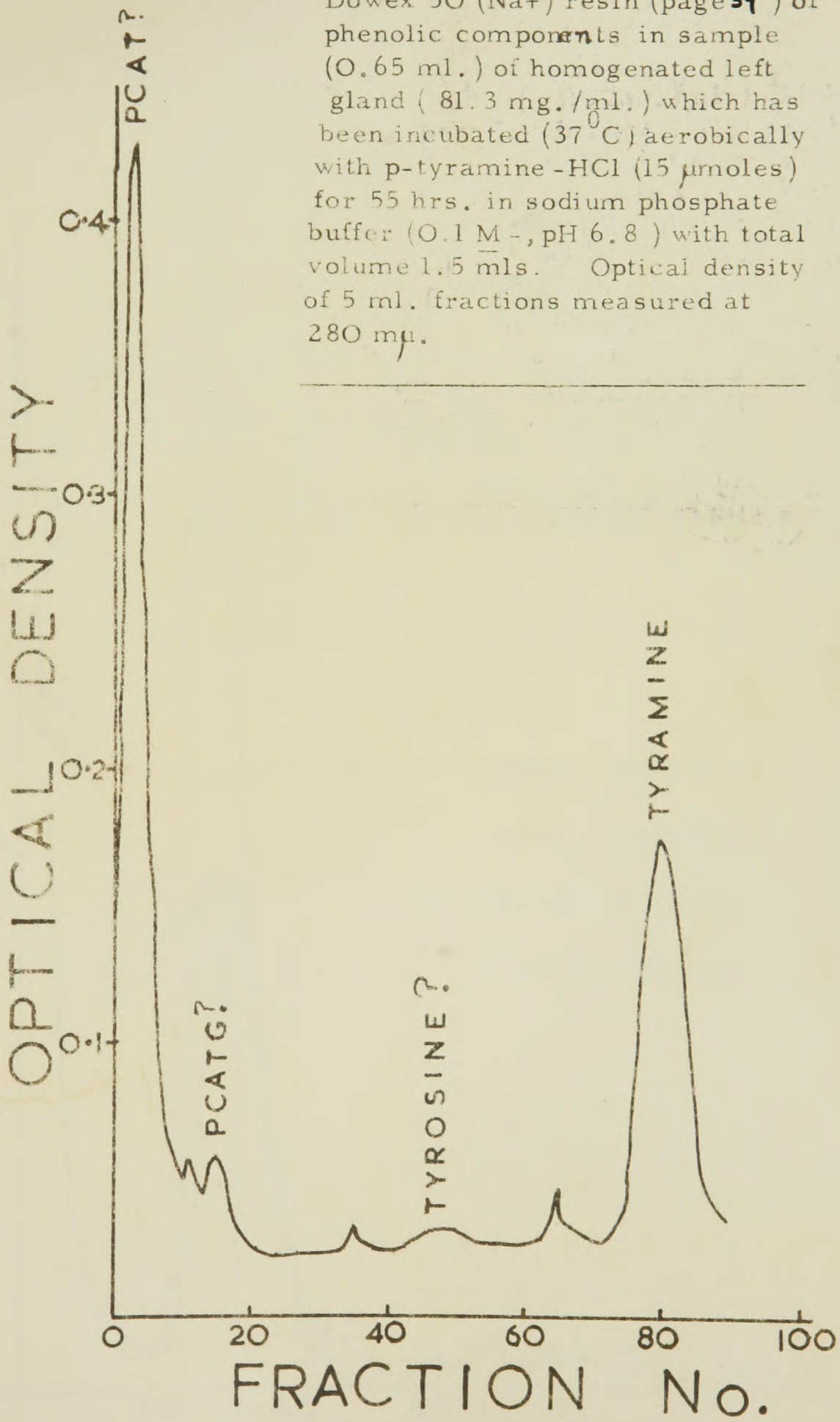
catechol (Cat) see page 161), another phenolic substance, less concentrated, at 70-73 and a weaker spot at 64-66. 4-Methylcatechol (Rf% 77-79) as well as Cat. and Pcat. may be present, but could not be identified conclusively.

C. TYRAMINE OXIDATION BY GLAND HOMOGENATE

Prolonged incubation of p-tyramine-HCl (15 µmoles) with left gland homogenate (81.3 mg. wet weight tissue) in phosphate buffer (0.1 M-, pH 6.8) and oxygen elicited a response after 31 hours. During the next 23½ hours (i.e. 1397 mins.) 260 µls. of oxygen-corrected for that (95 µls.) consumed by homogenate without tyramine - was taken up. The uptake by the control at the end of the entire incubation (3300 mins.) was found to be 302 µls.

Separation on a Dowex 50 column (fig. 52) of the homogenate and tyramine mixture after incubation indicated that if no other substance migrated together with tyramine, 30.5% of that added initially had disappeared. (0.91 mg. of tyramine had previously been run down the column and the elution peak area for this standard determined). If one atom of oxygen were required to oxidise a molecule of tyramine then the 4.5 µmoles unaccounted for would cause

FIGURE 52 Column chromatography on Dowex 50 (Na+) resin (page 39) of phenolic components in sample (0.65 ml.) of homogenated left gland (81.3 mg./ml.) which has been incubated (37 ^{0}C) aerobically with p-tyramine-HCl (15 μmoles) for 55 hrs. in sodium phosphate buffer (0.1 M-, pH 6.8) with total volume 1.5 mls. Optical density of 5 ml. fractions measured at 280 mμ.



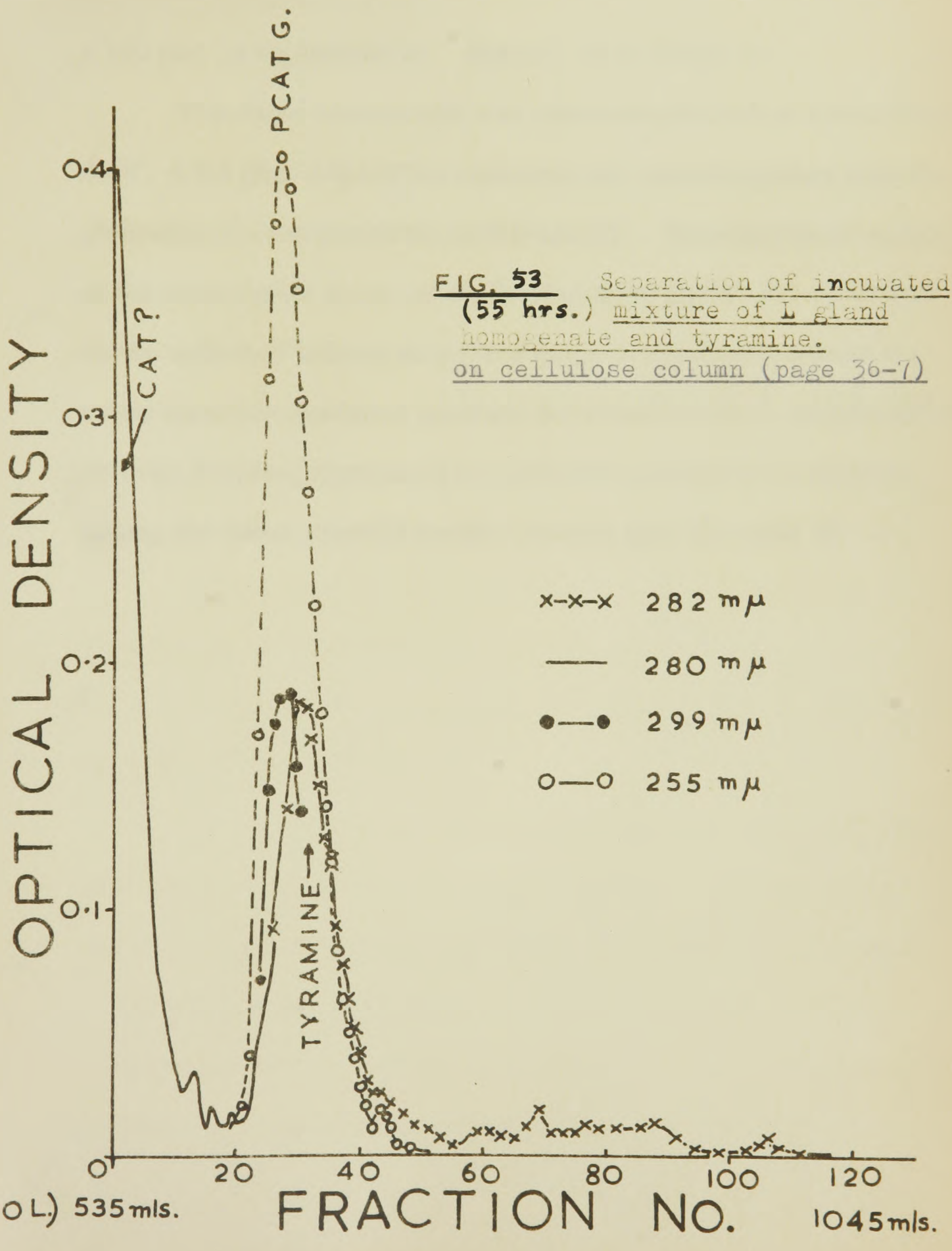


FIG. 53 (55 hrs.) Separation of incubated mixture of L gland homogenate and tyramine. on cellulose column (page 36-7)

± 100 µls. of O_2 instead of 260 µls. to be taken up.

The same homogenate was chromatographed on cellulose in BAW, 4:1:1 (fig. 53) but the tyramine was eluted together with the glucoside of protocatechuic acid (Peat G). Examination of samples of the homogenate by paper chromatography (BAW, 4:1:1) as well on the cellulose column described (page 37-8) failed to reveal the likely tyramine oxidation products (of noradrenaline, noradrenalone or even 3-hydroxytyramine) in sufficient quantity to be detected among the other phenolic amines present (see appendix 3).

CHAPTER EIGHT

DISCUSSION

p-Tyrosine-G-C^{14} and dopa-G-C^{14} in contrast to hydroquinone-G-C^{14} are incorporated into cuticle of Calliphora during tanning (Karlson, 1960). The results obtained by Fuzeau-Braesch (1959) using crickets support these findings since generally C^{14}-labelled tyrosine was utilised for melanin formation and was incorporated into the peptide chains of the cuticular proteins. However, he claimed that it did not participate in tanning.

Recent work has shown that in the left colleterial gland of Periplaneta protocatechuic acid and its glucoside are synthesised from tyrosine-G-C^{14} injected into the haemocoel (Brunet, 1961, unpublished work).

The mechanism by which the biosynthesis of protocatechuic acid occurs in insects is not yet understood. Richards (1951) and Hackman (1958) suggested similar pathways (see fig. 30) such that from dopa by successive oxidative deamination, decarboxylation and oxidation protocatechuic acid is formed. Their hypothesis was based on the claims that in cuticle the diphenolic acids,

shown in figure 30, are present. These compounds were not chromatographically detectable in extracts of colleterial gland, though (especially if transient) their absence cannot be vouchsafed.

Decarboxylation of p-tyrosine.

There is some evidence that in homogenate of left colleterial gland there is a heat labile system capable of bringing about the decarboxylation of tyrosine. Few decarboxylases have been demonstrated in insect or invertebrate tissue (Bheemeswar, 1958; Frontali, 1961). However, if it indeed exists and the system is found to be truly enzymic, this decarboxylase may play a significant role in the biosynthesis of the diphenols responsible for sclerotin formation (fig. 54).

The evidence concerning the nature of the reaction is however conflicting. Using three different solvent systems, formation of tyramine (after prolonged aerobic incubation of homogenated left gland and tyrosine,-albeit in the absence of buffer and pyridoxal-6-phosphate,) was demonstrated chromatographically. Spectrophotometrically the substance formed closely resembled tyramine (Table 11), and the amount formed appeared to account for the tyrosine disappearance (page 169-170, fig. 44).

The reaction was stimulated by the presence of right gland in the gland preparation (fig. 34) but absence of oxygen and presence of buffer appeared to some extent to have the opposite effect (fig. 51).

Phosphates retard some dec arboxylases possibly by interaction or competition with pyridoxal-6-phosphate, the co-factor. That the β-glucosidase-containing tissue influenced the cockroach system suggests that presence of the liberated protocatechuic acid might be responsible. The pH optimum for tyrosine decarboxylase in Streptococcus faecalis is 5.0 - 5.5 (Schales, 1951; Epps, 1944) (The optimum for the aspartate system in silk worm tissue was between pH 5.5 and 8.0 (Bheemeswar, 1958)). If it is as low as this in colleterial gland cells, the retarding effect of bicarbonate or phosphate buffer at pH 6.8 would be partially explained. The requirements of the system have yet to be studied. Sodium azide, but not DDC, retarded amine formation (fig. 37).

Difficulty was experienced in obtaining consistent and reproducible manometric evidence for the existence of the reaction. Boiled tissue preparations were inactive. The CO_2 evolved by left gland homogenate though small was greater in the presence than in the absence of p-tyrosine (fig. 50). Again right gland tissue affected the reaction profoundly (fig. 50), and again prolonged incubation was necessary before induction was brought about. That the influence of the right gland is caused by the acid reaction of the free diphenol is less likely in the presence of buffer of pH 6.8.

Although there is evidence that p-tyrosine is present in colleterial gland cells, the amount available would not account for approximately 300 µls. of CO_2 suddenly evolved when left and right glands were homogenized and incubated in bicarbonate buffer with and without protocatechuic acid. To produce this evolution about 2.20 mg. of tyrosine would be required. But this amount of tissue (158 mg. wet weight) contains at the most 0.6-0.7 mg. of endogenous protocatechuic acid (after hydrolysis of the glucoside) (see figs. 40, 44) and very much less tyrosine (figs. 32, 52).

Therefore if tyrosine is being decarboxylated, the extent of the reaction cannot be assessed manometrically because some other substances must also be contributing to the gas evolution. During the aerobic incubation of left gland with tyrosine, the 35.8% of the slow phenolic-amino component (at one time thought to be dopa) disappeared (fig. 44) but were this a result of decarboxylation, the amount of CO_2 from this source would not account for much of that measured (fig. 39). L-dopa when added to gland homogenate failed to increase CO_2 production.

Since right gland preparation, when incubated with left gland in bicarbonate buffer, is responsible for stimulating CO_2 evolution, possibly the pK of free protocatechuic acid is higher than that of its glucoside, which is itself firmly attached to the protein such that

dialysis (water) fails to remove it. Liberation of the stronger acid in a solution of bicarbonate would generate CO_2. (CO_2 evolution was noted and measured (137.2 µls.) upon tipping protocatechuic acid (15 µmoles) into the buffer, without prior mixing. When homogenated gland was present, less gas (112 µls.) was produced and still less (88 µls) if the tissue preparation has been previously heated (at 98°C for 10 minutes).)

The tissue, producing 0.6-0.7 mg. (about 4 µmoles) of protocatechuic acid would account for only 30-40 µls. of CO_2 on the basis of the figures (in parenthesis) quoted above.

If action of β-glucosidase of right gland is indirectly responsible for most of the gas evolution, the long induction period before CO_2 is produced (fig. 39) is not actually characteristic of the action of the heat labile right gland enzyme, even in presence of buffer (fig. 51). Auto-hydrolysis of the glucoside, it will be noticed (fig. 51), is quite rapid at 37°C. This long lag is reminiscent of that experienced with some phenoloxidase preparations (fig. 23).

The source of all the CO_2 produced endogenously is therefore not known. Perhaps manometric experiments on the decarboxylase system cannot yield quantitative results unless the endogenous phenolic substances can be safely eliminated. But decarboxylases are

in any case difficult to estimate manometrically (Schales, 1951).

However, there is good evidence that after prolonged aerobic incubation of homogenated left gland with antibiotic and p-tyrosine (in the absence of buffers or pyridoxal-6-phosphate) a phenolic amine, which strongly resembled tyramine (chromatographically and spectrophotometrically) was produced at the expense of the amino acid (figs. 43, 44). Amberlite IRC (H^+) resin was used to separate the amine from the other phenolic compounds and amino acids in solution.

It is very likely then that tyrosine is decarboxylated under these conditions but the significance of the reaction if indeed it is enzymic and exists in vivo can, at the moment, only be speculated upon (fig. 54). The inefficiency of the reaction in vitro could be a result of the unavoidable dilution of the gland cell contents. This problem concerning the existence of the decarboxylase may soon be resolved when generally and C^1_2, C^{14}-labelled tyrosine are used as substrates.

The oxidation of tyramine.

If in fact tyrosine is first decarboxylated before oxidative deamination of the side chain takes place, - instead of vice versa, as shown in figure 30 - a monoamine oxidase or dehydrogenase should be present in the left gland cells. It was found, after long incuba-

tion, that tyramine greatly increased the oxygen uptake of homogenated left gland. This oxidation had still not ceased 23 hours after it appeared to have begun and by that time more oxygen had been consumed (260 µls.) than was necessary to convert all the tyramine (4. 5 µmoles) that had apparently disappeared, (determined with Dowex 50 (Na^+) column (Fig. 52)). Possibly the intermediate formed was in its turn oxidised as fast as it was synthesised (see fig. 54). Further study of the system is required to ascertain the products of this reaction.

In the experiment shown in fig. 43, the amount of tyramine found after incubation was in fact less than expected. 1 µmole of added tyrosine gave rise to 0. 62 µmoles of tyramine (fig. 44)(although about 0. 77 µmoles of tyr had disappeared.) Perhaps some of the tyramine had been further metabolised. Only 0. 60 µmoles of the amino acid was lost directly judging from the amount contained in the boiled control after incubation (page 170).

The most likely products (see fig. 54) of an hydroxylation or dehydroxylation are respectively 3-hydroxytyramine (Imaizumi, Yoshida, Hiramatsu and Omori, 1958), or octopamine (norsynephrine) (Pisano, Creveling and Udenfriend, 1960). Neither of these substances nor noradrenaline could be detected by the methods

described in chapter seven. The amounts involved at any time, in the absence of specific inhibitors, would be very small and the other substances present would interfere, (see appendix 3).

Attractive though the hypothesis is that tyramine is further metabolised by gland homogenate, a further possible cause of the increase in oxygen uptake must be duly considered. Tyramine may suddenly increase the oxygen uptake of left gland preparation if it reacts with protocatechuic acid quinone formed by the phenol-oxidase after slow autohydrolysis of the glucoside has taken place (fig. 54). As a result of the substitution the quinone would be reduced to the diphenol which could immediately be reoxidised, (fig. 4). If decarboxylation followed monosubstitution (Mason, 1955) and reoxidation, further substitution with tyramine could occur - followed by yet further oxidation.

If substitution indeed explained the apparent oxidation of tyramine, the long induction period before the reaction became noticeable manometrically would be a function of the rate of autohydrolysis of the endogenous glucoside (which is normally quite rapid at 37°C - fig. 51). If the oxidation of tyramine is real, prolonged incubation may have been necessary because of the dilution of the cell contents and/or shortage of cofactors or electron carriers.

To test the possibility outlined above other amines or free amino acids (other than tyrosine and dopa) should be tested. Tyrosine did increase oxygen uptake slightly (fig. 15), when protocatechuic acid was added. Dopa did not, possibly because it is a competitive inhibitor of the phenoloxidase (Table 4). Glucoside-free homogenate preparations should be tested(for tyramine dehydrogenase or monoamine oxidase activity) before any further speculations can be made.

If free amines are formed in vitro in incubated homogenates perhaps some of the unknown substances formed after prolonged aerobic incubation of gland homogenates (detected by chromatography) are mono- and di- substituted phenols or their quinones.

Nevertheless N-acetyltyramine, (Butenandt, Groschel, Karlson and Zillig, 1959), 3-hydroxytyramine, noradrenaline and adrenaline (Ostlund, 1953; Dresse, Jeuniaux and Florkin, 1960) have been detected in insects in significant amounts to arouse speculation concerning their biosynthesis.

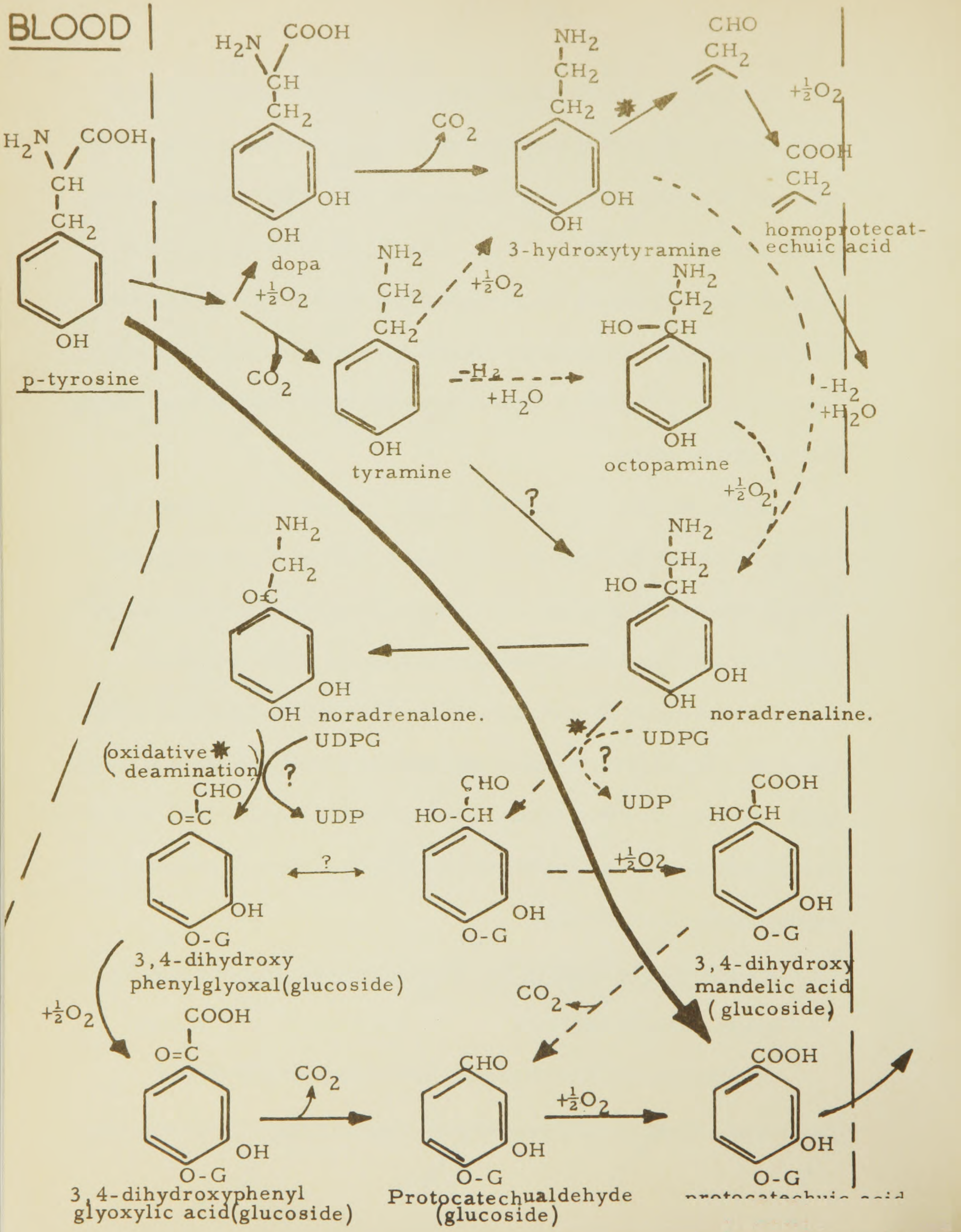
Hypothetical pathways for the biosynthesis of protocatechuic acid. (see fig. 54).

Tyramine may be oxidised to form 3-hydroxytyramine (Imaizumi, Yoshida, Hiramatsu and Omori, 1958; Grass and Siser,

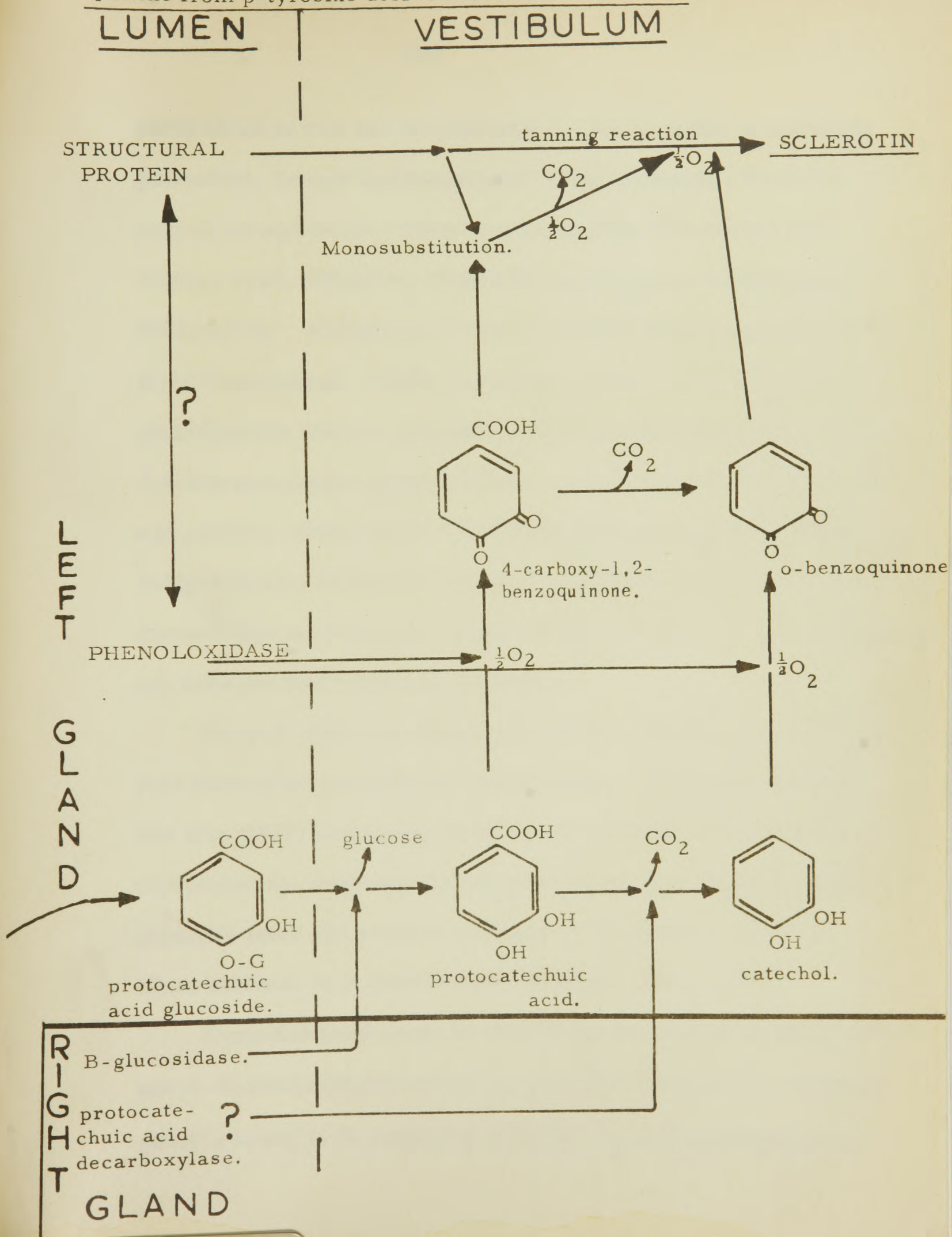
FIGURE 54. Hypothetical pathway for the biosynthesis of the tanning-

CELL

BLOOD



quinone from p-tyrosine derived from the haemocoel.



1959) which in turn can be converted to noradrenaline in mammals (Rosenfeld, Leeper and Udenfriend, 1958; Imaizumi, Yoshida, Matsumoto and Osawa, 1958; Senoh, Gerling, Udenfriend and Witkop, 1959; Goldstein, Friedhoff, Simmons and Prochoroff, 1959, 1960). Noradrenaline can be readily deaminated and oxidised (ZuckerKandl, 1959b; Herzmann, 1959). 3,4-dihydroxyphenylglycolic aldehyde (Leeper, Weissback and Udenfriend, 1958), 3,4-dihydroxyphenylglycol (Axelrod, Kopin and Mann, 1959; Kopin and Axelrod, 1960a,b) and 3,4-dihydroxymandelic acid (Axelrod, 1958; Goodall, Kirschner andRosen, 1959) are now known to be formed from noradrenaline in mammals. However, these substances have not been identified in insects.

Several other hypothetical alternatives exist for removing the side chain from tyramine or noradrenaline. Imaizumi, Yoshida, and Kita (1958) have proposed that noradrenalone may give rise to protocatechuic acid via dihydroxyphenylglyoxal, dihydroxyphenylglyoxylic acid, and protocatechualdehyde as shown in figure 54. Their evidence in support of these claims is slight so far.

Tyramine itself could be oxidatively deaminated (Thölen, 1959) and 3,-hydroxytyramine which has not been identified in colleterial gland is known to be converted in animals to 3,4-dihydroxyphenyl

acetaldehyde (Holtz, Heise and Ludtke, 1939; Fellman, 1958; Goldstein, Friedhoff, Pomerantz and Simmons, 1960) which can then be oxidised to the corresponding acid (Fellman, 1959; Goldstein, Friedhoff and Simmons, 1959; Goldstein et al., 1960). The latter substance, homoprotocatechuic acid may be decarboxylated in insects (Malek, 1960) presumably to yield homocatechol (4-methylcatechol) or, if further oxidised at the time of decarboxylation, protocatechuic acid may be formed (figure 30).

These hypothetical but already known pathways can serve as a guide to further investigation, but little interpretation can be made of them at this stage.

Formation of the glucoside(s).

Since the phenoloxidase in left colleterial gland cells can oxidise any o-diphenol once deaminated (fig. 27), the pathways shown in figure 54 have quite arbitrarily included the presence of a glucosetransferase system purporting to function as a precaution against oxidation by phenoloxidase at the time of oxidative deamination. However, this Phenol + UDPG $\longrightarrow$ UDP + phenol-β-glucoside system has not been identified yet in the left gland cells (Kent, 1958). Locust fat body has recently been found to contain this UDPG-glucosetransferase system (Trivelloni, 1960) - in this case most

probably existing as a detoxifying mechanism. The hepatic caecae of Periplaneta are known to contain the enzyme too (Dutton and Duncan, 1960).

In view of these facts it is possible that synthesis of the phenolic glucoside, ultimately to be the precursor of the tanning quinone, may occur outside the colleterial glands in the fat body or even in the walls of the alimentary canal. In support of this supposition the following points can be make:-

1) incorporation of the L-p-tyrosine-C-C^{14} into the protocatechuic acid glucoside present in colleterial gland is not very rapid i. e. it takes about 20-30 hours (Brunet, 1961).

2) except the glucoside, no intermediates such as dopa, 3-hydroxytyramine, noradrenaline and octopamine - labelled or unlabelled - have yet been identified in gland cell extracts.

3) the circulation of the blood or lymph in the haemocoel is very efficient (Wigglesworth, 1953; Brunet, 1960).

The techniques that have been developed and used in this investigation of the phenolic compounds would be well-suited for determining the site and mode of (C^{14}-labelled) glucoside synthesis in the insect and for identifying the intermediates on the pathway from (C^{14}-labelled) tyrosine to (labelled) protocatechuic acid. If the

colletérial gland is not the site of diphenol and glucoside synthesis, as it was thought to be until recently, investigation of the reactions caused by incubating gland preparations have been largely fruitless.

Decarboxylation of protocatechuic acid.

There is good evidence that catechol is formed during incubation of left gland homogenate (fig. 32). Again presence of right gland enhances the reaction responsible (figs. 32, 33, 34). Difficulty was encountered in demonstrating manometrically that catechol is formed by decarboxylation of protocatechuic . The CO_2 evolution during incubation of homogenated left and right glands was unaffected by the addition of the diphenolic acid (fig. 39), but that of right gland (corrected for boiled control) was increased. Protocatechuic acid is itself also slowly decarboxylated it seems (fig. 50). So whether a decarboxylase exists in right gland or not cannot be decided yet.

Formation of catechol by homogenated gland is much more rapid under aerobic conditions in the absence of buffer (c. f. fig. 32 and 51). This would suggest that decarboxylation of protocatechuic acid mostly takes place after its oxidation by phenoloxidase (fig. 54).

Whether the tanning agent is actually o-benzoquinone or 4-

carboxy-1,2-benzoquinone cannot be decided. Since the quinone of protocatechuic acid would be decarboxylated after monosubstitution anyway (Mason, 1955) the nature of the tanning agent is immaterial with regard to sclerotin formation.

Catechol has not been detected in oothecal fluid. Until further experiments are conducted it must be assumed that catechol formation is induced during prolonged incubation of gland preparation.

Catechol is oxidised more slowly than protocatechuic acid (or hydroquinone) (Tables 1 and 3). Therefore it is unlikely that phenoloxidase of left gland acts only after decarboxylation of protocatechuic acid (fig. 51), although in presence of detergent and excess catechol oxidation was much more rapid (fig. 16). At one time prior decarboxylation was thought necessary to explain the long induction period of the phenoloxidase, but this is not the likely explanation (page 130).

CONCLUSION

As already mentioned earlier, further study of the nature of the phenoloxidase and of the biosynthesis of the tanning agent in Periplaneta is necessary before attempted application of these findings to insect cuticle. Use of labelled precursors and specific inhibitors would seem to be the most profitable avenue of

approach.

The occurrence of phenolic glucosides may well be more widespread in invertebrates, since diphenols are thus protected at the site of phenolase activity. Certainly the process of tanning by quinones derived from diphenols as a means of hardening certain structures might be more widespread in nature, than has been envisaged. Diphenols have been found in fluke eggs (see Mansour, 1958), and the capsule or "maiden's purse" in dogfish (Brunet, 1960). Their hardening is probably due to tanning by a diphenol-phenoloxidase system too. In some invertebrates, such as molluscs, hardening of the periostracum or like structures is very likely augmented by quinone-tanning.

The importance of expanding knowledge on the metabolism of phenolic compounds need not be overemphasised. The role played by these substances in pigment formation, tanning and possibly in respiratory processes, and the pharmacological and physiological effects of some of them lends a dynamic quality to the study of their metabolism.

APPENDICES.

APPENDIX I

Correlation of the protein (T. C. A. precipitate) concentrations of left gland 'exudate' with a) their QO_2 values and with b) the figures for µls. O_2 uptake/ml. of preparation/hour determined for each 'exudate' when incubated (37°C) in the presence of phosphate buffer (0.1M-, pH 6.8), protocatechuic acid (3 µmoles) and in an atmosphere of oxygen.

TCA ppt mg/ml.	(a) QO_2	(b) µls. O_2/ ml./hour	TCA ppt mg/ml.	(a) QO_2	(b) µls. O_2/ ml./hour
Supernatants			2.5	0.80	2.00
1.3	5.40	6.87	7.5	0.65	4.87
0.1	19.40	1.94	15.0	0.46	6.82
0.8	17.08	15.18	5.0	1.03	5.15
2.4	3.44	8.26	4.5	1.77	7.96
0.9	1.88	1.65	3.0	1.46	4.38
0.4	2.02	0.81	8.8	1.96	17.15
1.5	5.92	8.88	12.0	1.88	22.58
0.6	4.46	2.68	5.8	1.10	6.37
0.7	14.90	10.42	6.0	0.97	5.78
0.2	10.80	2.16	5.7	3.55	20.14
0.6	5.10	3.06	7.2	2.94	21.19
0.6	-	3.06	0.9	3.72	3.35
0.2	-	4.00	1.6	1.30	2.08
Precipitates			1.6	11.00	17.60
0.2	3.80	0.76	4.5	-	9.14
1.5	3.16	4.74	3.4	-	14.49
1.0	1.44	1.44	2.0	-	16.77
0.4	4.85	1.94	2.0	-	16.65
0.3	6.57	1.97	2.4	-	8.74
4.4	1.60	7.05	4.2	-	5.55
3.4	0.82	2.79			

Correlation of T.C.A. ppt.		vs.	µls O_2/ml/hr.
Totals	127.1		308.42
n		41	
S.S.	833.73		3877.6068
C.F.	394.01		2320.0706
T.S.S.	439.72		1557.5362

S.P		1336.035
C.F.		956.102
		+379.933

$$r = \frac{+379.933}{\sqrt{439.72 \times 1557.5362}}$$

$$= \frac{+0.459}{} \quad (39 \text{ D.F.})$$

exact t = 3.23

Supernatants

	T.C.A.ppt	vs.	µls O_2/ml./hr
Totals	10.3		68.97
n		13	
S.S.	12.72		587.0051
C.F.	8.00		365.9124
T.S.S.	4.72		221.0927

S.P.		69.341
C.F.		54.115
		+15.226

$$r = \frac{+15.226}{\sqrt{4.72 \times 221.0927}}$$

$$+0.471 \quad (11 \text{ D.F.})$$

exact t = 1.77

Appendix I (Continued) 16 (fig. 29)

Correlation of T.C.A. ppt. vs. QO_2

Totals	109.6		147.23
n		33	
S.S.	773.36		1407.6127
C.F.	364.01		656.8688
Total sum of squares:	409.35		750.7439

sum of products 228.974
C.F. 488.982
260.008

$$\text{Correlation coefficient (r)} = \frac{\text{Sum of products}}{\sqrt{\text{Product of sum of squares}}}$$

$$= \frac{-260.008}{\sqrt{409.35 \times 750.74}}$$

$$= -0.469 \quad (31 \text{ D.F.})$$

$$\text{exact t (student)} = r\sqrt{\frac{n-2}{1-r2}}$$

$$= 2.961$$

Appendix 2

Resolution by paper chromatography (BAW, 4:1:1) of small volumes (0.01 - 0.05 mls.) of 5 ml. fractions containing known amounts of reference phenolic compounds eluted from a cellulose column (44 cms long, flow rate - 1.66 mls/min) in BAW, 4:1:1 at tube 7 - 135 mls. collected after sample run on. Detection with 1% ferricyanide (Fe^2) spray 2 ml. sample contained

Catechol (4.4 mg), protocatechuic acid (6.2 mg.) tyramine -HCl (6.88 mg.), L-p-tyrosine (1.4 mg), 3-hydroxytyramine (7.6 mg.) and L-dopa (3.94 mg.)

NOTE

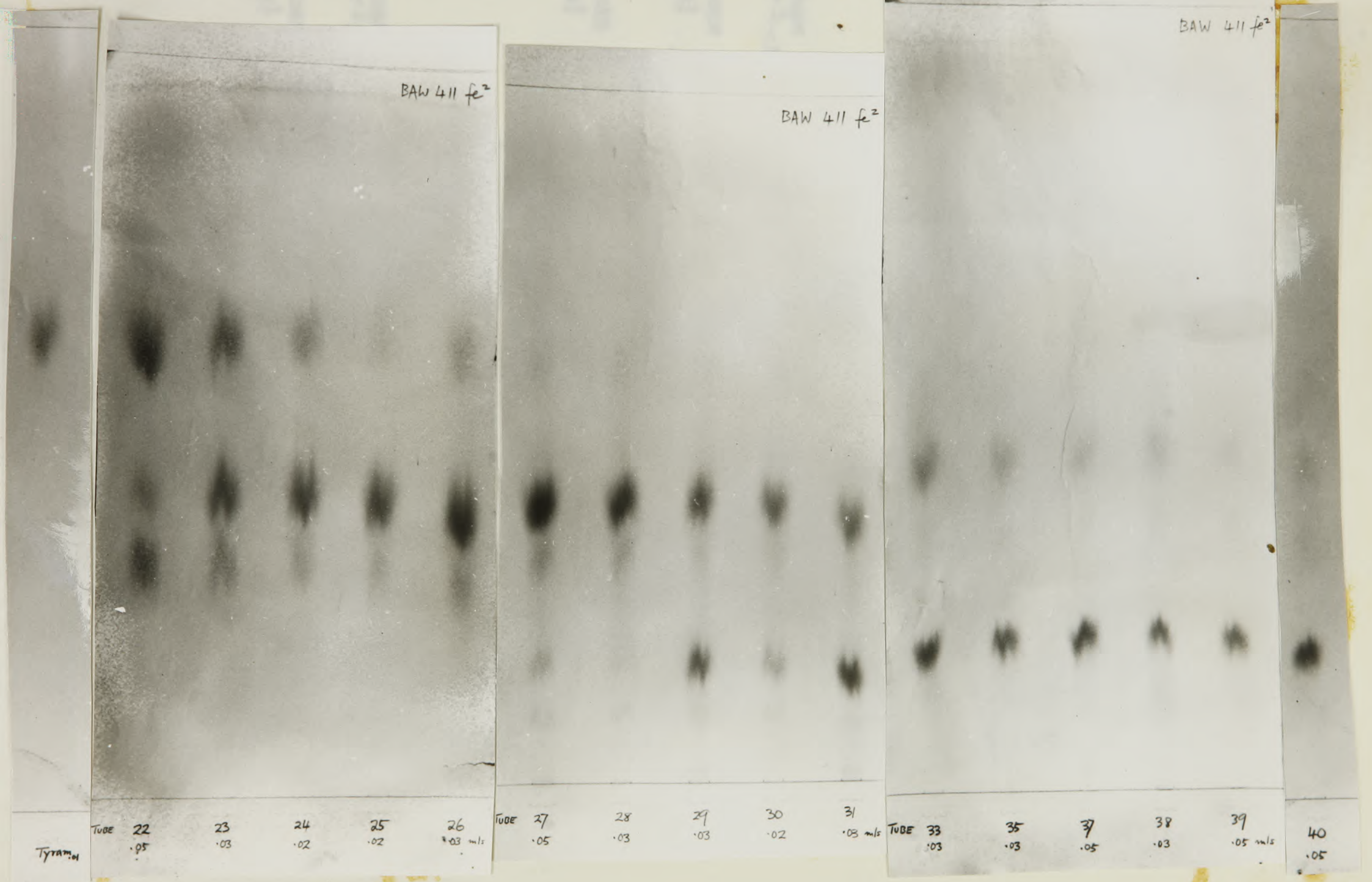
On cellulose (standard grade, ashless) tyrosine precedes 3-hydroxytyramine. The opposite is true on Whatman No.1 paper in the same solvent (BAW, 4:1:1).

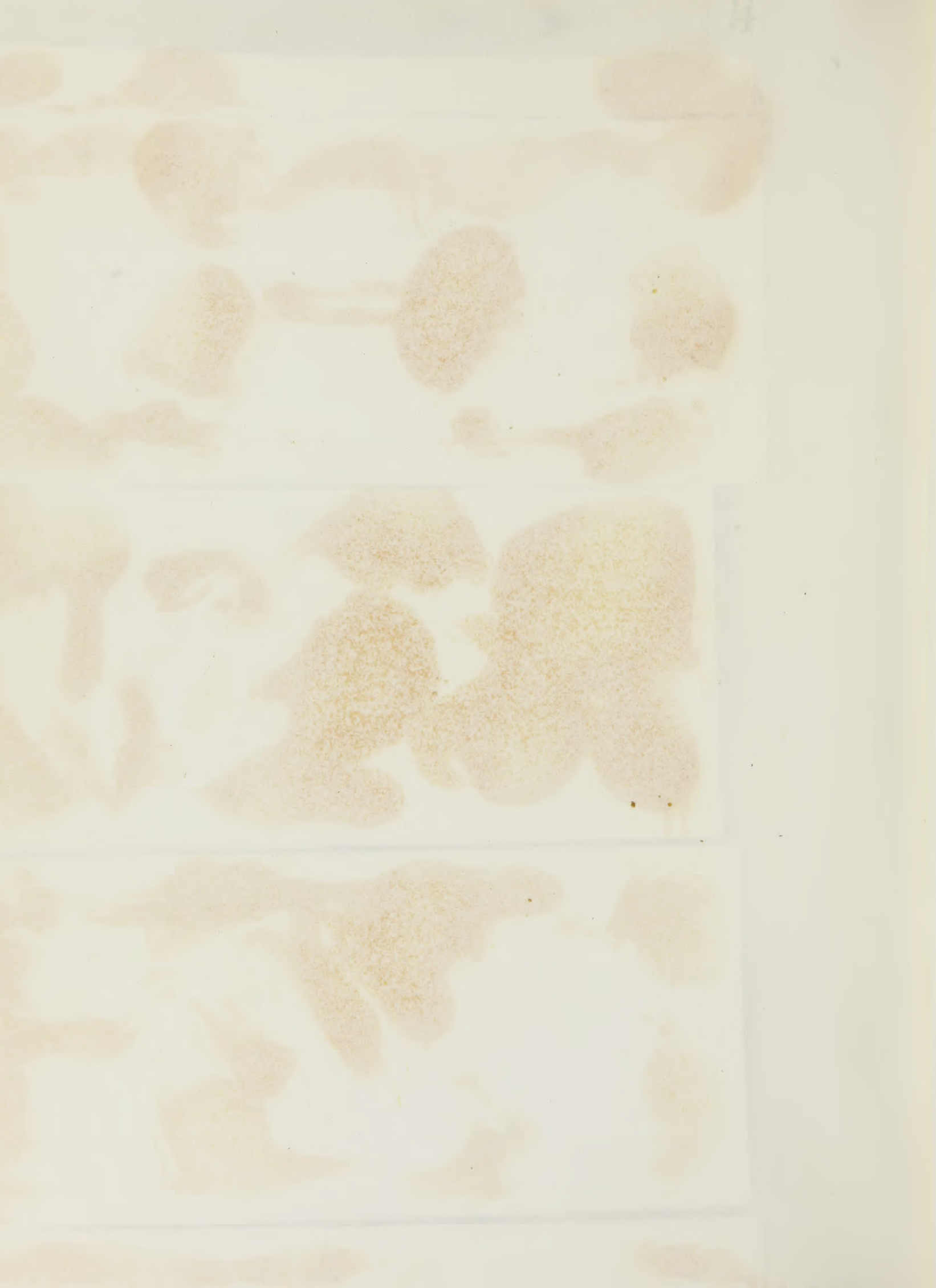

BAW 411 fe²
7 .05
8 .05
9 .01
10 .01
11 .01 m/s


BAW 411 fe²
TUBE 12 .01
13 .01
14 .01
15 .02
16 .05 m/s


BAW 411 fe²
TUBE 17 .02
18 .02
19 .03
20 .03
21 .03 m/s


BAW 411 fe²
Dopam .01
Tyr+ Dopa


BAW 411 fe²
BAW 411 fe²
BAW 411 fe²
Tyramine
TUBE 22 .05
23 .03
24 .02
25 .02
26 .03 mls
TUBE 27 .05
28 .03
29 .03
30 .02
31 .03 mls
TUBE 33 .03
35 .03
37 .05
38 .03
39 .05 mls
40 .05



Appendix 2 cont.

Amount and spectral properties of reference compounds placed on cellulose column (3 x 155 cms) in total of 6 mls. of BAW, 4:1:1 (see fig. 49)

	catechol	protocatechuic acid	tyramine-HCl
	1.9 mg.	3.2 mg.	2.6 mg.
$\varepsilon\lambda$max (10^{-3}-Molar)	2.13	5.97	1.84
λmax (mμ)	281	261 299	282
λmin (mμ)		284	
	3-hydroxy tyramiae-HCl	p-tyrosine	L-dopa
	2.4 mg.	2.7 mg.	13.7 mg.
$\varepsilon\lambda$max (10^{-3}-Molar)	2.99	1.414	1.36
λmax (mμ)	286	282	287

Appendix 3 cont.

Compounds	Solvents Descending: BAW Rf%	Phenol/water Rf%	BEW Rf%	2% acetic Rf%	BEB Rf%	Ascending: APW Rf%	Propanol/N-HCl Rf%
Phenols							
catechol	81		84	72	62		
hydroquinone	81		82		88		
pyrogallol	64(16)						
4-methylcatechol	78						
Phenolic acids							
protocatechuic	74-76		43-72	47	25	85	93
homoprotocatechuic	71					76	
3,4-dihydroxyphenyl-propionic	76						
3,4-dihydroxymandelic	47					44	
2,5-dihydroxyphenyl-pyruvic lactone	78						
gallic	49						
Amino acids							
L-p-tyrosine	36	63	15	84	(58)	48	58
o-tyrosine	47	74				52	64

Appendix 3

Separation of reference compounds by paper chromatography (Whatman No. 1).....

(descending) in n-butanol: acetic acid: water, 4:1:1 (BAW)/2% acetic acid, phenol saturated with water (74g:24g)/ n-butanol: ethanol: borate, 1:1:1 (BEB)-borated paper/n-butanol: ethanol: water, 4:1:1 (BEW)

(ascending) (in collaboration with Dr. P.C.J. Brunet) in amyl alcohol: pyridine: water, 2:2:1 (APW) / n-propanol: N- HCl, 3:1

detection with ferricyanide or ninhydrin.

dopa	21-22	35	06	69-70		36
L-tryptophane	49	79				
L-aspartic	25	17				
alanine		61				
serine	30	35				
glutamic	28	30				
Amines						
tyramine	62	75-80		90	63,72	63
3-hydroxytyramine	45			89	60,69	42
octopamine	63			85-88	62,70	51
noradrenaline		29(22)		85-87		31
glucosamine		22				

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Addendum

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