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To my husband Crawford and my parents for their constant support, encouragement and enthusiasm throughout this project.

SOME BIOLOGICAL PROPERTIES OF PYOCYANIN AND ITS DERIVATIVES.

ANN V. ARMSTRONG.

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CONTENTS.

CONTENTS

CONTENTS.

FIGURES.

FIGURES.

FIGURES

FIGURES

FIGURES.

TABLES.

TABLES.

TABLES.

TABLES.

PLATES. following page.

REVIEW of the LITERATURE

In the mid-nineteenth century post operative infections were widespread due, in part, to the fact that dressings were crude and often heavily contaminated. Occasionally, a bluish-green colour was imparted to these dressings. In 1850, Sédillot found that it was possible to transfer this phenomenon from one patient to another, probably due to dressings contaminated by infected handlers. This was probably one of the first recorded instances of a hospital cross infection. Fordos (1860) extracted such bluish-green dressings and isolated a blue crystalline material which was named "pyocyanin". Lücke (1862) investigating the same problem suggested that the small rod-shaped organisms observed in wound discharges could be the source of pyocyanin.

These rod-shaped organisms were subsequently isolated from wound discharges and several names were proposed by independent workers,

Bacterium aeruginosum (Schroeter 1872);

Bacterium pyocyanea (Gessard 1882);

Pseudomonas pyocyanea (Migula 1895); and

Pseudomonas aeruginosa (Migula 1900).

However Rhodes (1961) proposed that the name Pseudomonas aeruginosa should be given to the type species of the genus Pseudomonas.

Gessard (1890, 1891, 1892) showed that the bluish-green pigment was a mixture of two separate pigments:- 1) Pyocyanin a bluish-green non-fluorescent pigment soluble in both chloroform and water, 2) Fluorescein a greenish-yellow fluorescent pigment insoluble in chloroform and soluble in water.

INCIDENCE of PSEUDOMONAS INFECTIONS

From the literature, it is apparent that Pseudomonas aeruginosa has been implicated as a primary or secondary invader in a variety of human infections. Williams and Cameron (1896) described a generalised Pseudomonas aeruginosa infection with lesions in the respiratory tract, the eye, joints and kidneys. Dudgeon and Sargent (1905) found that Pseudomonas aeruginosa was present as a secondary invader in cases of peritonitis. The complete division of Pseudomonas aeruginosa infectivity into primary or secondary is not completely valid. There appear to be a number of factors which may influence the susceptibility of a host to Pseudomonas aeruginosa and in many cases the primary infection may be secondary, or vice versa. However, in this review an attempt has been made to divide pseudomonas infections into primary and secondary.

A. Primary infections caused by Pseudomonas aeruginosa

Lilley and Bearup (1928) isolated the organism from local lesions in the ear, eye, skin, lung, urinary tract, meninges, and bone joints; at the same time it was isolated from infants suffering from diarrhoea, dysentery or a typhoid-like fever. It was shown by Pons (1927) that Pseudomonas aeruginosa caused typhoid-like diseases in patients living in tropical countries. Salvin and Lewis (1946) isolated Pseudomonas aeruginosa from forty-five per cent of patients suffering from an ear infection, although it was not isolated from the ears of healthy individuals.

In all of the investigations mentioned above little or no indication concerning the mode of action of Pseudomonas aeruginosa was proposed; the reports merely stated that the organism was isolated from a variety of sites in the human body. It has become increasingly apparent that infections caused by Pseudomonas aeruginosa are more common and more serious when other predisposing conditions prevail in the human body. Children, especially premature babies, appear to be highly susceptible to this type of infection. The pathogenicity of Pseudomonas aeruginosa in the newborn has been extensively studied. In general

it seemed that the organism entered the body via the umbilicus, the gastro-intestinal tract or respiratory tract. (Schaffer and Oppenheimer 1948; Neter and Weintraub, 1955; Hoffman and Finberg, 1955; Asay and Koch 1960; Rogers, 1960).

Neter and Weintraub (1955) found that the majority of infections in the newborn were either symptomless or generalised and fatal. Few cases were recorded where patients showed symptoms and recovered. Eggers and Wöckel (1958) described generalised infections of the newborn caused by Pseudomonas aeruginosa with involvement of the ear. Asay and Koch (1960) and Jacobs (1964) described outbreaks of Pseudomonas aeruginosa infections in infants, characterised by manifest lesions of the skin, mouth and throat. It was significant that these children with severe infections had previously received broad spectrum antibiotics. These authors postulated that treatment with these antibiotics altered the patient's resistance in some way, and thus increased their susceptibility to infection. It was not possible to prove this hypothesis but it was shown that such antibiotic treatment could remove competing bacteria and thus favour the extensive proliferation of Pseudomonas

aeruginosa. This was previously stated by Geppert, Baker and Copple (1952). Williams, Williams and Hyams (1960) found that individuals whose resistance was already lowered, because of other diseases, tended to develop severe Pseudomonas aeruginosa infections. Five patients died of a Pseudomonas aeruginosa infection and it was suggested that treatment with corticosteroids and broad spectrum antibiotics increased the patient's susceptibility. Pseudomonas aeruginosa was found in large numbers in the ward, by sampling the air, and it was thought that the source of these organisms was from bronchial secretions of patients. Bassett, Thompson and Page (1965) described a number of infections caused by Pseudomonas aeruginosa in a premature baby and special care unit which occurred over a period of two and a half years. Illness was more common among infected premature babies than among full term babies; the latter often showed no clinical symptoms. The signs of infection observed during a fourteen month survey were as shown in Table I. Of the non-infected premature babies 89% showed no signs of clinical infection whereas of the infected premature babies only 19% showed no signs of infection. This difference between the two groups led the authors to postulate a causal relationship between Pseudomonas aeruginosa and the infections of premature babies.

	PREMATURE BABIES		MATURE BABIES
	NOT INFECTED	INFECTED	INFECTED
NUMBER	93	70	56
AVERAGE BIRTH WEIGHT	5lb 0oz (2.3Kg)	4lb 6oz (2Kg)	-
SIGNS OF INFECTION			
TEMPERATURE FALL	5 (5%)	37 (53%)	10 (18%)
VOMITING	4 (4%)	38 (54%)	19 (34%)
POOR COLOUR	4 (4%)	29 (41%)	15 (27%)
LOOSE STOOL	4 (4%)	17 (24%)	10 (18%)
NO SIGN OF INFECTION	83 (89%)	13 (19%)	29 (52%)
RESPIRATORY DISTRESS SYNDROME	3 (3%)	19 (27%)	13 (23%)

TABLE 1: Clinical signs in babies infected with Pseudomonas aeruginosa and uninfected premature babies during a fourteen month period. Bassett, Thompson and Page (1965).

Six fatal cases were described, in three of which the pathogenicity of the organism was clear cut:-

Patient 1. birthweight 4lbs 6ozs. Pseudomonas aeruginosa was isolated from the urine on the seventh day and the child died on the seventeenth day after birth. At post mortem bilateral renal vein thrombosis and renal cortical necrosis was observed and Pseudomonas aeruginosa was isolated from the kidneys.

Patient 2. birthweight 3lbs 7ozs. Pseudomonas aeruginosa was isolated from the throat 12 hours after birth and signs of infection became apparent on the fifth day. Meningitis was diagnosed on the eighth day and the child died on the twelfth day. The post mortem showed the presence of green pus over the meninges, from which Pseudomonas aeruginosa (pyocine Type P, serological type 6) was isolated.

Patient 3. birthweight 3lbs 15ozs. This child died five weeks after birth. Pseudomonas aeruginosa (pyocine Type P, serological type 6) was isolated from throat and rectal swabs taken ante mortem and from a tracheal swab taken post mortem.

In premature babies the predisposing factor to Pseudomonas aeruginosa susceptibility appears to be that they are premature babies. This point is supported by

the fact that in the survey mentioned above, fatal infections involved the excretory, respiratory and nervous systems. This interesting piece of work indicates the limited knowledge concerning the mode of action of Pseudomonas aeruginosa, indeed, well-defined criteria of infection are not easily discernible. On the other hand, predisposing factors to Pseudomonas infections in adults have been determined, for example, broad spectrum antibiotic and cortico-steroid treatment.

B. Secondary infections caused by Pseudomonas aeruginosa.

An association between Pseudomonas aeruginosa and other bacterial organisms in some cases of dysentery or typhoid like fevers was suggested by Tobie (1946) and in cases of infantile diarrhoea by Hunter and Ensign (1947). In 1958 twenty-three cases of septicaemia caused by Pseudomonas aeruginosa were reported by Forkner, Frei, Edgcomb and Utz; of these, thirteen patients with acute leukaemia were undergoing treatment with anti-leukaemic drugs and this may have been a predisposing factor to Pseudomonas aeruginosa infection since these drugs lowered the patients' resistance and hence increased susceptibility to infection. Kwantes (1960) described Pseudomonas aeruginosa associated with cases of otitis.

Pseudomonas aeruginosa was also found in association with several cases of severe urinary tract infection. (Edgar and Dickinson 1962). It would be interesting to compare these reports with those described under primary infection.

The secondary invasion by Pseudomonas aeruginosa as a complication of extensive burns has been recognised as a problem for many years. Such infection is usually airborne probably due to the long periods of exposure of the burn area to the air during treatment (Colebrook and Francis, 1941); (Bourdillon and Colebrook, 1946). It was shown by Cason and Lowbury (1960) that infection of burns with Pseudomonas aeruginosa was controlled by local application of a cream containing neomycin, chlorhexidine and polymyxin. Systemic administration of polymyxin has been of little use in controlling invasive infection by this organism. (Tumbusch, Vogel, Butkiewicz, Graber, Larson and Mitchell, 1961; Kefalides, Arana, Bazan, Velarde and Rosenthal, 1964; Jones, Jackson and Lowbury 1966).

Jackson, Lowbury and Topley (1951) maintained that colonization of burns by Pseudomonas aeruginosa and coliform bacilli resulted in rejection of skin grafts and prevented the normal healing process of the skin. In

addition, such infections were sometimes fatal; five patients died after colonization of their burns by Pseudomonas aeruginosa. Lowbury and Fox (1954) believed that the burn lesions themselves were an important source of infection, but that airborne infection of other patients could occasionally occur (Lowbury 1954). In a controlled trial Lowbury (1954) found that infection with Pseudomonas aeruginosa was less frequent for burns that were dressed in air conditioned rooms than for those that were not. This finding confirmed the work of Bourdillon and Colebrook (1946) who by using the slit sampler, found that heavy bacterial contamination of the air in ward dressing-rooms and operating-rooms was largely derived from reservoirs inside the room such as blankets and contaminated dressings from infected burns. Rabin, Graber, Vogel, Finkelstein and Tumbusch (1961) isolated Pseudomonas aeruginosa from the burn lesions of thirty-eight patients who later died. The same organism was also isolated from the blood of two patients who had developed endocarditis. The antibiotic gentamicin was used successfully in the treatment of serious burns infected with Pseudomonas aeruginosa with accompanying septicaemia. (Stone, Martin and Kolb, 1964, and Stone, Martin, Huger and Kolb, 1965).

In a survey carried out by Barber and Waterworth (1966) on gentamicin, it was shown that this antibiotic had consistently high activity against Pseudomonas aeruginosa; this activity exceeded both that of either streptomycin or kanamycin.

Jones and Lowbury (1967) showed that parenteral administration of carbenicillin and gentamicin protected burned mice against invasive infection with strains of Pseudomonas aeruginosa. The use of carbenicillin in the treatment of patients whose burns were infected with Pseudomonas aeruginosa gave promising results.

Groups of mice were infected by inoculation of small burns with two strains of Pseudomonas aeruginosa; about 70% died with Pseudomonas septicaemia. Both carbenicillin and gentamicin protected the mice if treatment was started at time of infection or 24 hours later.

When patients with burns were treated with carbenicilli and probenecid 10 out of 23 patients had Pseudomonas aeruginosa eliminated from their burns. This was significantly higher than the untreated controls (1 out of 23). This result was interesting but a

much larger investigation would be required before its significance can be fully appreciated.

Lindberg, Brame and Moncrieff (1968) showed that invasive infection by Pseudomonas aeruginosa in severe burns was controlled by topical application of 10% sulphamylon (p-amino-methyl-benzene sulphonamide, either as HCl or acetate). This cream substantially eliminated lethal burn wound sepsis but the high concentration of active ingredient and its breakdown product produced undesirable side effects. Sulphadiazene and sulphamethoxazole were effective in controlling infections in rats when applied topically. These compounds in combination with sulphamylon achieved an effective but lower blood level of sulphonamide and breakdown product.

4% sulphamylon in combination with 1% sulphamethoxazole was more effective than 10% sulphamylon in controlling burn wound sepsis. The development of agents effective in control of thermal injury was desirable when one considered that invasive infection by Pseudomonas aeruginosa with accompanying cellulitis, invasion of the walls of small blood vessels and occasionally septicaemia was often a fatal complication of serious burns. (Fraenkel 1906, Jackson, Lowbury and Topley 1951, Rabin, Graber, Vogel, Finkelstein and Tumbusch 1961, Teplitz, Davis,

Mason and Moncrieff 1964a, Teplitz, Davis, Walker, Raulston, Mason and Moncrieff 1964b, Sevitt 1964); antibiotic treatment was likely to fail even if the organisms are removed since irreparable damage had often occurred by the time invasion was recognized (Jones, Jackson and Lowbury, 1966).

HOSPITAL CROSS INFECTION with PSEUDOMONAS AERUGINOSA

Cross infection in hospitals with any organism has always presented serious problems and not the least of these is cross-infection with Pseudomonas aeruginosa of patients who are seriously ill or who have extensive burns. However it is only in recent years that Pseudomonas aeruginosa has been considered of any importance as a cause of hospital fatalities (Finland, Jones and Barnes 1959, Finland 1960, Williams, Williams and Hyams 1960, Barber 1961), yet many cases of cross infection in hospitals by this organism have been described.

The distribution of Pseudomonas aeruginosa does not readily account for its cross infective activity since it is not normally present on the skin although it is occasionally found in the faeces of healthy individuals. In addition it is rarely present in the air or dust unless infected patients are present. This sometimes accounts for the spread of infection in a burns ward (Lowbury and Fox 1953, 1954; Ringen and Drake 1952). Twenty-four cases of infantile diarrhoea

recorded in a nursery were caused by carriers among the mothers and nursing staff. The latter had previously drunk milk infected with Pseudomonas aeruginosa and cross infected the young infants (Ensign and Hunter, 1946). Florman and Schifrin (1950) described a similar outbreak in a hospital infants ward where the nursing staff caused the cross infection. The growth of Pseudomonas aeruginosa in pharmaceuticals was the cause of a large proportion of hospital cross infections. Meningitis caused by Pseudomonas aeruginosa was recorded by Botterell and Magner (1945) after two patients received intrathecal injection of penicillin and by Evans (1945) and Weinstein and Perrin (1948) after spinal anaesthesia of patients.

Ridley (1958) isolated Pseudomonas aeruginosa from bottles of eyedrops, many of which were "sterile", and also from eye ointments. Allen (1959) described eye infections including post-operative ophthalmitis culminating in blindness which developed after the use of contaminated eyedrops and lotions.

Aycliffe, Bowbury, Hamilton, Small, Asheshov and Parker (1965) isolated Pseudomonas aeruginosa from handcreams and attributed cases of meningitis, urinary

and wound infections in a neurosurgical unit to the pre-operative use of a shaving brush contaminated with Pseudomonas aeruginosa. Similarly contaminated eyedrops accounted for pseudomonal infection in 15 out of 25 patients undergoing ophthalmic operations. In 6 of these patients there was loss of the eye (Aycliffe, Barry, Lowbury, Roper-Hall and Walker 1966) In 1966, 7 cases of infection of the urinary tract in children after cytoscopy were traced to contamination of the chlorhexidine solution used for disinfecting the bladder-irrigation reservoir. (Mitchell and Hayward). This infection was traced back to the stock solutions of chlorhexidine in use in the hospital. Burdon and Whitby (1967) showed that aqueous solutions of "Hibitane" (chlorhexidine) and "Savlon" (cetrimide and chlorhexidine mixture in the ratio 10:1) were frequently contaminated with organisms identified as Pseudomonas species. Noble and Savin (1966) reported the isolation of Pseudomonas aeruginosa from a steroid cream containing 0.1% chlorocresol, although Pseudomonas aeruginosa is sensitive to 0.1% chlorocresol in aqueous solution. On investigation it was found that chlorocresol was divided between the oil and water phases of the cream in such a way that the aqueous portion had insufficient chlorocresol to exert an inhibitory effect; the pseudomonas grew in the

aqueous phase. This cream was widely used in the treatment of a variety of skin disorders and subsequently minor infections were recognized over a period of several months. Stricter precautions in the preparation and sterilization of the cream in the dispensary eliminated this source of infection.

Sussman and Stevens (1960) isolated Pseudomonas aeruginosa from the cellulose wadding used in splints and plasters and believed that this organism was responsible for an outbreak of infection in an orthopaedic ward.

In 1942, Nelson found that coliform bacilli grew and survived in bottles of distilled water with cork stoppers. Lowbury (1951) has described the isolation of Pseudomonas aeruginosa from 2% soap solution and various antiseptics (for example Dettol, 1% cetrimide, and calamine lotion) contained in cork stoppered bottles. Since Pseudomonas aeruginosa can survive such conditions, living cultures can be directly transferred to open wounds and quickly set up an infection.

Plotkin and Austrian (1958) and Anderson and Keynes (1958) also found Pseudomonas aeruginosa in cork stoppered bottles of soap solutions and various antiseptics, particularly quaternary ammonium compounds and chloroxylenol. They showed that organisms survived

in fissures in the cork stoppers and on removal of the cork thus contaminated solutions in the bottle. Linton and George (1966) showed that a substance, possibly a tannin, present in cork inactivated the disinfectant chlorhexidine. An outbreak of Pseudomonas septicaemia was traced by Plotkin and Austrian (1958) to the use of swabs for disinfection of the skin. These were left soaking in "Zephiran" solution prior to insertion of intravenous cannulas. The solution contained the organism and failed to inhibit its growth since the cellulose fibres of the swabs absorbed the disinfectant and inactivated it.

Colonization of sinks and water taps by this organism was also suggested as a possible source of cross infection (Wilson, Nelson, Phillips and Boak 1961).

Blood and infusion fluids were often heavily contaminated with Pseudomonas aeruginosa and caused severe, fatal infection following transfusion (Pittman 1953, Stevens, Legg, Henry, Dille, Kirby and Finch 1953, McEntegart 1956, Gilat, Hertz and Altmann 1958, Bourgain, Bonnel and Raby, 1958). In addition to growth in liquids Hoffman and Finberg (1955) believed that conditions of high humidity encouraged growth of Pseudomonas aeruginosa. This was confirmed

by Emmanouilidou-Arseni and Koumentakou (1964).

Contaminated instruments (for example, catheters and cytoscopes) and urinals were also a source of cross infection if improperly sterilised. When strict sterilisation procedures were implemented and a closed drainage system of the bladder in prostatectomy was introduced, following an outbreak of Pseudomonas aeruginosa infections in a urological ward, there was a significant reduction in the outbreak (Pyrah, Goldie, Parsons and Raper 1955).

Air is not usually contaminated with Pseudomonas aeruginosa but Lowbury (1954) showed that airborne cross infection of burns often occurred. Contamination by Pseudomonas aeruginosa of ventilation plants in operating theatres was the cause of post-operative cross infections. (Asay and Koch 1960). Anderson (1959) had already isolated Pseudomonas aeruginosa from the humidifying water in a ventilation plant from which it was sprayed into the air and was the subsequent cause of post-operative cross infections.

Pseudomonas aeruginosa was isolated from electrical aspirators by Rogers (1960) when investigating an outbreak of respiratory infections in infants following surgery.

In 1965, Bassett, Thompson and Page described 6 fatal and numerous less serious neonatal infections caused by the presence of Pseudomonas aeruginosa in resuscitation

equipment. The aspirators were difficult to disinfect or even clean and were found to be contaminated with the same type of Pseudomonas aeruginosa isolated from the majority of infected babies including those which died.

Tinne, Gordon, Bain and Mackey (1967) reported an outbreak of respiratory infection in a cardiac surgery unit in which three patients died. Pseudomonas aeruginosa, pyocine type 10 was isolated from the anaesthetic equipment. The three fatal cases had undergone open heart surgery followed by post operative tracheotomies and prolonged mechanical ventilation. They had also received corticosteroids and broad spectrum antibiotics. As mentioned previously these have been cited as possible predisposing factors in Pseudomonas aeruginosa infections.

THE PRINCIPLES OF PSEUDOMONAS AERUGINOSA PATHOGENICITY

The problems of cross infection in hospitals and the difficulties encountered in the treatment of such infections indicates that the isolation of a toxic factor or factors for the pathogenicity of Pseudomonas aeruginosa is of prime importance. Many attempts have been made to isolate chemical constituents from the cells of this organism and the characteristics of some products have been determined. However, the role of these cellular constituents in Pseudomonas aeruginosa pathogenicity still remains inconclusive.

In 1888, Bouchard described substances produced by Pseudomonas aeruginosa which were antibacterial. Emmerich and Loew (1899) and Emmerich, Loew and Korschun (1902) noted that old Pseudomonas aeruginosa culture supernatant fluids were bactericidal to many micro-organisms; and that ten-fold concentration caused rapid and complete lysis of thick suspensions of Vibrio cholerae and Bacillus anthracis. It was suggested that the organism produced an enzyme, responsible for the lytic action, this was termed Pyocyanase. Pyocyanase is now known to lyse many bacteria, including Escherichia coli, Salmonella typhi, Corynebacterium diphtheriae

Vibrio comma, Streptococcal and Staphylococcal species. In addition it also inactivated the toxins of Clostridium tetani and other bacteria very rapidly (Anderson 1948). Although the antibacterial properties of pyocyanase were accepted, its enzymic properties were challenged by Klimoff (1901) and Dietrich (1901), since it was heat-stable and soluble in ether, chloroform and benzene. This suggested that pyocyanase was not an enzyme and, in fact, this was found to be so by Anderson (1948) who showed that it was lipoidal in nature, its activity depending largely on the presence of unsaturated fatty acids. Raubitschek and Russ (1908) extracted cultures of Pseudomonas aeruginosa and obtained alcohol-soluble substances which possessed bactericidal activity against Bacillus anthracis; however the alcohol-insoluble fraction also possessed some bactericidal activity. It was believed that this action was due to "lipoids" present in the alcohol extract. Fukuhara (1909) and Ohkubo (1910) confirmed that alcoholic and acetonic extracts of Pseudomonas aeruginosa cultures were bactericidal, haemolytic and antitoxic. Hosoya (1929) succeeded in crystallising the "lipoid" and Hettche (1933, 1934) isolated pyocyanic acid, a free acid with some antibacterial activity, from

the cells of Pseudomonas aeruginosa.

The chloroform-soluble blue pigment of Pseudomonas aeruginosa, Pyocyanin, was shown to be antibacterial by both Hettche (1932) and Kramer (1935). This pigment was thermostable and strongly bacteriostatic towards both Gram- positive and Gram- negative bacteria but had little effect against moulds or yeasts. A degradation product of pyocyanin, α-hydroxy-phenazine was less active against bacteria but possessed considerably more activity against yeasts and fungi. Friedheim (1934) showed that pyocyanin was an oxygen carrier; increased oxygen consumption was obtained when pyocyanin was added to washed suspensions of either living bacteria (such as Pseudomonas aeruginosa, Staphylococcus aureus, and pneumococcus type III) or red blood corpuscles. Read and Boyd (1932) also found that pyocyanin was a significant oxygen carrier in metabolic reactions. In 1937 Young found that pyocyanin initially caused an increase in glucose respiration, quickly followed by inhibition of respiration, of slices of cerebral cortex in oxygen.

In 1941, Schoental isolated three antibacterial substances from the chloroform extracts of Pseudomonas aeruginosa cultures. These substances were not

enzymic because of their properties, namely, 1) solubility in alcohol, 2) resistance to boiling and 3) resistance to the action of proteolytic enzymes. They were

1) Pyocyanin

2) α- oxyphenazine

3) colourless bacteriolytic substance.

Pyocyanin and α- oxyphenazine were pigmented; however α-oxyphenazine showed more activity against both Gram-positive and Gram-negative cells and was also less toxic than pyocyanin to tissue cells. It was suggested that the colourless bacteriolytic substance was similar to the "pyocyanic acid" described by Hettche. Tables 2 - 6 illustrate these results.

Young (1947) isolated four fractions from Pseudomonas aeruginosa which possessed antibiotic activity. These were a) pyocyanin, b) α-oxyphenazine, c) a fluorescent residue, and d) a substance present in ether extracts of acidified cultures.

The latter two showed more activity against Gram-positive organisms.

Pseudomonas aeruginosa can be differentiated from Pseudomonas fluorescens since it produces zones of β-haemolysis on 2% defibrinated bovine red blood agar (Salvin and Lewis 1946). Christie (1948) stated that

SAMPLE	Conc^{n}. mg/ml	Solvent	Yield %	O_2 uptake in µl/30min. before and after mixing of test substance. Before	30min.	60min.	90min.	Inhibition of O_2 uptake between 30–60min. per cent.+
Unextracted	10.0	-	-	78	8	6	4	- 92
Extract	0.1	$CHCl_3$	13	77	12	0	0	-100
Residue	20.0	-	13	89	89	130	135	+ 46
Residue	8.0	C_6H_6	4.5	120	76	100	101	- 17
Residue	8.0	Petrol ether	2.0	162	66	45	42	- 72
H_2O/Control	-	-	-	123	159	195	240	+ 58

* The culture received six additions of 1% glucose.

\+ = 0 to - 100 = per cent inhibition
0 to + 100 = per cent normal respiration.

TABLE 2: Inhibition of the respiration of Vibrio cholerae by a three months' old broth* culture of Pseudomonas pyocyanea and its fractions, obtained by extraction with different solvents, Schoental (1941).

Sample	Conc$^{n.}$ mg/ml	Yield %	O_2 uptake in µl /30 min. and after mixing of test substance			Inhibition of O_2 uptake between 30-60min. % +
			Before	30min.	60min.	
Bacterial cells (5 days growth)						
Chloroform Extract	0.5	17	195	27	3	- 98
H_2O/Control	-	-	216	225	313	+ 45
Ground bacterial cells						
Unextracted	4.0	-	69	33	27	- 61
Chloroform Extract	0.5	35.5	84	28	28	- 73
Extracted residue	20.0	-	51	88	105	+115
H_2O/Control	-	-	81	84	75	- 7

+ - see Table 2.

TABLE 3: Inhibition of the respiration of Vibrio cholerae by Pseudomonas pyocyanea dried bacteria, cultures and different fractions obtained from them by extraction with chloroform, abstracted from Schoental (1941).

Substance	Concn. mg/ml	O_2 uptake in µl before and after addition of substance									
		Vibrio cholerae				Inhibition	Staphylococcus aureus				Inhibition
		Before	30min.	60min.	90min.	%	Before	30min.	60min.	90min.	%
Pyocyanin	0.5	70	15	1	3	- 99	74	21	9	4	- 88
	0.1	65	30	22	19	- 66	102	100	76	63	- 25
	0.05	68	42	31	16	- 54	-	-	-	-	-
	0.01	63	60	42	20	- 33	-	-	-	-	-
	0	62	90	97	118	+ 57	-	-	-	-	-
α-oxyphenazine	1.0	241	46	7	3	- 98	-	-	-	-	-
	0.5	-	-	-	-	-	76	22	10	6	- 87
	0.1	279	46	45	12	- 84	62	26	16	15	- 74
	0.05	261	105	66	50	- 75	-	-	-	-	-
	0	279	256	348	314	+ 25	79	111	159	183	+101
Crude III lytic factor	1.0	249	0	0	0	-100	82	21	15	9	- 82
	0.5	213	18	2	1	- 99	84	30	24	15	- 71
	0.1	54	17	18	15	- 67	78	48	64	72	- 18
	0.05	54	22	20	29	- 63	78	96	115	129	+ 47
	0	54	39	81	99	+ 50	89	145	248	252	+178
Purified III lytic factor	0.1	63	10	0	0	-100	-	-	-	-	-
	0.05	63	14	9	10	- 86	-	-	-	-	-

TABLE 4: Inhibition of the respiration of Vibrio cholerae and Staphylococcus aureus by different substances from Pseudomonas pyocyanea, abstracted from Schoental (1941).

Strain of bacteria	Growth during 24hr at 37°C	
	Control	0.1% α-oxyphenazine
C. diphtheriae gravis	+++	-
B. anthracis	+++	-
Neisseria meningitidis	+++	-
V. cholerae	+++	-
V. metchnikovi	+++	-
V. El-Tor	+++	-
Strep. pneumoniae (Type I)	+++	-
Strep. haemolyticus	+++	-
Strep. faecalis	+++	± ?
Staphylococcus aureus	+++	-
Staphylococcus bovine sp.	+++	-
Bact. shigae	+++	-
Bact. sonnei	+++	-
Bact. flexneri V	+++	++
Bact. typhosum H	+++	-
Bact. typhosum O	+++	-
Bact. paratyphosum A	+++	++
Bact. paratyphosum B	+++	+
Bact. coli	+++	+ ?
B. subtilis	+++	-
Proteus vulgaris	+++	-
Chr. prodigiosum	+++	-
Br. melitensis	+++	-
B. megaterium	+++	-
Br. abortus (6 days)	+++	-
Bact. friedlanderi	+++	+
Cl. welchii	+++	-
Cl. septique	+++	-
Cl. tetani	+++	-
Sarcina lutea	+++	+

TABLE 5: Growth inhibition of different bacteria by α- oxyphenazine, Schoental (1941)

Substance	Staphylococcus bovine	Streptococcus haemolyticus	Pneumococcus	V. Cholerae	B. aertrycke
α-oxyphenazine	1: 20,000	1: 20,000	1: 20,000	1: 40,000	1: 2000
Proflavine	1: 50,000	1: 100,000	1: 4,000	1: 100,000	1: 10,000
Purified III	1: 20,000	1: 2,000	1: 5,000	1: 100,000	-

TABLE 6 A: The concentration of some antibacterial substances, inhibiting the growth of bacteria, in serum broth during 24 hours at 37°.
Schoental (1941).

Substance	Concentration mg/ml								
	5.0	1.0	0.5	0.2	0.1	0.075	0.05	0.025	0.
Pyocyanin	..	-	-	-	±	+	++	+++	+++
α -oxyphenazine	..	-	-	++	+++	+++	+++	+++	+++
Bacteriolytic substance III	-	±	+	+++	+++	+++	+++	+++	+++
Proflavine	..	..	-	-	-	-	++	+++	+++

.. = not tested

\- = no growth

\+ = few cells surrounding the explant (migrating ?)

+++ = growth similar to the control.

TABLE 6B: Effect of antibacterial substances on the growth of fibroblasts in tissue culture. Schoental (1941).

strains of Pseudomonas aeruginosa which haemolysed sheep red blood cells were pathogenic. Haemolysis of horse red blood cells was used by Terry (1952) to distinguish Pseudomonas aeruginosa from other Pseudomonas species.

The haemolysins produced by Pseudomonads were used to divide cultures into 2 groups by Liu (1957)

1) type A pathogenic cultures which produced a fast-acting heat-stable haemolysin from Pseudomonas aeruginosa and Pseudomonas pseudomallei, and
2) type B non-pathogenic cultures which produced a slow-acting, heat-labile haemolysin from Pseudomonas aereofaciens and Pseudomonas chloraphis.

The action of these haemolysins on human, ox, sheep and rabbit red blood cells was studied and it was found that the haemolysin of Pseudomonas aeruginosa was inhibited by normal animal sera including that of man and that human red blood cells appeared to be the most sensitive to the action of this haemolysin. The haemolytic titre of the haemolysin from Pseudomonas aeruginosa remained exactly the same after heating for 15 minutes at 100^{o} and its toxicity for mice (i/p injection 0.5ml) was very similar. Heating reduced the titre and toxicity of the other haemolysins. It would appear that haemolysin production cannot be used for species differentiation

but haemolysin production in Pseudomonas aeruginosa is probably of importance in its pathogenicity. Berk (1962) produced haemolysin from Pseudomonas aeruginosa by two different methods (1) extraction of blood agar media after bacterial growth, and 2) saline washing of cellophane plate grown cells) and apart from slight variations in heat stability both preparations appeared to be similar. Complete haemolysis of sheep red blood corpuscles was obtained after exposure to these haemolysins over a wide pH range (pH 5.6-8.0) although the optimal pH was below pH6.

Liu, Abe and Bates (1961) studied the pathogenic role of six fractions obtained from Pseudomonas aeruginosa. These fractions were designated as follows:-

IA. pyocyanin

IB. dialysable substances other than pyocyanin

II. haemolysin

III. extracellular enzymes (for example, lecithinase lipase, protease

IV. slime, and

V. residual bacterial cells.

Table 7 illustrates the procedure used for the separation of the six fractions.

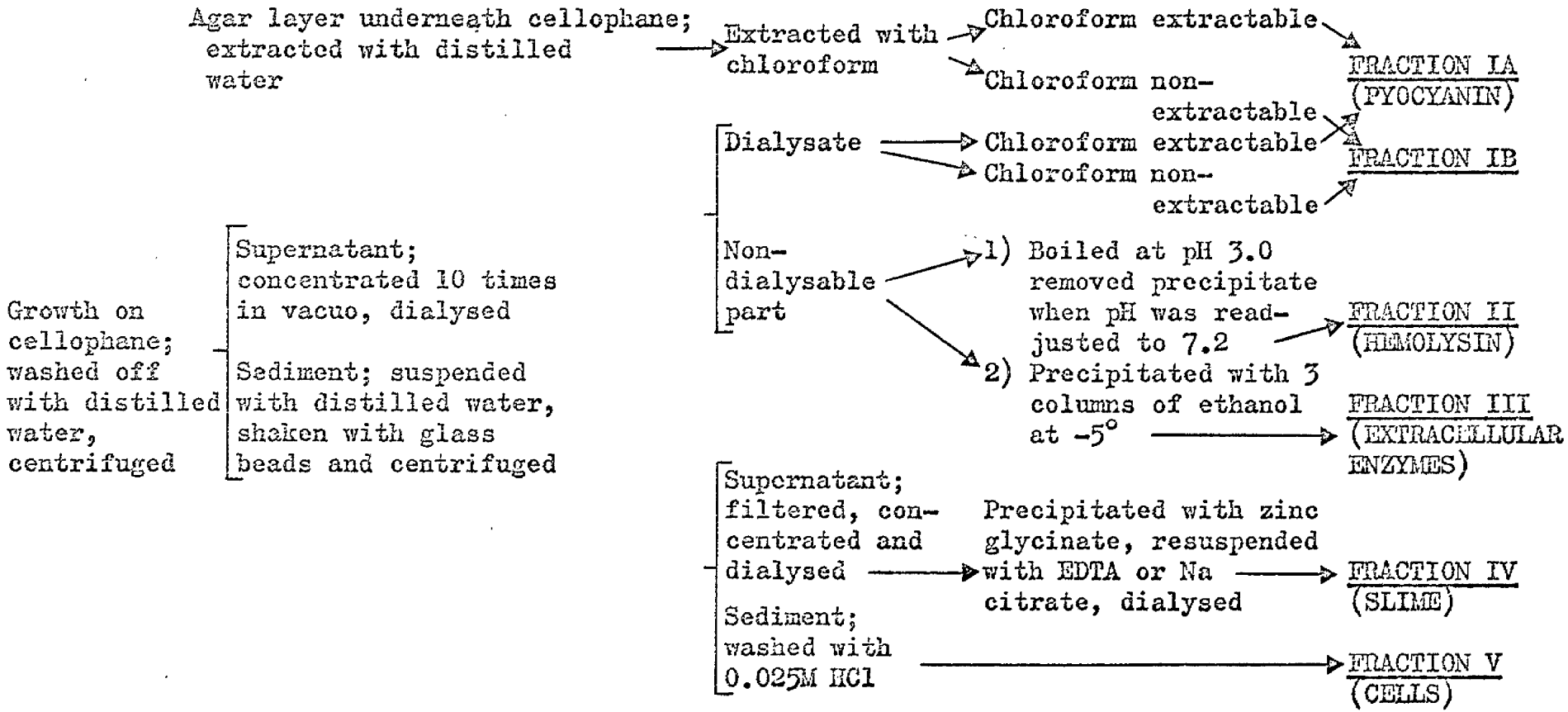


TABLE 7: The technique used for the separation of six fractions of *Pseudomonas aeruginosa*. (Lui, Abe and Bates, 1961)

Fraction IA was toxic to mammalian cells cultured *in vitro* but its toxicity *in vivo* was reported to be negligible.

Fraction IB was necrotic to plant tissue cells. A small pin prick was made on one spot of the vein of a tobacco leaf and a drop of each fraction was placed on this area. This fraction IB was the only one capable of eliciting a necrotic lesion in plant tissue. However it did not produce any change in the skin of the rabbit.

Fractions II and III were toxic to animals and contributed to the lethality of generalised infections as well as being involved in the production of local lesions. Fraction III was found to be antigenic whereas fraction II was not.

Fraction V was relatively non toxic, and the authors suggested that an endotoxic substance within the cell was not significant in the pathogenesis of this species, as has been found in the enteric bacteria.

Fraction IV, a slime. This appeared to be the most important fraction in the pathogenicity of *Pseudomonas aeruginosa*. It was antigenic and also toxic for mice.

The results obtained with these fractions are shown in Tables 8 and 9.

	Fractions[x]	Hemolysin	Lecithinase	Protease	Lipase	Toxicity to mammalian cells.
Pseudomonas aeruginosa, p - A - 1	1 A	0	0	0	0	+*
	1 B	0	0	0	0	+
	II	32	0	0	0	≠
	III	64	16	64	16	++
	IV	0	0	0	0	+
	V	0	0	0	0	0
Pseudomonas aeruginosa. P - A - 7	1 A	0	0	0	0	+
	1 B	0	0	0	0	+
	II	64	0	0	0	++
	III	128	32	128	32	++
	IV	0	0	0	0	+
	V	0	0	0	0	0

* indicates delayed toxicity resulting in the rounding of Hela cells and destruction after several hours.

≠ indicates instantaneous dissolution of the Hela cells.

x - see Table 7.

The haemolysis of Fraction II showed up readily at 20° as well as at 37°, and the activity was not inhibited by boiling, cyanide or EDTA. The haemolysis of Fraction III was observed only at 37° and the activity was inhibited by boiling, cyanide or EDTA.

TABLE 8: In vitro activities of various fractions of Pseudomonas aeruginosa, Liu, Abe and Bates (1961).

Fractions[x]	Dilutions	Mortality of mice in days						
		1	2	3	4	5	6	7
IA	1:1	0/5	0/5	0/5	0/5	0/5	0/5	0/5
IB	1:1	0/5	0/5	0/5	0/5	0/5	0/5	0/5
II	1:1	4/5	4/5	5/5				
	1:2	1/5	1/5	2/5	2/5	3/5	3/5	3/5
	1:4	0/5	0/5	0/5	1/5	1/5	1/5	1/5
III	1:1	5/5						
	1:2	1/5	1/5	2/5	2/5	3/5	3/5	3/5
	1:4	0/5	0/5	0/5	0/5	1/5	1/5	1/5
IV	1:1	5/5						
	1:2	4/5	5/5					
	1:4	1/5	2/5	2/5	2/5	3/5	3/5	3/5
V	1:1	0/5	0/5	0/5	0/5	1/5	1/5	1/5
	1:2	0/5	0/5	0/5	0/5	0/5	0/5	0/5

x - see Table 7.
All fractions were adjusted so that materials from one plate would be dissolved or suspended in 3 ml of saline; each dilution (0.5 ml) was injected intraperitoneally. The denominators indicate the number of mice used; the numerators indicate the number of mice that died.

TABLE 9: In vivo activities of various fractions of strain P - A - 7 expressed as the mortality of mice after intraperitoneal injections, Liu, Abe and Bates (1961)

Pulverer and Korth (1962) stated that Pseudomonas aeruginosa enzymes, such as oxidases, proteases and catalases were of significance in its pathogenicity.

Liu (1966a) attempted to separate the lecithinase and protease of Pseudomonas aeruginosa in order to observe their pathogenic effects on animals. He found that the lecithinase was responsible for oedema and induration of the skin while protease was responsible for the haemorrhagic and necrotic lesions. After intraperitoneal and/or intravenous injection lecithinase primarily caused necrosis of the liver whereas haemorrhagic lesions of the intestines and lungs were produced by protease.

Liu (1966b) also showed the presence of a lethal toxin produced by Pseudomonas aeruginosa in vivo which appeared to be protein in nature. This toxin was produced by extraction of lesions produced in rabbit skin by widespread injections of Pseudomonas aeruginosa. Immunization of animals with this toxin resulted in the production of antibodies that could neutralise the effects of the toxin but could not protect animals from further infection. A similar lethal toxin was

produced in vitro - 1) by growing the organism on the surface of agar plates and 2) by collecting filtrates from vigorously aerated shaking cultures of Pseudomonas aeruginosa in rabbit serum and in broth.
Both preparations, i.e. in vivo and in vitro toxin, killed mice with symptoms of shock, lowered the blood pressure of rabbits and appeared to be distinct from any previously described products of Pseudomonas aeruginosa.

It has been suggested by this author that the lethal effect of Pseudomonas infections involving a large area of skin was probably due to the absorption of the lethal toxin produced in the skin and not due to the effects of the resultant bacteraemia.
However as already stated by Liu (1966a) the formation of skin lesions was mainly due to the production of protease and lecithinase and was not due to this lethal toxin. These toxin preparations have as yet not been investigated biochemically.

Rugstad (1966,1967) described the finding and subsequent purification of a Kininase, a kinin-inactivating enzyme produced by Pseudomonas aeruginosa. The kinins have been mentioned as possible mediators in

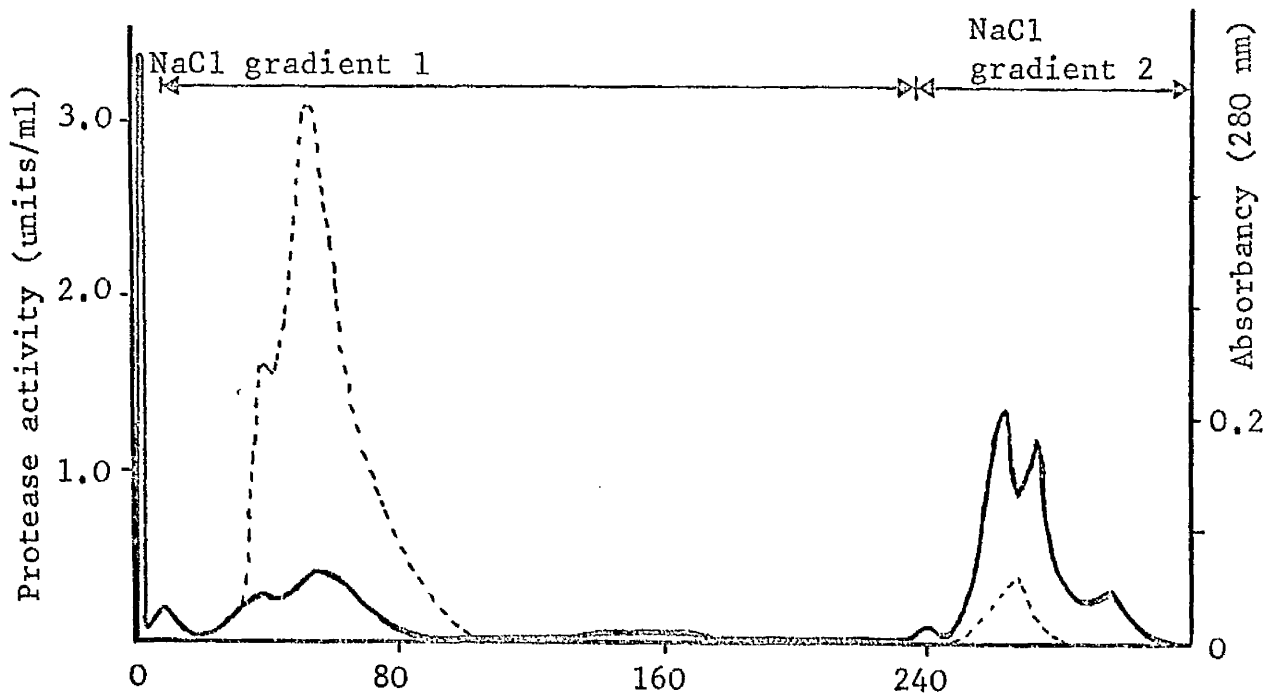
the inflammatory response. This kininase was found to be stable in blood from humans and rats; no loss of activity was detected after *in vitro* incubation with blood at 37° for 24 hours. Thus this kininase could diffuse fairly easily from a focus of infection without being destroyed or inactivated, and in this way, reduce the amount of free kinins in the vicinity of an infected area. It is possible therefore that the ability of *Pseudomonas aeruginosa* to eliminate cellular kinins in this way may be related to its pathogenicity.

Johnson, Morris and Berk (1967) isolated a protease showing elastase activity from a highly proteolytic strain of *Pseudomonas aeruginosa*. Table 10 summarises the purification procedure and Figure 1 shows column chromatography of partially purified agar extracts on DEAE-Sephadex A-50. Protease Peak I exhibited elastase activity. Through a series of chemical and column (DEAE-Sephadex pH8.6) steps the elastase fraction was isolated and purified one hundred fold. This partially purified elastase produced dermonecrosis in rabbits whereas no such dermonecrosis was obtained with the non-proteolytic major protein component eluted prior to the elution of elastase (see figure 1).

Treatment	Volume Initial	Volume Recovery	Protease Units/ml	Protease Total units	Protein mg/ml	Specific Activity	% Recovery	Fold Purification
Precolumn								
Crude supernatant fluid	- ‡	400	8.7	3480	8.8	0.98	-	-
1M Manganese chloride (5% by volume), centrifuge 30-70% Ammonium sulphate	400	190	19.6	3724	2.3	8.5	107	8.5
44-64% acetone dialysis	190	70	48.0	3360	1.65	29.0	91	29.0
DEAE Sephadex Column								
Sample applied	25	-	48.0	1200	1.65	29.0	-	-
Peak I (tubes 50-60)	-	-	32.0*	-	0.032	100.0	-	100.0
Peak II (tubes 254-266)	-	-	0.16*	-	0.165	10.0	-	10.0
Total Protease (Peaks I and II) +	-	-	-	862	-	-	70	-

* Assay following dialysis
\+ Uncorrected for salt influence.
‡ Data are not relevant.

TABLE 10: A summary of the purification of an extra-cellular protease from a 3 day agar culture of Pseudomonas aeruginosa, Johnson, Morris and Berk (1967).



----- Protease activity. ——— protein

NaCl buffer gradient 1. 200 ml 0.02M Tris at pH 8.6 + 200 ml 0.02M Tris with 0.04M NaCl at pH 8.6 in constant volume mixing bottle.

NaCl buffer gradient 2. 0.02M Tris with 0.5M NaCl at pH 8.6 was fed to the mixing bottle containing the remaining volume of buffer system 1.

Figure 1 Column chromatography of partially purified agar extracts on DEAE-Sephadex A-50, from Johnson, Morris & Berk, 1967.

The results obtained in mice toxicity tests with the various fractions are shown in Table II.

Purified protease (LD_{50} equivalent to six protease units) and the non proteolytic fraction caused death within 24-48 hours when a single dose was administered to mice intraperitoneally in concentrations of 50 - 100 µg protein whereas crude preparations exhibited little or no lethality below 600-1000µg protein per mouse. In other words the dose levels were lowered with increasing purity. However it was difficult to state whether mouse lethality was due solely to the protease moiety or whether it was due in part to foreign proteins still present in the purified enzyme fraction.

It would appear that this paper adds little to the already confused knowledge concerning Pseudomonas aeruginosa pathogenicity.

In 1968, Kucera and Lysenko isolated extracellular proteinases of Pseudomonas aeruginosa toxic for larvae of the greater wax moth Galleria mellonella L. Lysenko (1963 a, b, c, d) had shown that Pseudomonas aeruginosa was highly virulent for Galleria larvae; he had also shown that this organism produced a toxic

	Specific activity	Units of activity	µg Protein	Mortality of mice * 1h.	2h.	16h.	48h.
Crude protease from agar extracts	0.79	3.2	4100	0/6	0/6	6/6	
		0.8	1000	0/6		6/6	
		0.4	500	0/6	0/6	2/6	3/6
Partially purified elastase	83.5	16.7	200	6/6			
		8.35	100	0/6	6/6		
		4.17	50	0/6	0/6	0/6	2/6
		2.08	25	0/6	0/6	0/6	0/6
Non-enzymatic protein fraction	-	-	220	0/6	0/6	4/6	6/6
			110	0/6	0/6	4/6	4/6
			55	0/6	0/6	0/6	0/6

* The denominators indicate the number of mice used and the numerators indicate the number of mice that died.
For the proteinase assay, the unit of activity was defined as the hydrolysis of one equivalent milligram of protein, from casein substrate, per millilitre of enzyme per minute under the conditions specified in the assay.

For the elastase assay, the unit of activity was defined as the release of 1 mg of orcein, from orcein-elastin under the conditions specified in the assay.

TABLE 11: Mouse toxicity studies of samples having varying degrees of proteolytic activity and purity, Johnson, Morris and Berk (1967).

substance the toxity of which was closely related to proteolytic activity. The toxic substance was later isolated and identified as a proteinase (Lysenko 1964).

The proteinases were separated from the culture filtrate by means of ammonium sulphate precipitation, chromatography on DEAE cellulose, gel filtration on Sephadex G-75 and preparative disc electrophorsis. Five proteinases were isolated and characterised by pH optima, molecular weights, elastase and collagenase activities.

Lysenko and Kucera (1968) investigated isolated purified proteinases of Pseudbmonas aeruginosa for toxity to Galleria mellonella L; they also observed the changes produced in the haemolymph of Galleria larvae by these proteinases. These authors found the most toxic proteinase fraction exhibited collagenase activity, and also showed specific activity to some proteins of the globulin part of blood proteins of the larvae.

It was found difficult to determine which of these properties accounted for the damage and consequent death of the insects.

From this review of factors responsible for or associated with the pathogenicity of Pseudomonas aeruginosa it is apparent that there are many factors contributing to the pathogenic effect of Pseudomonas aeruginosa and that most authors have widely differing views as to the relative importance of the various factors.

At the present time however it would appear that little is known of the mechanism by which this organism produces its pathogenic effects on the host despite the increased frequency with which infection by this organism is encountered.

Investigation has been initiated from various angles, including a search for

1) an endotoxin moiety similar to that which is of prime importance in enteric bacteria
2) an exotoxin
3) various extracellular enzymes
4) abnormal conditions in the host; for example, the presence of a previous infection or shock condition.

The results of these investigations have not produced a clear cut answer and the overall picture is complex and confused.

It would seem from the literature that a complexity of factors work in conjunction to produce the familiar pathogenic effects. Although it is conceivable that many of these factors could play important roles in the disease process, few of them, with the exception of the haemolysin and protease, have been extensively purified and studied *in vivo*.

Results presented in this thesis throw new light on this problem.

THE PREPARATION AND PROPERTIES OF PYOCYANIN.

Pyocyanin was first crystallised in 1860 by Fordos, who was interested in the blue colour imparted to bandages and dressings by pus. The bandages were soaked in ammonified water and a greenish-blue solution was obtained. This solution was shaken with chloroform, and the chloroform layer was separated and evaporated to dryness in air. The residue was mixed with distilled water which dissolved the blue material and some unknown substances, leaving behind the chloroform-soluble fatty material. The distilled water extract was mixed with chloroform, and the chloroform layer was separated and evaporated to dryness. The pooled dried residues were treated with a few drops of hydrochloric acid; a colour change of blue to red occurred. After subsequent chloroform extraction, the unknown substances were dissolved in the chloroform leaving behind a chloroform insoluble red substance. Treatment of this insoluble red substance with barium carbonate in chloroform liberated some blue material into the chloroform. Evaporation of this chloroform solution yielded a minimal amount of long dark-blue prismatic crystals, which Fordos called Pyocyanin.

This method was crude and yielded minimal amounts of product insufficient for further study. The purity of the final product was doubtful to say the least; Fordos himself admitted that the solution of pyocyanin contained some pus.

In 1887, Wasserzug showed that the formation of pyocyanin can be prevented by several substances, e.g. 5% potassium nitrate, 8% potassium chlorate, 0.5% ammonium tartrate, 5% sodium chloride and by many disinfectants at non-inhibitory concentrations.

Gessard (1890, 1891, 1892) showed that the bluish-green pigment consisted of two different substances - a) pyocyanin, a bluish chloroform-soluble material described by Fordos (1860), and b) fluorescein a greenish-yellow material, insoluble in chloroform but soluble in water.

In 1899, Boland noticed that solutions of pyocyanin in chloroform became yellow after exposure to sunlight. This was believed to be due to oxidation of pyocyanin to pyoxanthose by chlorine released from the chloroform. This may be identical to the substance "pyoxanthose" described by Fordos (1862).

Browning, Cohen and Gulbransen (1920) and Browning, Cohen, Gaunt and Gulbransen (1922) studied the antibacterial properties of acridine and phenazine compounds, in particular a large number of amino- and methylamino- phenazonium compounds. The antibacterial effect against Staphylococcus aureus and Escherichia coli was tested both in peptone water containing 0.35% NaCl and in ox serum heated at 55°. It was found that of the phenazine compounds the methyl chloride derivative was more effective than the hydrochloride derivative against both organisms. The methylchloride derivatives of the amino compounds were particularly effective in serums. However, similar amino compounds in the acridine group were found to be much more effective.

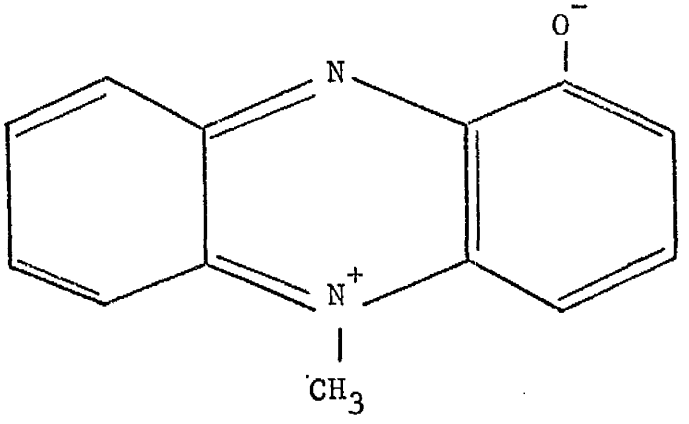
Wrede and Strack (1924) isolated pyocyanin in a crystalline form. Broth cultures were extracted with chloroform and the chloroform extract concentrated to a small volume, in vacuo. Careful addition of petroleum ether to the concentrated chloroform solution yielded dark blue needles. The solvents were removed carefully with a pipette and the dark-blue crystals were dried. A purer preparation of pyocyanin crystals was obtained in the following way. A chloroform extract of a broth

culture of Pseudomonas aeruginosa was evaporated in vacuo; the residue was heated to approximately 50° with approximately 20 ml water until dissolved. The solution was filtered into ice water, and after grinding with a glass rod, abundant crystallisation occurred; the yields of pyocyanin were about 1 mg/litre.

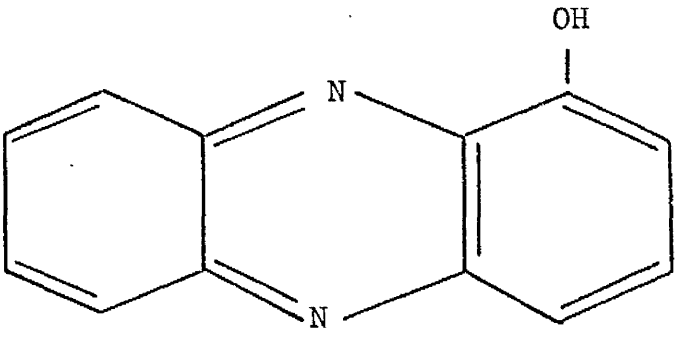
Wrede and Strack (1928) synthesised α-oxyphenazine using 1-methoxyphenazine as starting material (see materials and methods Page 86.)

Wrede and Strack (1929) synthesised pyocyanin, starting from either phenazine, 1-methoxyphenazine or 1-hydroxyphenazine (see Figure 2). Subsequently, Wrede (1930) showed that pyocyanin was a derivative of phenazine. Wrede and Strack (1924) stated that pyocyanin readily dissolved in warm water, chloroform, nitrobenzol, pyridine and phenol. It was fairly resistant to acids and red coloured salts were formed; in the presence of oxygen it was less resistant to alkali treatment and was rapidly broken down.

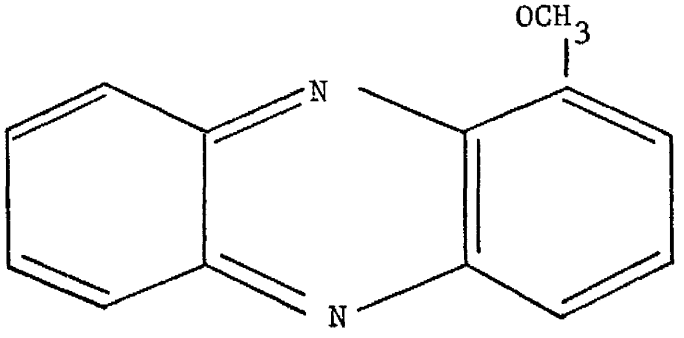
McIlwain (1937) showed that pyocyanin could also be prepared by the photochemical oxidation of phenazine methosulphate.



Pyocyanin



1-Hydroxyphenazine



1-Methoxyphenazine

Figure 2 The structure of pyocyanin, 1-hydroxyphenazine and 1-methoxyphenazine

Wrede and Strack (1929) believed that pyocyanin was a dimer (Figure 3) but Michaelis (1935) stated that it was probably a monomer (Figure 2).

Jensen and Holten (1949) considered that pyocyanin was a resonance hybrid of the mesomeric forms of N-methyl 1-hydroxyphenazine (Figure 4).

Hilleman (1938) found that pyocyanin was easily hydrogenated to the 9, 10 dihydro derivative which was phenolic in character. He showed that condensation of the 9, 10 dihydro derivative of pyocyanin with oxalyl chloride $\begin{pmatrix}COCl\\COCl\end{pmatrix}$ produced a cyclic amido ester which eliminated 9 methyl-1-hydroxy-phenazine as a possible structure for pyocyanin, because the presence of a methyl group at the 9C position would prevent formation of the cyclic amido ester.

Schoental (1941), during an investigation into the antibacterial activity of Pseudomonas aeruginosa, succeeded in isolating a biological preparation of pyocyanin. The method employed was essentially the same as that of Wrede and Strack (1924) with the exception that 48 hour old agar cultures containing 2% glycerine were extracted with chloroform. The yields were much higher (2 4mg/litre) than Wrede and Strack obtained using

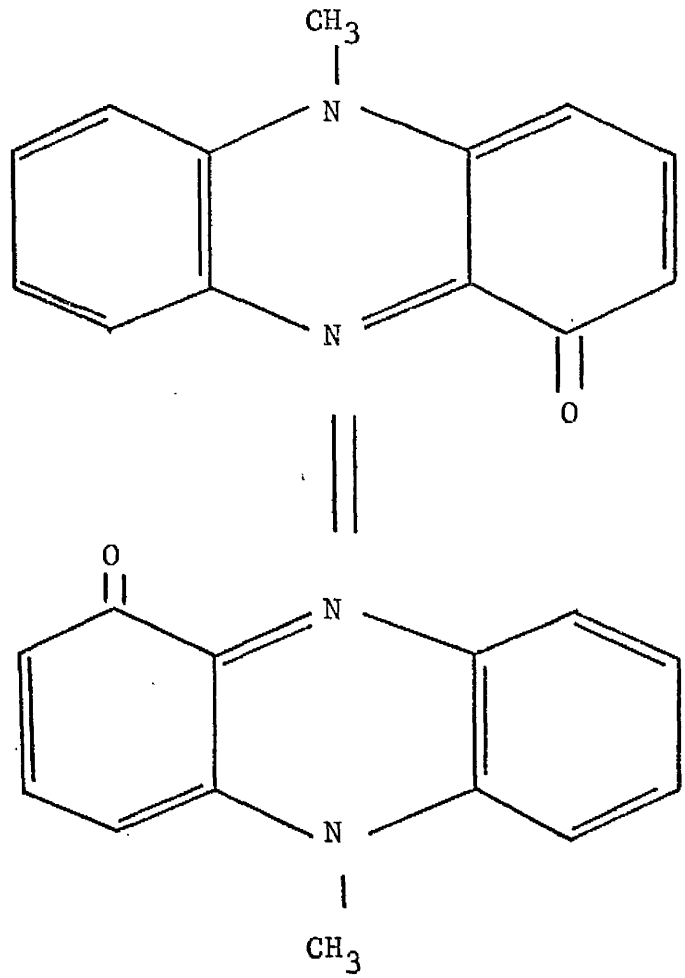


Figure 3 The dimeric structure of pyocyanin

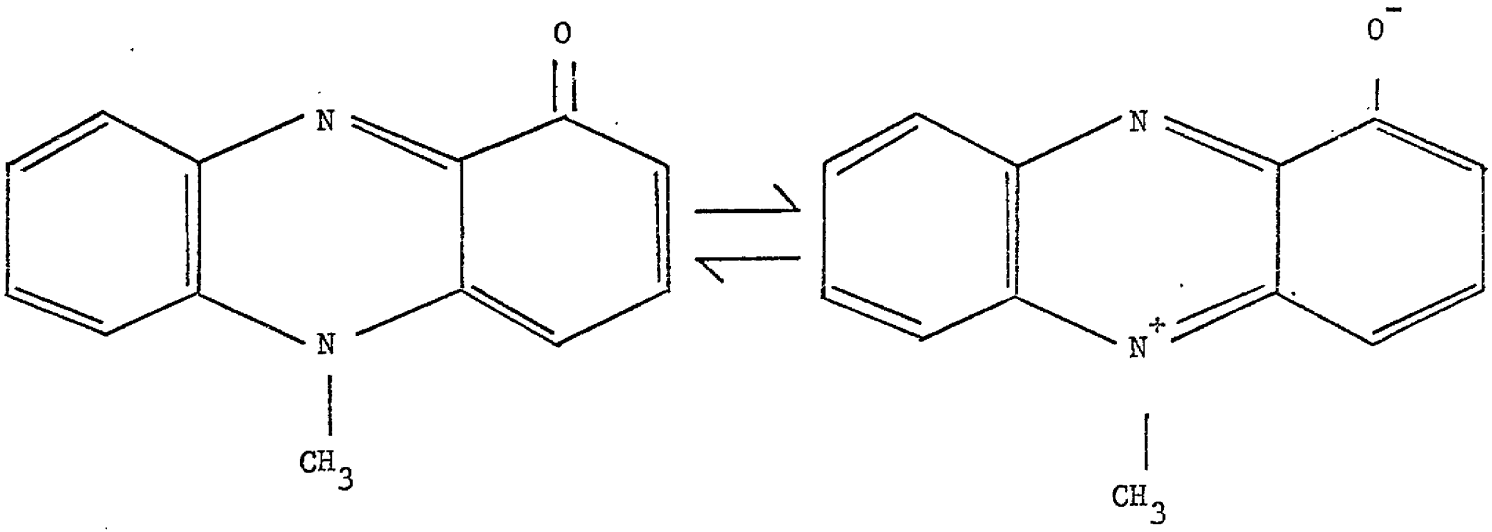


Figure 4 Pyocyanin - a resonance hybrid of the mesomeric forms of N-methyl 1-hydroxyphenazine

broth cultures. However in spite of its high bactericidal properties pyocyanin had little therapeutic value because of the high toxicity and instability.

Schoental (1941) also found that cultures of Pseudomonas aeruginosa left incubating for longer than 8 - 10 days turned brownish-yellow. Chloroform extraction of cultures yielded a dark brown extract, (Table 12). A yellow substance was separated from the extract after column chromatography on aluminium oxide with alkali as eluate. The column fraction was acidified and after purification, yellow prismatic needles were obtained; these were readily soluble in alkali producing a deep red-violet solution. The same substance was obtained if an alkaline solution of pyocyanin was allowed to stand in air, the colour gradually changing from blue to red-violet; acidification and purification yielded yellow crystals. The properties of these crystals suggested that they were identical to α-oxyphenazine, synthesised by Wrede and Strack (1928). The properties noted included the colour reactions of the crystals (yellow at pH 2 - 9; red-violet above pH 10; pink below pH 2),

Brownish-yellow substance

↓ + $CHCl_3$

brown extract

↓ on Al_2O_3 column

yellow substance separated

↓ elute with dilute alkali

red-violet eluate

↓ + HCl

yellow flocculent precipitate

↓ + $CHCl_3$ (evaporated to dryness)

yellow crystals

↓ + alkali

deep red-violet solution.

Pyocyanin (blue)

↓ + Alkali

red-violet substance

↓ + HCl

yellow flocculent precipitate

↓ + $CHCl_3$ (evaporated to dryness)

yellow crystals.

TABLE 12: Preparation of α-oxyphenazine (i.e. 1-hydroxyphenazine) Schoental (1941).

its phenolic character, solubility in alkali, crystalline form, sublimation and melting point not depressed by admixing of pure α-oxyphenazine. Schoental (1941) showed that α-oxyphenazine was not only bactericidal to many bacteria but caused less toxicity and irritation to tissue cells. Stokes, Peck and Woodward (1942) reported that α-oxyphenazine possessed considerable fungistatic action against pathogenic fungi, such as Microsporium gypseum, Trichophyton gypseum and Candida albicans and yeasts, while pyocyanin was considerably less effective. These results may offer some hope for future treatment of surface or localised fungus infections in man. Surrey (1946) repeated successfully the work of Wrede and Straok (1929) and obtained pyocyanin in 58% yield by treatment of 1-hydroxyphenazine with methyl sulphate. (Figure 17). 1-methoxyphenazine was prepared by condensation of pyrogallol monomethyl ether and o-phenylenediamine in the presence of lead dioxide, glacial acetic acid and benzene. A light yellow crystalline solid was obtained. Acid hydrolysis of 1-methoxyphenazine resulted in its demethylation to give 1-hydroxyphenazine, an orange coloured solid.

Alkylation of 1-hydroxyphenazine yielded pyocyanin; 1-hydroxyphenazine was heated with methyl sulphate after which the methosulphate was extracted exhaustively with chloroform and pyocyanin was obtained in the form of dark blue needles (See page 88.).

An improved method of preparation of 1-hydroxyphenazine was outlined by Hegedus (1950). Reduction of 2, 6, 2' trinitrodiphenylamine followed by oxidation with ferric chloride yielded 69% of 1-aminophenazine. Acid hydrolysis of the 1-aminophenazine with aqueous phosphoric acid at 150° yielded 53% 1 hydroxyphenazine. Melo (1949) prepared 1-hydroxyphenazine by condensation of pyrogallol with o-phenylenediamine.

One of the most interesting characteristics of pyocyanin is that it can undergo a reversible reduction to a colourless leuco compound at pH values greater than 6, two electrons being concerned thus -

oxidant + 2 electrons ⟶ Reductant.

At pH values below 6 (i.e. in acid solutions) pyocyanin is red, and its reduction is effected in two stages, each involving one electron; during the first reduction stage a green compound is formed and during the second stage a colourless leuco compound is

formed thus -

oxidant + 1 electron ⟶ semireductant ....(1)

semireductant + 1 electron ⟶ reductant..(2)

See Figure 5 for the changes in structure of pyocyanin which occur during this reaction, (Hewitt 1950).

From the literature it is apparent that a great deal of interest has been evoked by pyocyanin and its derivatives. A large number of chemical syntheses have been attempted with varying degrees of success. However few if any theories as to the biological action of these compounds have been put forward. It would seem that most of the research done on pathogenicity of Pseudomonas aeruginosa and its products has been carried out using biological preparations of pyocyanin or its derivatives and not chemically pure products.

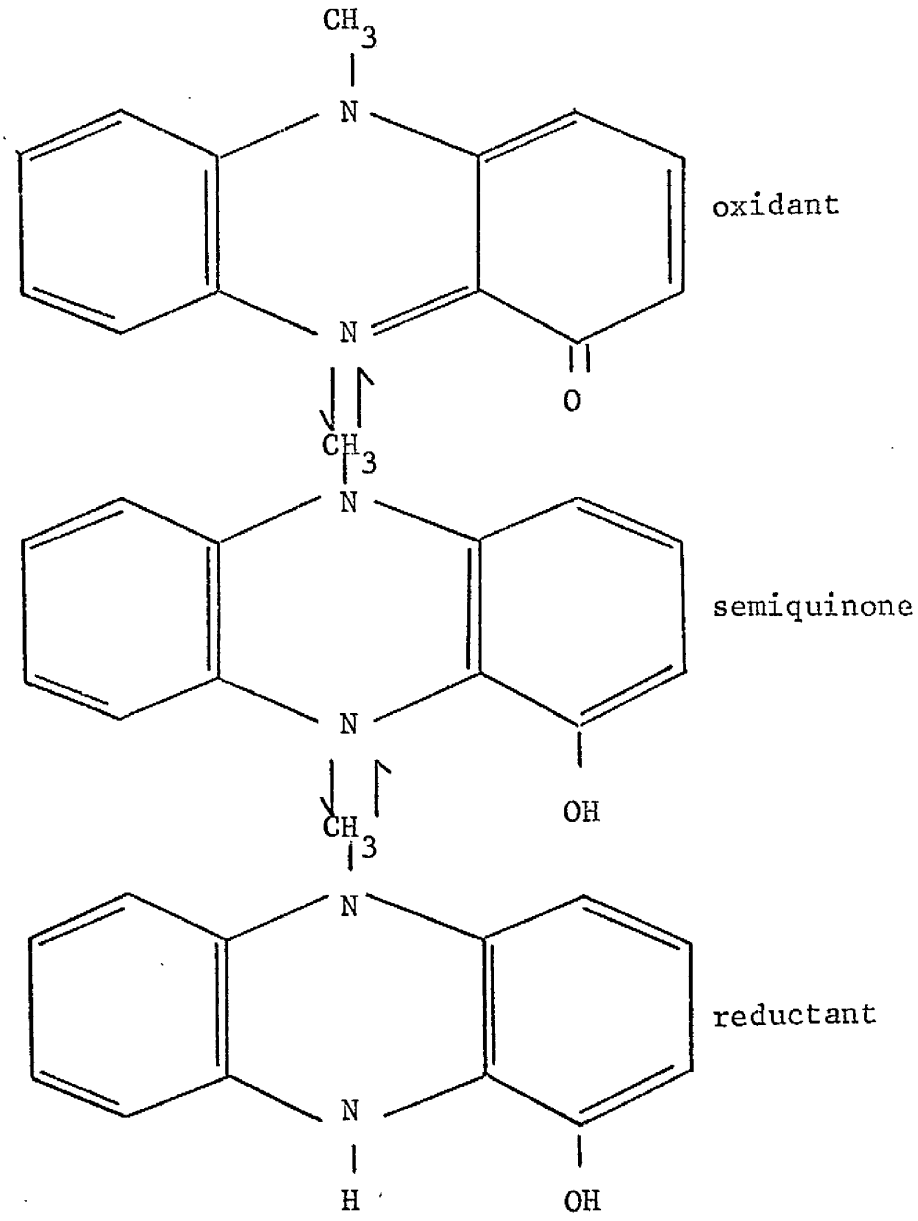


Figure 5 The changes in structure when pyocyanin is reduced

THE MITOCHONDRION

The mitochondrion is an intracellular organelle found in eucaryotic cells (Figure 6). It can be considered as an energy transducing apparatus in which electron flow is coupled to the synthesis of Adenosine triphosphate (ATP) or to the translocation of solutes. If this is so, the origin of the electrons is immaterial as long as their energy is adequate to drive the coupling process (Green 1966). Fernandez-Moran, Oda, Blair and Green (1964) showed that the mitochondrion was composed of two closely interlocking membrane systems -

a) an outer membrane system that enclosed the mitochondrion, and

b) a system of folded inner membranes that radiate into the interior from the periphery - the cristae. The cristae were composed of smooth membrane when they were positively stained and observed under the electron microscope.

If however, they were negatively stained with phosphotungstate they appeared to be covered with spherical sub-units (Hall and Palmer 1969). Stasny and Crane (1964) showed that the inner membrane was made up of these sub-units which can be resolved into a "membrane forming basepiece and a detachable knob".

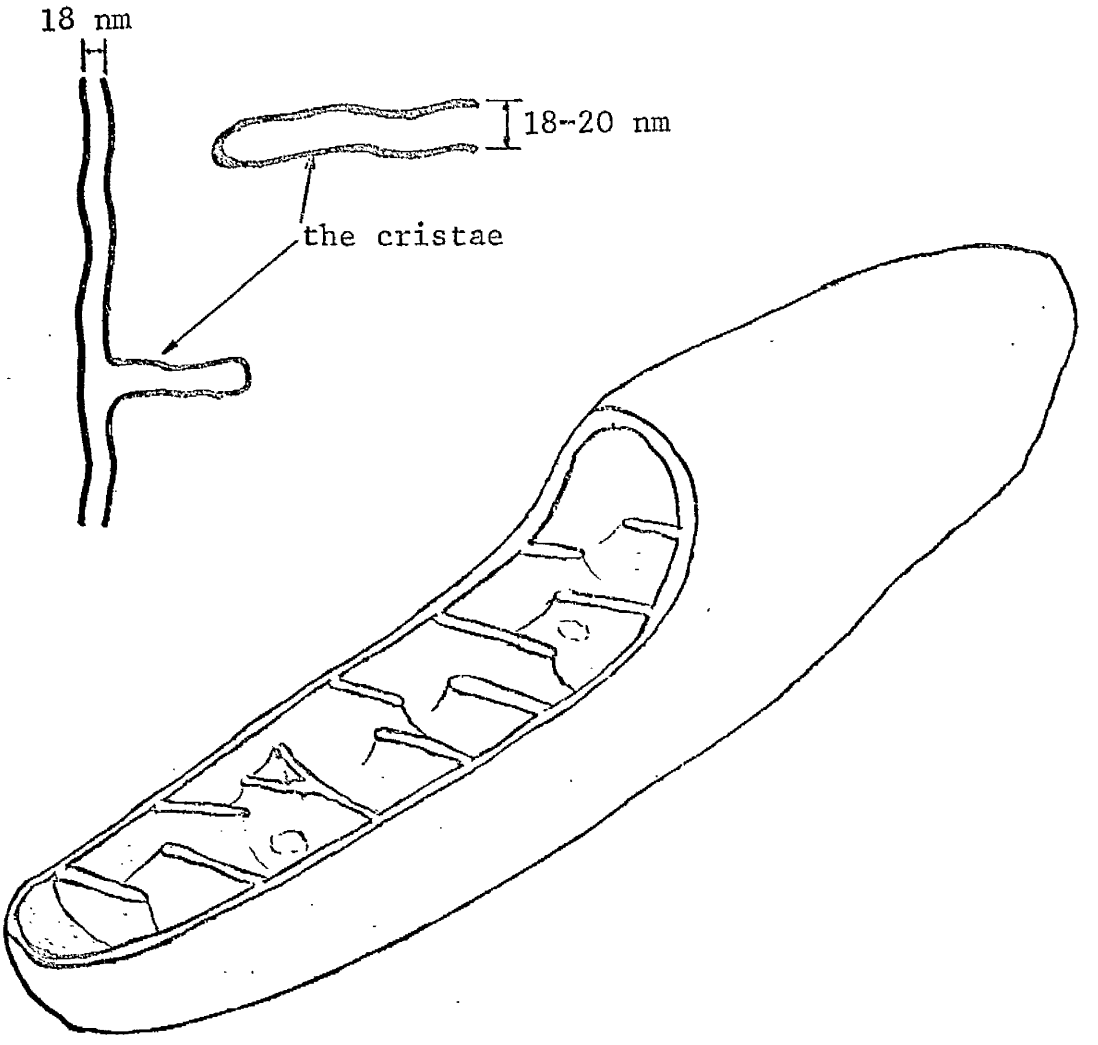


Figure 6 A diagrammatic representation of a liver mitochondrion

The inner membrane contains the complete electron transport chain and all the systems required for the coupling of electron flow to synthesis of ATP or to ion translocation.

Bachman, Allmann and Green (1966) showed that the outer membrane of mitochondrion contained 10 - 20% of the total protein of the mitochondrion while the inner membrane contained the remainder. Both membranes are lipoprotein systems, the lipid content and composition being virtually the same (Fleischer and Fleischer, 1966). The outer membrane contained

a) all the enzymes of the Kreb's citric acid cycle (other than succinic dehydrogenase),

b) the enzymes that carry out β-oxidation of fatty acids, and the synthesis of long chain fatty acids, and

c) enzymes involved in the synthesis of phospholipids.

Earlier Sanadi, Gibson, Ayengar and Jacob (1956) showed that the enzymes catalysing phosphorylation of ADP which accompanies oxidation of α-ketoglutarate (substrate level phosphorylation) and Sekuzu, Jurtshuk and Green (1963) showed that β-hydroxybutyric dehydrogenase were both present in the outer mitochondrial membrane.

Schaitman and Greenawalt (1968) believed that the inner membrane enclosed an apparently structureless matrix containing many soluble enzymes.

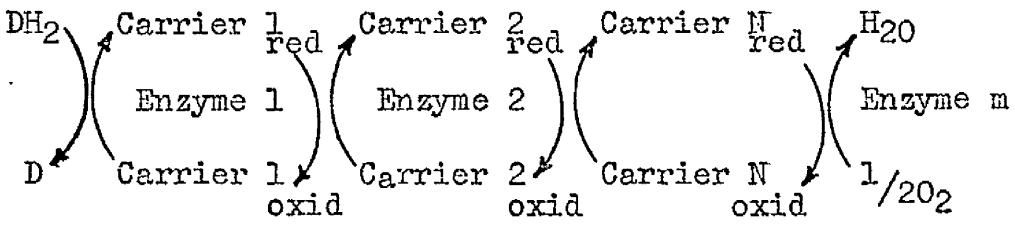
Mitochondria contain a relatively large amount of lipid (more than 90% is phospholipid) as an integral part of the membrane system, and the isolated submitochondrial particles and purified complexes still retain this component. Mitochondrial phospholipids exhibit a particularly high degree of unsaturation which appears to be of functional significance. The function of phospholipid appears to be twofold

1) to stabilize the active conformations of the various proteins of the respiratory chain both individually and collectively and
2) to permit their interaction with other essential components such as ubiquinone (CoQ), factors required for oxidative phosphorylation, and the structural proteins).

Biological oxidations ~~precede~~ proceed as follows:- substrates are dehydrogenated by dehydrogenases, which transfer the reducing equivalents to either a terminal acceptor (usually oxygen) or to another carrier, which

which is in the reduced state. This intermediate carrier is reoxidized either a) by reoxidation by other carriers and eventually by a terminal acceptor or b) by oxidation by a second substrate which is itself reduced in the process.

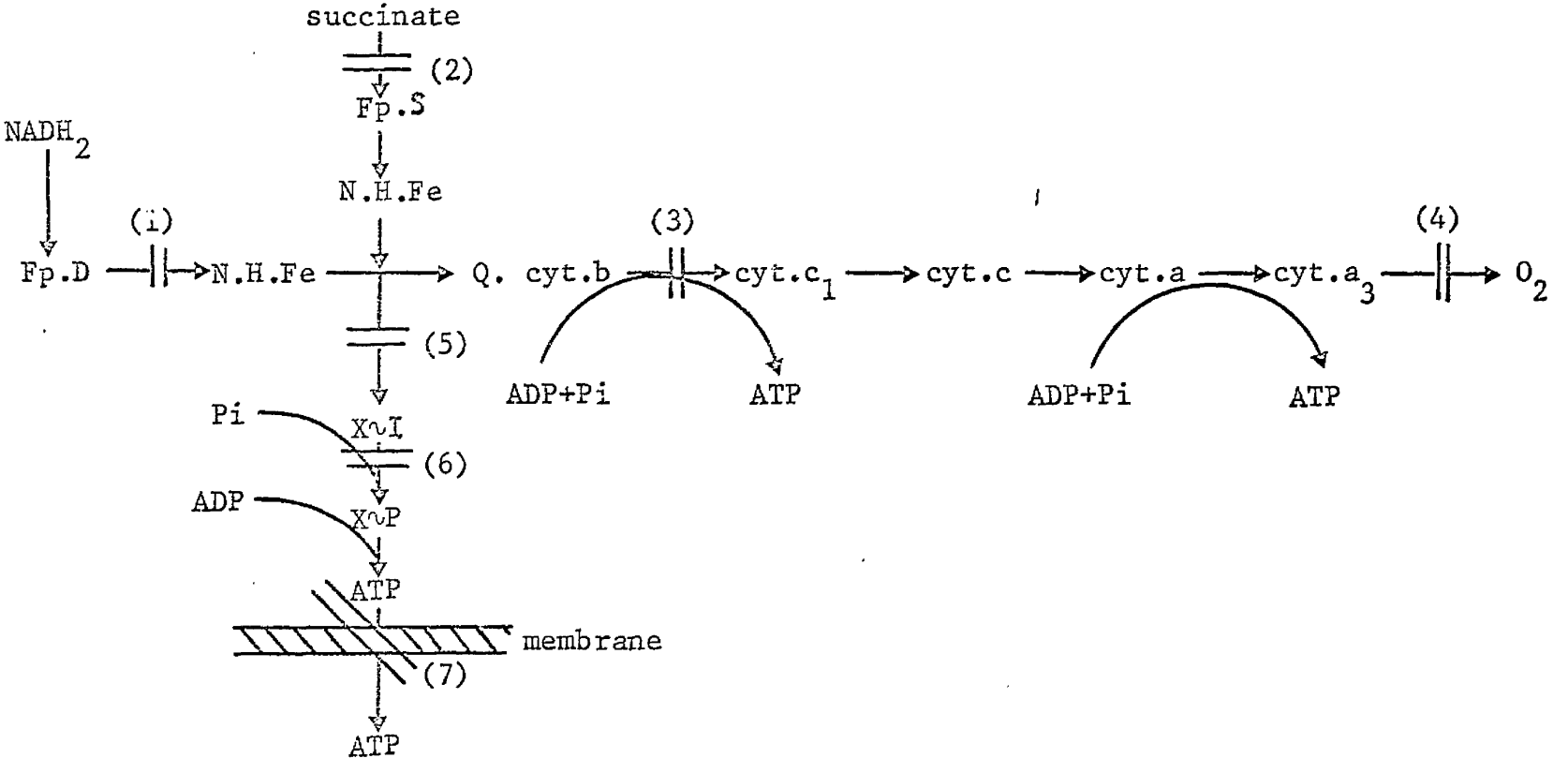
Where several respiratory carriers act in series the resulting chain is called (i) a respiratory chain, or (ii) an electron transfer system. This system intervenes between the substrate with its primary dehydrogenase at one end and the terminal acceptor at the other end.



or $DH_2 \xrightarrow{E1}$ carrier 1 $\xrightarrow{E2}$ carrier 2 $\cdots$ Carrier N $\xrightarrow{EM}$ O_2

In mitochondrial systems these carriers have been identified and include nicotinamide (or pyridine) coenzymes, flavin nucleotide coenzymes, coenzyme Q, cytochromes, non-heme iron and copper (Figure 7).

The elucidation of the electron transport chain has been made possible by the study of isolated mitochondrion or submitochondrial particles. There have been several different approaches to this problem and these have



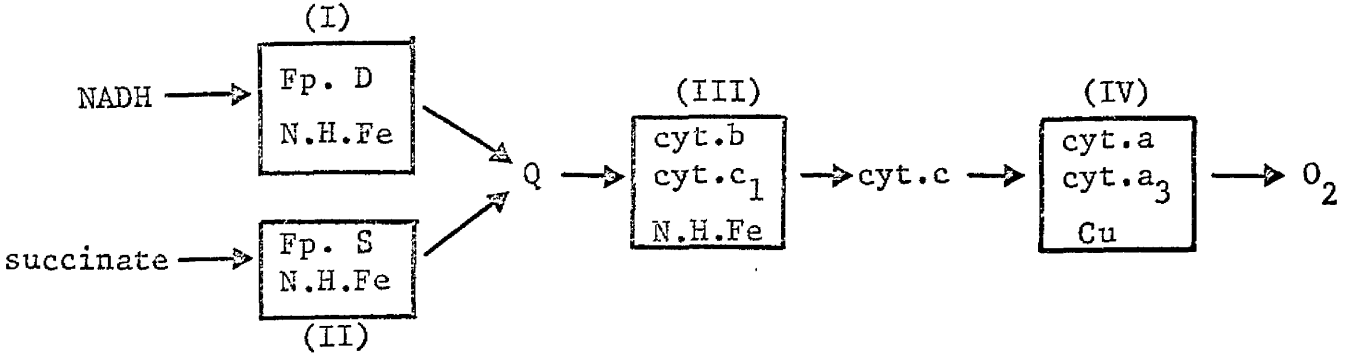
The sites of action of inhibitors are shown. (1) Amytal and rotenone; (2) malonate; (3) antimycin A; (4) CN^-,N_3^-,CO; (5) dinitrophenol; (6) oligomycin; (7) actractylate. Fp.D denotes NADH dehydrogenase; Fp.S, succinic dehydrogenase; N.H.Fe, non-haem iron; Q, ubiquinone.

Figure 7 Diagrammatic representation of the respiratory chain and the phosphorylation reaction

contributed to the present state of information. The most detailed account of the composition of the various components and their order in the chain was obtained by Chance and Williams (1956) who, by the application of spectroscopic techniques measured the content of various suspected carriers (such as NAD, flavoproteins cytochromes) and the kinetics of their reduction and reoxidation in various respiratory particles.

Lehninger (1964) and Green (1966) gave details of their attempts to isolate from mitochondria the enzymes involved in substrate oxidation, oxidative phosphorylation and other respiration-linked activities in order to determine those reactions which are responsible for, and essential to, electron transport.

After removal of the components not directly concerned with electron transport, submitochondrial electron transport particles were fractionated into four complexes which were isolated as relatively pure lipoproteins by Green and Goldberger (1966) and Hatefi (1968). They found that each of the four complexes isolated contained two or more components of the electron transport chain and lipid material. (Figure 8). The latter was



Fp.D denotes NADH dehydrogenase; Fp.S, succinic dehydrogenase; N.H.Fe, non-haem iron. After Green (1966) and Hatefi (1968). I, NADH-Q reductase; II, succinate-Q reductase; III, Q-cytochrome c reductase; IV, cytochrome c oxidase.

Figure 8 Schematic representation of the arrangement of the complexes in the mitochondrial electron transport system

shown to be essential for the activity of the complex (Fleischer, Brierley, Klouwen, and Slauterback 1963; Fleischer, Fleischer and Stoeckenius 1967).

These four complexes, plus the two mobile carriers CoQ and cytochrome C (which become almost completely detached during isolation of the complexes) accounted for all the oxidation reduction reactions of the mitochondrial electron transport system.

Inhibitors such as cyanide and carbon monoxide have been used to disrupt the chain at various points in order to determine the sequence of components and their mode of interaction. (Table 13).

Yamashita and Racker (1968) almost reconstituted the chain between succinate and molecular oxygen from soluble components. However addition of a particulate fraction of cytochrome b was necessary and it appeared that this fraction was the base on which the individual components are organised.

Hall and Palmer (1969) stated that the process of cellular respiration could be divided into three sections -

"a) the rearrangement and oxidation of carbohydrates to produce reduced coenzymes.

b) oxidation of these coenzymes by molecular oxygen

Class	EXAMPLES	Specifically affected complex or reaction
A	HCN, HN_3, CO	IV Cytc (Fe^{+2}) $\rightarrow$ O_2
B	Antimycin A	III Cytb (Fe^{+2}) $\rightarrow$ cyt C_1 (Fe^{+3}) (perhaps at NHI)
	2-Alykl-4 hydroxyquinoline-N-oxide	III Same as above
	3-(2-Methyloctyl) naphthoquinone	III ?
	BAL + O_2	III ?
C	Rotenone	I FMN $\rightarrow$ Q and/or cytb (Fe^{+3})
	Barbiturates (Amytal, seconal)	I Same as above
D	Thenoyltrifluoroacetone	II Peptide-FAD $\rightarrow$ Q and/or Cytb
E	Oligomycin	Inhibit respiration (NADH $\rightarrow$ O_2 or succinate $\rightarrow$ O_2) when coupled to phosphorylation
	Atractylate	Stimulate respiration when rate is limited by phosphorylation or blocked by oligomycin
F	Uncoupling agents (substituted phenols, phenylhydrazones, gramicidin, arsenate, dicoumarol	
G	Arsenite, Cd^{+2}	Inhibit respiration for α-ketoglutarate and pyruvate only by virtue of blocking dihydrolipoyl dehydrogenase. Inhibit phosphorylation, probably at or prior to site 3.

TABLE 13: The different classes of respiratory inhibitors, from Mahler and Cordes (1968).

via the components of the respiratory chain.

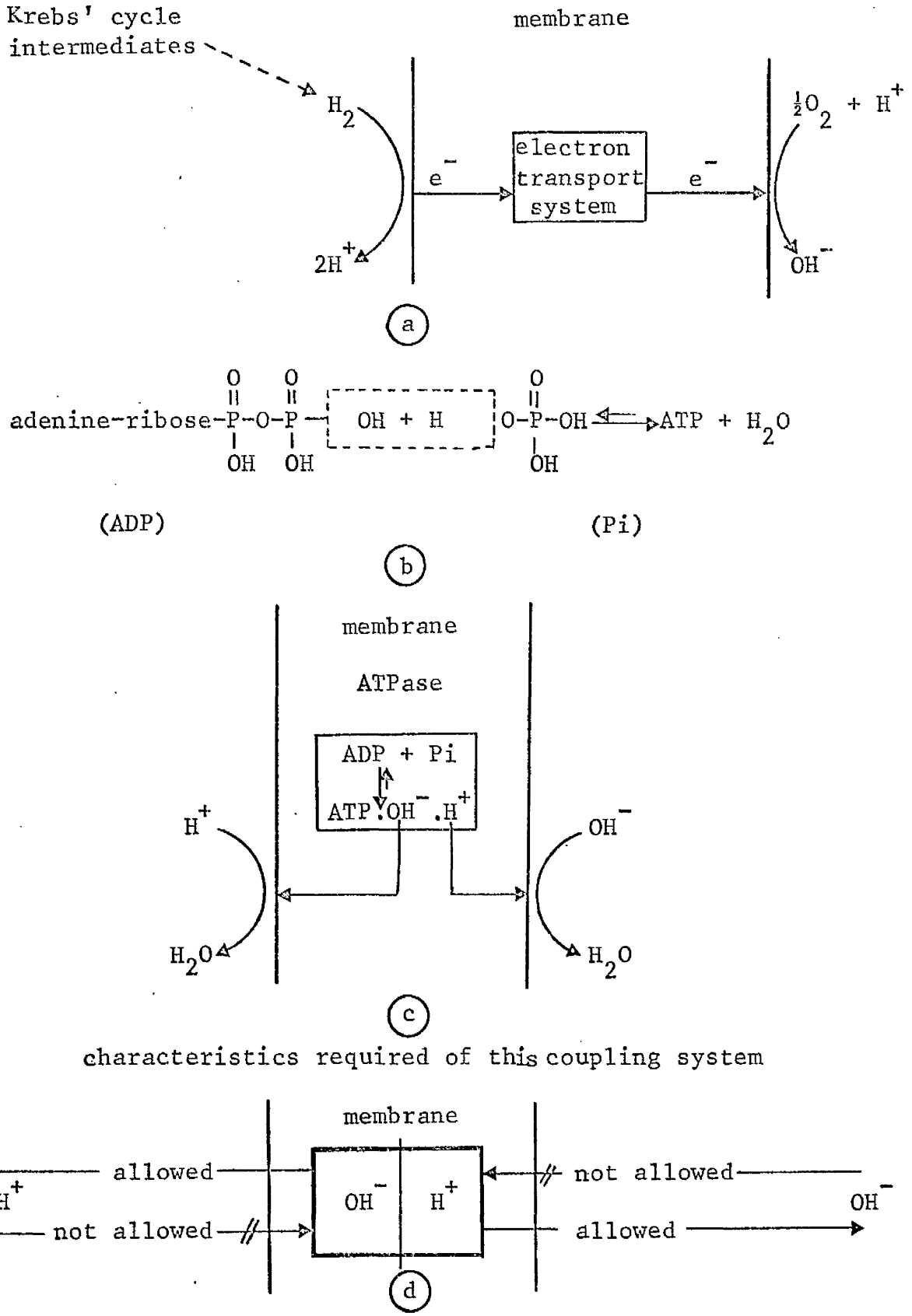
c) the synthesis of ATP during the oxidation of the coenzymes."

However b) and c) are still not completely understood.

Much of the knowledge of ATP formation in the respiratory chain has come from the use of uncoupling agents such as dinitrophenol and oligomycin. A hypothetical model was shown for the formation of ATP involving the intermediates $X \sim I$ and $X \sim P$ - this is known as the "chemical hypothesis". (Figure 7). Since it has proved almost impossible to determine the chemical nature of these intermediates, an alternative "chemiosmotic theory" was developed (Mitchell, 1961, 1966). This theory was based on the suggestion that ATP was synthesised by reversing the activity of the enzyme adenosine triphosphatase (ATP - ase), Figure 9.

$$ATP + H_2O \overset{\text{"ATPase"}}{\rightleftharpoons} ADP + H_3PO_4$$

Hall and Palmer (1969) believed that the "chemiosmotic" theory and "chemical" theory were not so distinct as at first thought, since the chemiosmotic theory results in a proposed mechanism



characteristics required of this coupling system

a, Production of a proton gradient by the electron transport system; the production and removal of H_2O occurring during the synthesis of ATP are shown in b and c; in d the necessary permeability characteristics of the "coupling membrane" are defined.

Figure 9 The coupling of electron transport to phosphorylation as envisaged by the chemiosmotic theory

of X ~ I formation which previously was only described vaguely. One of the most important effects of this theory was to cause research workers to review their ideas on oxidative phosphorylation and to strengthen the suggestion that association of enzymes with structural components of mitochondrial membrane was important for their function.

The position of ubiquinone (CoQ) in the electron transport chain was a problem in the early investigations since it was found to be a mobile component. CoQ is a tetrasubstituted benzoquinone (Figure 10). Wolf, Hoffman, Trenner, Arison, Shunk, Linn, McPherson, Folkers (1958); Crane, Hatefi, Lester and Widmer (1957) Folkers (1961), Green (1961), Morton (1961), Isler, Ruegg, Langemann, Schudell and Rysser (1961). In 1961 it was suggested by Green and Hatefi that coenzyme Q was the link just prior to cytochrome b at which complex I and II meet (Figure 8); it was a respiratory carrier capable of interacting with the flavoproteins of the respiratory chain.

This brief review illustrates the difficulties inherent in mitochondrial research today, since any studies using the isolated mitochondrion and electron transport pathway as experimental models

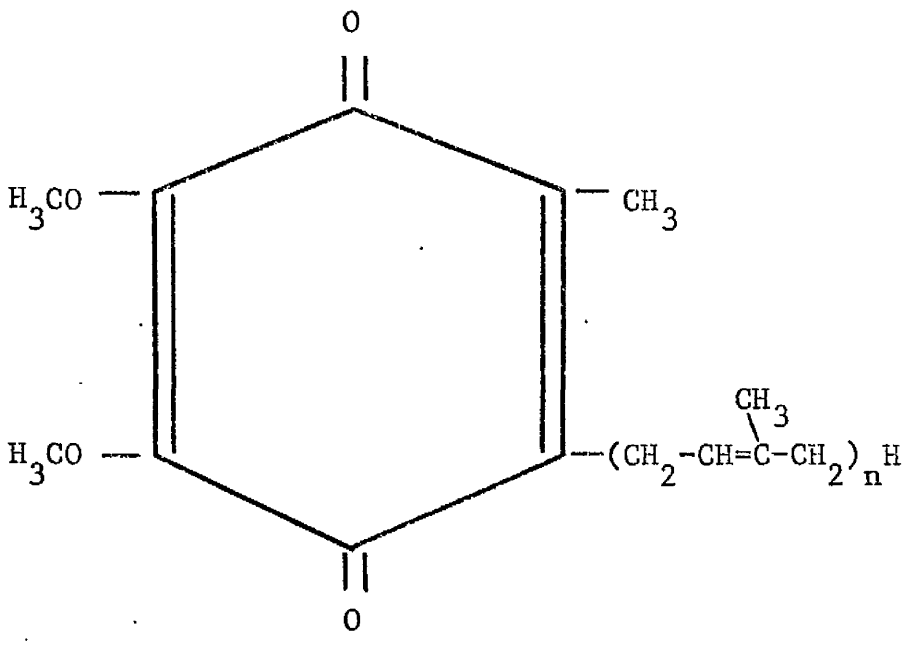


Figure 10 The structure of ubiquinone (Co Q)

is of necessity observing the mitochondrion in an unnatural state. Since little or nothing is known of the effect of the cytoplasm on the mitochondrion or vice versa it is very difficult to draw conclusions when one is studying the isolated mitochondrion.

The position of ubiquinone however, seems to be accepted and studies with ubiquinone have been facilitated by the fact that it is not so closely bound to the inner mitochondrial membrane as the other components.

PHENAZINES in BIOLOGICAL SYSTEMS

It is important to consider the biological action of phenazine compounds since these can be obtained by the degradation of pyocyanin and, in addition, pyocyanin can be synthesised from various phenazine compounds.

Semi-reduced phenazines are important in biological systems in which oxidation reactions are complex. Semi-reduction is an important process by which the formidable energy barrier to ordinary air oxidation via the electron transport system is lowered. Therefore it would be expected that some phenazines, because of their semi-reduced state, could act as transfer agent or electron carriers for oxygen in the metabolic processes of such biological systems. Ideally the phenazine should be one that forms a semi-reduced phenazine in perfect equilibrium with the cell constituents and the air, so that the phenazine is reduced as it aids the cell metabolic processes and is quickly reoxidised by the air. (Figure 11). However, ideal equilibrium is seldom obtained. Dickens and McIlwain (1938) showed that phenazines could act as carriers in the hexosemonophosphate metabolism in certain tissues. Steensholt (1946) found that oxygen consumption by liver tissue increased when placed in a Ringer's phosphate solution containing

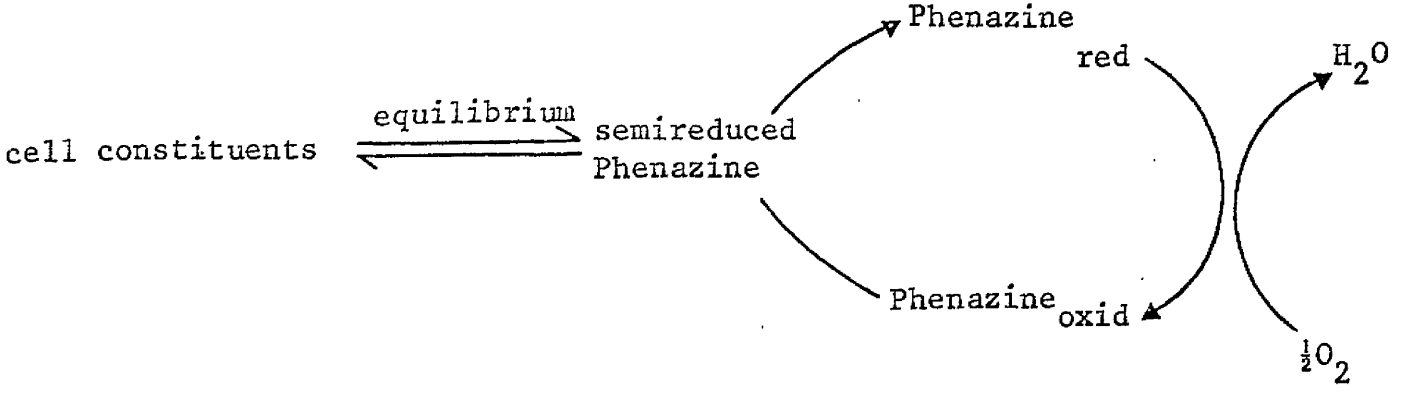


Figure 11 Phenazines in biological systems

1-methoxyphenazine or 1-hydroxyphenazine. Conversely, Phenazine itself was shown to have an antagonistic effect on the metabolism of Lactobacillus casei (Sarett 1946).

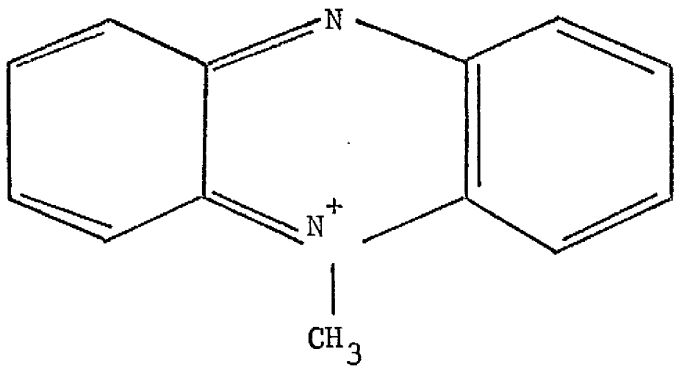
Phenazonium salts appeared to be better bacteriostatic agents than phenazines alone. Pyocyanin, for example, was found to be quite a good bacteriostatic agent but is also toxic. Escherichia coli was not inhibited by any of the phenazine compounds up to 10 mg/100 ml concentration. Staphylococcus aureus was inhibited by a number of phenazine derivatives. There was no relation between α substituted compounds and antibiotic activity. (Schales, Schales and Friedman 1945).

Carr, Vivian and Krantz (1943) showed that the phenazine nucleus was inert pharmacologically but that N-methyl phenazonium methosulphate possessed a marked pressor action, i.e. increased blood pressure by 40 mm in dogs, and a minimal dose of 1.5 mg / 100 gm body-weight caused convulsions in the rat. Pentobarbitol or ether served as an antidote for these convulsions.

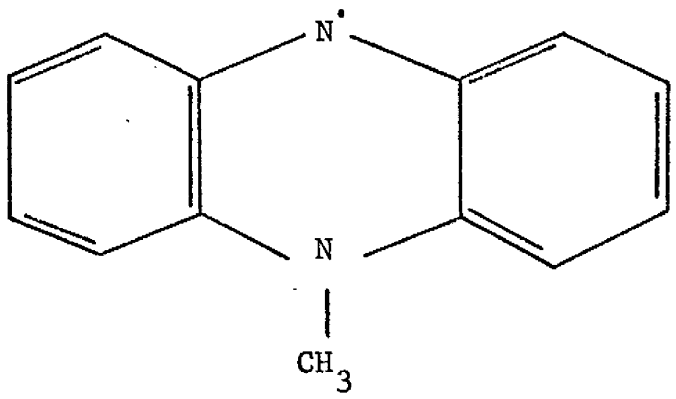
The phenazine-dyes have also been used as artificial electron carriers in order to purify and characterise respiratory chain-linked flavoprotein dehydrogenases. An interesting property of these flavoprotein dehydrogenases is their relatively high selectivity for electron carriers. Theoretical problems encountered in the use of a dye assay entails the need to ascertain that a particular electron carrier reacts directly with the dehydrogenase, in both particle-bound and soluble form, rather than by way of another catalyst. The assay must also measure the complete or almost complete turnover rate of the dehydrogenase, that is, the reaction rate of the dehydrogenase with the dye must equal, or almost equal, the rate of the substrate-flavoprotein reaction, otherwise misleading results are obtained. Rarely is this requirement met. However, Singer and Kearney (1963) showed that phenazine methosulphate was probably measuring the full activity of the dehydrogenase since the same turnover number was measured for succinate-dehydrogenase with coenzyme Q_{10} and phenazine methosulphate, while other assays measured, showed considerably lower turnover numbers.

It seems likely therefore that Phenazine methosulphate and its derivatives due to their rapid and direct action are the acceptors most suited for assay of succinic dehydrogenase from aerobic cells in both soluble and particulate preparations (Figure 12).

An assay of Phenazine methosulphate activity was first introduced by Kearney and Singer (1956). At that time a manometric assay using mitochondria and a fixed concentration of phenazine methosulphate was used. However, Massey and Singer (1957 a and b) and Giuditta and Singer (1958) soon recognised that the apparent K_m for the dye may vary widely due to a) the source of the enzyme, b) the method of isolation and c) the degree of purification. Therefore, the true activity of a given sample must be based on V_{max} with respect to the dye. Redfearn and Dixon (1961) and Arrigoni and Singer (1962) found that in the manometric assay, the reoxidation of the leuco dye may eventually limit the rate of reduction. This limitation on the manometric use of phenazine methosulphate may be overcome by substituting oxygen for air or by lowering the temperature. Arrigoni and Singer (1962) suggested an adaptation of the assay



oxidised



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reduced (coloured) dye which can be oxidised and polymerised to other products.

N-Methylphenazinium methosulphate (phenazine methosulphate,P.M.S. This dye is useful for measurement of oxygen uptake; reoxidation of the reduced dye can be linked to cytochrome c reduction; it interacts directly with pyridinoproteins without intervention of the flavoproteins.

Figure 12 The structure of Phenazine methosulphate and its action on the electron transport chain, Mahler and Cordes, 1967

method, namely spectrophotometric measurements with cytochrome C as terminal oxidant which removed the limitations. Another problem encountered in such assays was the fact that intact mitochondria showed a strong permeability barrier to N-alkylphenazonium dyes and related nitrogenous compounds (Singer and Lusty 1960). Damage to the mitochondrial membrane by freezing and thawing or treatment with calcium ions removed, partly but not entirely, this permeability barrier. It has been recommended by Arrigoni and Singer (1962) that brief digestion with crude Naja naja venom (from Naja naja snake, or hooded cobra, India) or purified phospholipase A will render mitochondrial systems fully permeable to the phenazine methosulphate without damaging the flavoprotein.

Keilin and King (1960) and Giuditta and Singer (1958) investigated the inhibitory effect of cyanide on phenazine methosulphate using heart muscle particles and they confirmed that 50% of the activity towards phenazine methosulphate was abolished by cyanide and since this inactivation is manifest in all particulate preparations of the heart enzyme, including "succinate-co-enzyme Q reductase" Giuditta and Singer proposed that

phenazine methosulphate accepted electrons at two points in the respiratory chain of heart:- 1) the flavoprotein itself (cyanide insensitive reaction) and 2) another site in the immediate vicinity of the dehydrogenase (cyanide sensitive reaction). If the enzyme is extracted from the heart and used in the soluble form for the phenazine methosulphate assay cyanide has no effect on it; that is the cyanide sensitive site was lost by extraction of the enzyme in the soluble form.

Katagiri, Endo, Tada, Kimura, Sunagawa, Niinomi, Sato, Nomura, Hori, Nishiyama, Matu, Shiratori, Matsura and Yoshio (1967 a) synthesised a series of phenazine compounds containing mono-oxide and di-oxide derivatives; fifty-two of these compounds were tested for antimicrobial and cytotoxic activity on Bacillus subtilis, Mycoplasma Spp., Hela cells and Ehrlich Ascites tumour cells and the results were as follows:-

1) the cytotoxic test - growth inhibition rate was calculated using an electric micro-cell counter which proved to be much more accurate and

sensitive than any other previous method. No special correlation was observed between the cytotoxicity and the structure of the compounds tested.

2) the antimicrobial test - compounds having antibacterial activities were more numerous in the α substituted position than in the β substituted position. Eight of the eleven di-oxide compounds tested inhibited the growth of Ehrlich Ascites tumour cells, but results indicated that phenazine derivatives are ineffective against mycoplasmas. (Katagiri, Endo, Tada and Nikaido 1966).

The antitumour activity of these phenazine di-N-oxides was also tested. (Katagiri, Endo, Tada, Nikaido 1967 b). Most of them prolonged the survival time of mice with an Ehrlich ascites tumour. Comparison was made of the antitumour activity of the parent phenazine compounds, phenazine mono-N-oxide and phenazine di-N-oxide using a series of analogues respectively. All the parent compounds and almost all the phenazine mono-N-oxides showed no antitumour activity with doses in mice of 50 mg/kg/day. However several analogues of phenazine di-N-oxides showed the

antitumour effect after injecting the same doses. No significant difference in cytotoxicity against Hela cells was observed with any of the phenazine analogues. It was postulated that the antitumour effect of phenazine di-N-oxides was correlated with their ability to inhibit DNA synthesis, since these compounds were able to induce the λ phage from lysogenic E. coli K12 (λ).

PSEUDOMONAS RESPIRATORY SYSTEMS

Horio (1958) and Higashi (1960) extracted and partially purified the five respiratory components from Pseudomonas aeruginosa :-

(i) two cytochromes

(ii) a Pseudomonas "blue protein"

(iii) a Cytochrome oxidase

(iv) a Pseudomonas hydroquinone oxidase.

Horio (1958) and Horio, Higashi, Matsubara, Kusai, Nakai and Okunuki (1958) concluded that, in the electron transfer system of Pseudomonas, cytochrome C-551 and Pseudomonas "blue protein" are functional at a site corresponding to the cytochrome C of animal mitochondria (Figure 7).

Pandya and King (1966) used fractions, from sonically disintegrated cells of Pseudomonas aeruginosa, obtained by differential centrifugation, to determine the distribution of certain respiratory enzymes. In particular a search was made to determine whether the organism contained ubiquinone, menaquinone or both. Enzymes oxidising menaquinone were found and reductases coupling NADH with menaquinone and ubiquinone were present at approximately equal concentrations regardless of which of these quinone types was present.

Cartwright and Smith (1967) and Cartwright and Buswell (1967) have described the properties of vanillate o- demethylase induced in a Pseudomonad (strain T) during growth on ferulate. This enzyme showed interesting specificity with respect to orientation of the substituents in the phenyl nucleus and for optimal activity it required reduced nucleotide and an unstable unidentified cofactor. Their work was extended to examine other induced dealkylases and also to attempt to solve difficulties encountered in the attempts to isolate and identify the unstable cofactor of vanillate O-demethylase.

Cartwright and Buswell (1969) described some properties of cell-free extracts of a soil pseudomonad; these extracts were obtained by ultrasonic disruption of bacteria grown for 15 hours at 25° with forced aeration on 4 - methoxybenzoate and subsequent centrifugation for 15 minutes at 18,000 g to remove intact cells and cellular debris. In addition to other enzymes, a 4 - methoxybenzoate-o- demethylase was induced in the organism during growth on 4 - methoxybenzoate. Their results suggested that the apoenzyme was distinct from and less specific than vanillate-o-demethylase whereas the cofactor

requirements of the two demethylases appeared to be identical. Again the cofactor of 4 - methoxybenzoate-o-demethylase was believed to be unstable. The elimination by demethylation of a one carbon unit was suggestive of tetrahydrofolate and/or cobalamin as cofactors and the apparent lability of the endogenous cofactor was consistent with the properties of the former. However experiments designed to establish the participation of these factors in demethylation were inconclusive.

The cell-free extracts were examined by Warburg manometry for oxidative activity towards a range of methoxy- and hydroxy-benzoates. The enzymic nature of the reactions was shown by lack of oxygen uptake in the absence of extract or when a heat treated (100° for 5 mins.) extract was used. It was found that the rate of 4-methoxy-benzoate oxidation was proportional to the protein concentration. The effect of protein concentration on the oxidation of 4-methoxybenzoate by unsupplemented extract, and extract supplemented with NADPH, is shown in Figure 13.

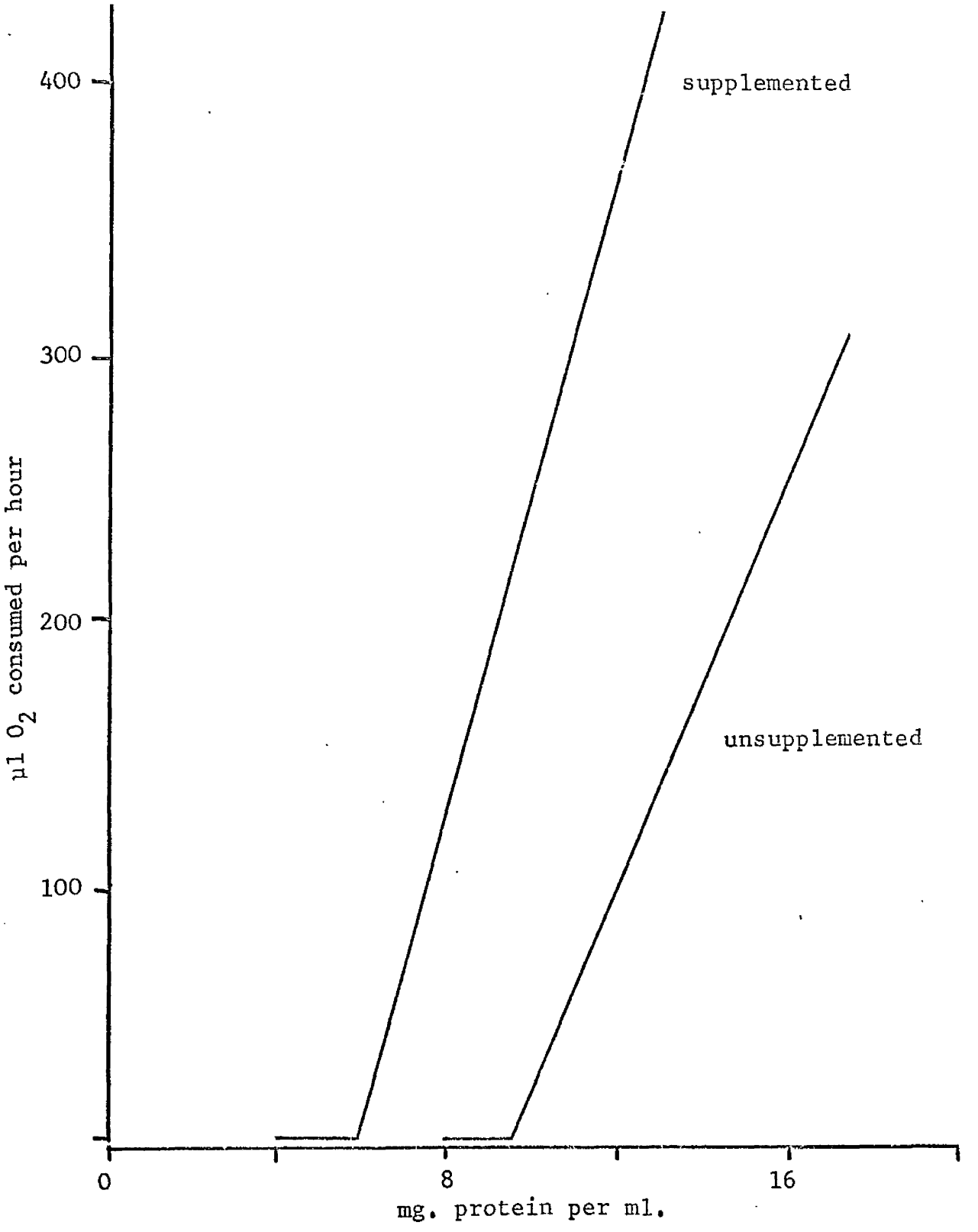


Figure 13 The effect of protein concentration on the oxidation of 4-methoxybenzoate by unsupplemented and supplemented cell-free extracts

PRODUCTS of PSEUDOMONAS AERUGINOSA affecting THE MAMMALIAN RESPIRATORY SYSTEM

The reduction of oxidised cytochrome to reduced cytochrome can be catalysed by a number of dehydrogenases specific for the substrate which is the hydrogen donor. Thus the dehydrogenases for the oxidation of α-glycerophosphate to glyceraldehyde phosphate, or of succinic acid to fumaric acid, or of lactic acid to pyruvic acid or of formic acid to carbon dioxide, all transfer hydrogen to cytochrome. Some of them can make use of acceptors other than cytochrome; for instance methylene blue or pyocyanin are acceptors for glycerophosphate dehydrogenase but riboflavin, the flavoproteins, glutathione or ascorbic acid cannot serve this purpose; methylene blue can also accept hydrogen from succinic, lactic and formic dehydrogenases.

Phenazine methosulphate, which is closely related to pyocyanin, has been shown to be the acceptor most suited for assay of succinic dehydrogenases (Figure 12; and page 54).

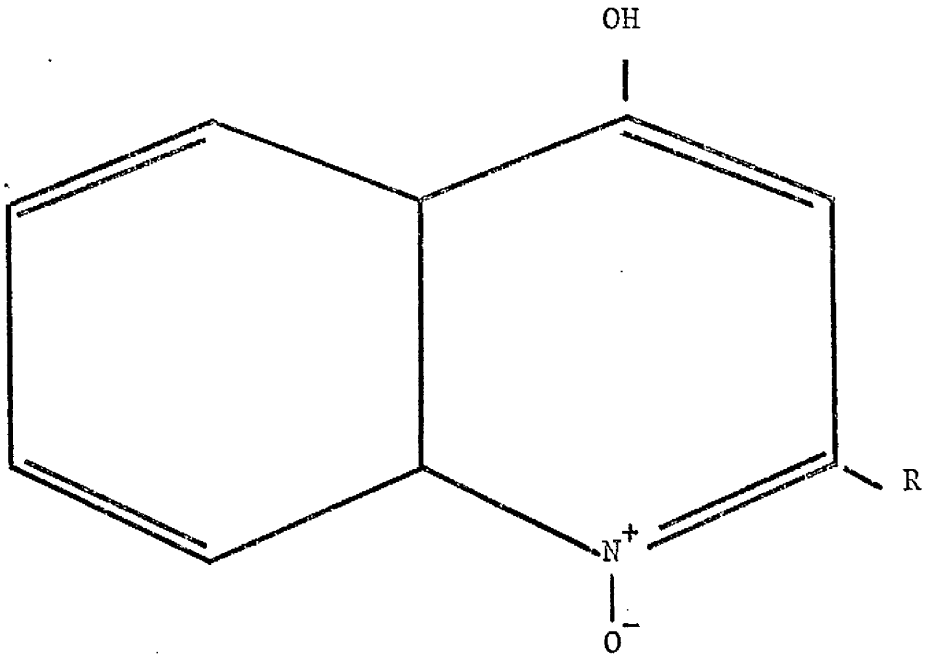
Pyocyanin acted as an electron acceptor (or oxygen carrier) in the formation of phosphoglyceric acid from glucose, and also in the oxidation of α-hydroxyglutaric acid to α-ketoglutaric acid in the presence of a

dehydrogenase occurring in animal tissues. Anderson (1948) stated that pyocyanin acted as an hydrogen carrier (that is electron donor) in the reduction of cytochrome. Keilin and Hartree (1940) suggested that the antibiotic effect of pyocyanin could be due to inhibition of the succino dehydrogenase enzyme.

The 2-alkyl-4-hydroxyquinoline-N-oxides (Figure 14) were isolated from Pseudomonas aeruginosa by Lightbrown and Jackson (1956); these compounds have been used as respiratory inhibitors in experiments investigating the sequence of components of the electron transport chain (Figure 7) and have been shown to be potent inhibitors of complex III, that is Cytb (Fe^{+2}) $\longrightarrow$ $CytC_1$ (Fe^{+3}) (Figure 8).

Cruickshank and Lowbury (1953) found that, by using a modification of Medawar's (1948) skin culture technique, pyocyanin showed toxicity against human skin cells and leucocytes in vitro; it was suggested that this might explain the local pathogenic effects caused by Pseudomonas aeruginosa in burns. However, pyocyanin was shown to be non-toxic when injected into the skin of animals (Jackson, Lowbury and Topley 1951).

In 1960, Berk, Nelson and Pickett showed that heat-killed cells of Pseudomonas aeruginosa inhibited the



where R = CH_3

CH_3CH_2

$CH_3\ (CH_2)_n$

Figure 14 The structure of 2-alkyl-4-hydroxy quinoline N-oxides

endogenous and succino oxidase activities of mouse monocytes but did not affect their cytochrome oxidase activity. Monocytes from mice immunized with heat-killed Pseudomonas aeruginosa showed less inhibition of such activity and it was suggested that cellular immunity was related, in some way, to the sensitivity of mammalian cells.

Nelson and Berk (1960) investigated aspects of cellular immunity and stated that the overall effect of Pseudomonas aeruginosa on mammalian tissue cells was one of direct toxicity. Their studies showed that the organism, living or dead, and its component parts (including Piromen, a chemically defined nucleic acid polysaccharide fraction) had a toxic effect on the respiration of mammalian cells. They proposed an hypothesis to explain the development of Pseudomonas lesions, which involved an alternation between toxic destruction of cells and bacterial multiplication. This hypothesis could also explain why Pseudomonas aeruginosa lesions still persist even after antibiotic treatment since a toxic substance as yet unspecified would be unaffected by the antibiotics, and yet could still cause the destruction of the cells, which lead

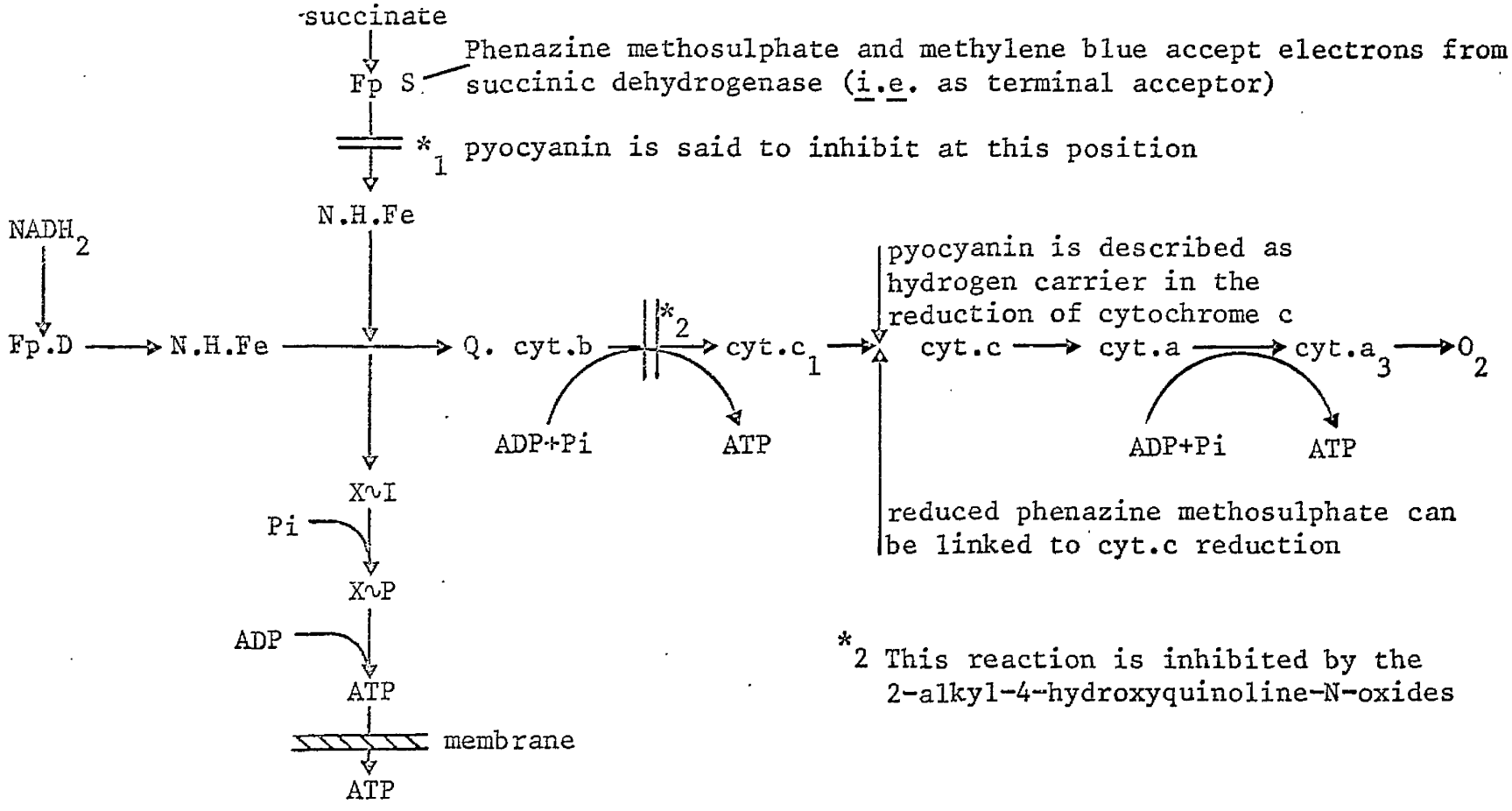
to lesions.

Berk and Nelson 1962, showed that trace amounts of Piromen partially inhibited acid phosphatase and β-glucuronidase activities of mouse monocytes. The action on alkaline phosphatase was not clear cut. All these enzymes were inhibited by a particulate fraction obtained by centrifugation at 35,000 rpm for 30 minutes from disrupted cells of Pseudomonas aeruginosa.

It was thought that these results accounted for the toxicity of intact Pseudomonas aeruginosa cells.

Although numerous lines of research have been followed since 1941, the exact mode of action of pyocyanin and its phenazine derivatives on cellular respiration has not been determined. There have been many widely varying reports as to the role of pyocyanin in electron transport :- it has been described both as an electron donor and acceptor, a terminal oxidase and an inhibitor, all of these functions taking place at different sites along the chain. Nor is the position of phenazine dyes any more clear cut, as shown in Figure 15.

This investigation attempts to solve some of these problems and also to correlate the biochemical and pathogenic action of Pseudomonas aeruginosa.



It has been postulated that a "blue protein" isolated from Pseudomonas aeruginosa is said to act as a terminal oxidase; however the site at which the "blue protein" acts in the respiratory chain has not been determined. Fp.D denotes NADH dehydrogenase; Fp.S, succinic hydrogenase; N.H.Fe, non-haem iron; Q, ubiquinone.

Figure 15 The role of phenazine compounds in the electron transport chain

Experiments were carried out to determine the site of action of pyocyanin and its derivatives in the electron transport chain; an hypothesis relating this to the pathogenic action of Pseudomonas aeruginosa will be presented.

M A T E R I A L S a n d M E T H C D S

STRAINS of ORGANISMS Pseudomonas species from hospital infections and from the National Collection of Type Cultures (Central Public Health Laboratory, Colindale Avenue, London N.W.9.) were used in this study. The Pseudomonas fractions used throughout this investigation were prepared from the following organisms:- Pseudomonas aeruginosa NCTC 6750, Pseudomonas diminuta NCTC 8545, Pseudomonas aeruginosa MSU 14388, Stobhill Hospital, Glasgow, Pseudomonas species MSU 5306, Western Infirmary, Glasgow, Pseudomonas hydrophila NCTC 7810, Pseudomonas species NCTC 7452 achromogenic.

These organisms were identified as Pseudomonas species using biochemical tests, stress being placed on the oxidase test and the breakdown of Hugh and Leifson's medium:-

i) Oxidase Test :- A 1% solution of tetramethylparaphenylene-diamine (British Drug Houses, Poole, Dorset) was freshly prepared and poured on to the surface of an overnight plate culture of the organism. The test was regarded as positive if the reagent turned blue within 15 - 30 seconds.

ii) Hugh and Leifson's Medium was prepared from dehydrated Bacto OF Basal medium (Difco Laboratories, Detroit, Michigan.). 9.4 gm of the granules were suspended in 1000 ml. cold distilled water and boiled to dissolve completely. The medium was autoclaved for 15 min. at 15lb/in.^{2}. A sterile solution of glucose was added to this medium to give a final concentration of 1% glucose.

Bacto Tryptone	2gm
NaCl	5gm
Dipotassium Phosphate	0.3gm
Bacto-Brom Thymol Blue	0.08g
Bacto-Agar	2g

Three 6 x $^5/_8$" test tubes of semi-solid Hugh and Leifson's medium were each stab inoculated with the organism; one tube was aerobically incubated at 37°C, the second tube was anaerobically incubated at 37°C, and in the third tube the surface of the medium was covered with a one inch layer of sterile paraffin oil and aerobically incubated at 37°C. Breakdown of the glucose during growth of the organism was revealed by a change in the colour of the indicator, bromothymol blue, from blue to greenish-yellow. In this way it was possible to determine whether glucose was broken down oxidatively or fermentatively.

PREPARATION of FRACTIONS from PSEUDOMONAS CULTURES.

I. Sloppy agar cultures

Media:- i) Sloppy agar standard medium

2 gm blood agar base (Oxoid No. 2)

5 gm nutrient broth (Oxoid No. 2, Oxoid Limited, London S.E.1)

200 mls distilled water.

ii) Sloppy agar King, Ward and Raney A (K.W.& R. A)

Peptone (Oxoid)	20 gm
Glycerol	10 gm
K_2So_4 (anhydrous)	10 gm
$MgCl_2$ (anhydrous)	1.4 gm
Water (distilled)	1000 ml.
Difco bactoagar	5 gm/ litre

This medium was used for the production of pyocyanin.

iii) Sloppy agar King, Ward and Raney B (K.W.& R. B)

Proteose peptone	20 gm
Glycerol	10 gm
K_2HPO_4	1.5 gm
$MgSO_4.7\ H_2O$	1.5 gm
Water (distilled)	1000 ml
Difco bactoagar	5 gm/ litre

This medium was used for the production of fluorescein.

The pH of these media was adjusted to 7.2 after the addition of agar. The media were autoclaved at 15lbs/in^2 for 15 minutes.

The organism was plated out on a solid agar plate which was incubated overnight at 37°C. The growth was harvested in 5 ml of the appropriate liquid medium (i, ii, or iii above); 0.5-1.0 ml of this emulsion was pipetted on to semi-solid sloppy agar and gently mixed. The plates were placed in sealed tins, packed with tissue paper. This procedure prevented the plates from drying out especially when incubated for periods longer than 24 hours. After incubation at 37°C for 24, 48 or 72 hours, the contents of each plate were frozen at -20° and subsequently allowed to thaw at room temperature. The extruded fluid was collected and centrifuged at 10,000 rpm for 10 minutes at 4° to remove the bacterial cells. The supernatant fluids were distributed in 20 ml amounts in universal containers and stored at -20°C until required.

Control supernatant fluids were prepared from uninoculated sloppy agar plates.

Fractions:-

PF 1 - supernatant fluid from standard sloppy agar

PF 2 - supernatant fluid from K.W. & R. A sloppy agar

PF 3 - supernatant fluid from K.W. & R. B sloppy agar

Control fluids corresponding to these fractions were

PF1C - from standard sloppy agar

PF2C - from K.W. & R. A sloppy agar

PF3C - from K.W. & R. B sloppy agar

II. Static liquid cultures

Media:- i) Nutrient broth Oxoid No. 2

Lab lemco Beef Extract	10 gm
Peptone (Oxoid L. 37)	10 gm
NaCl	5 gm

25 gm of the dehydrated medium was dissolved in 1 litre distilled water, mixed well and autoclaved at 15 lb/in^2 for 15 mins.

ii) King, Ward and Raney A liquid medium (K.W. & R. A)

iii) King, Ward and Raney B liquid medium (K.W. & R. B)

The organism was plated out on a nutrient agar plate and incubated overnight at 37°C. The growth was harvested in 5 ml of the appropriate liquid medium. This emulsion (0.5 ml) was added to 50 ml of either nutrient broth or liquid K.W. & R. A or liquid K.W. & R. B in a 250 ml conical flask. Cultures were incubated at 37°C for 18, 52, 76 and 120 hours. The cultures were centrifuged at 10,000rpm.

for 10 minutes at 4° to remove the bacterial cells; the supernatant fluids were retained and stored at -20° until required.

Control supernatant fluids were prepared from uninoculated media.

Fractions

PF 4 - supernatant fluid from nutrient broth

PF 5 - supernatant fluid from liquid K.W. & R. A

PF 6 - supernatant fluid from liquid K.W. & R. B

Control fluids corresponding to these fractions were

PF 4C - from nutrient broth

PF 5C - from liquid K.W. & R. A

PF 6C - from liquid K.W. & R. B

III. Shaking liquid cultures.

This method differed from that described in II above in that the flasks were shaken for 24 or 48 hours in order to increase the aeration of the cultures.

Fractions

PF 7 - supernatant fluid from nutrient broth

PF 8 - supernatant fluid from liquid K.W. & R. A

PF 9 - supernatant fluid from liquid K.W. & R. B

Control fluids corresponding to these fractions were

PF 7C - from nutrient broth

PF 8C - from liquid K.W. & R. A

PF 9C - from liquid K.W. & R. B

IV. Disintegrated Organisms

Organisms were grown in shaking liquid cultures (Method III). After centrifugation of the culture the bacterial pellet was resuspended in 50 ml of the appropriate liquid medium. Samples (4 ml) of this suspension were disintegrated in an MSE Mullard 60W Ultrasonic Disintegrator for 10 - 15 minutes. Carbol fuchsin staining after 5 minutes showed many broken cells and few whole cells (approximately 70% disintegration); after 15 minutes no whole cells were found. The disintegrate was centrifuged at 10,000 rpm for 10 minutes at 4°; the supernatant fluid was retained and stored at -20° until required.

Fractions

PF 10 - supernatant fluid from nutrient broth

PF 11 - supernatant fluid from liquid K.W. & R. A

PF 12 - supernatant fluid from liquid K.W. & R. B

Control fluids for use with these fractions were the same as those prepared in Method III.

PURIFICATION of PSEUDOMONAS FRACTIONS

Methanol Precipitation:- This procedure was carried out using both PF 1 and PF 36 (Page 82). The pH of the Pseudomonas fractions (30 ml) was adjusted to 4.0 with glacial acetic acid. The fraction was cooled to -6° in a freezing bath (A. Gallenkamp Ltd.), an equal volume of cold methanol was added slowly to prevent temperature rise and the mixture was left at -6° overnight.

If there was any precipitation, the mixture was centrifuged at 10,000 rpm for 10 minutes at -6°C; the sediment was resuspended in 3.0 ml sucrose phosphate buffer, pH 7.3 (solution III, see page 97) and tested manometrically for activity.

A sample (2 ml) of the methanol supernatant fluid was removed and the methanol was evaporated off under vacuum using gentle heat; the sediment was resuspended in 2 ml sucrose phosphate buffer, pH 7.3 and tested manometrically for activity.

The volume of methanol was adjusted in the remaining 58 ml of mixture to give Pseudomonas fraction: methanol (1:3 v/v). After leaving overnight at -6°, the mixture was spun at 10,000 rpm for 10 minutes at -6°.

The sediment was resuspended in 3.0 ml sucrose phosphate buffer, pH 7.3 and tested as before. Again, a sample (2.0 ml) of methanol was removed; the methanol was evaporated off as before and the sediment resuspended in 2.0 ml sucrose phosphate buffer pH 7.3 and tested manometrically. This procedure was repeated with Pseudomonas fraction: methanol 1:6 v/v and 1:9 v/v.

Fractions

PF 13 - precipitate from 1 volume methanol

PF 14 - precipitate from 3 volumes methanol

PF 15 - precipitate from 6 volumes methanol

PF 16 - precipitate from 9 volumes methanol

PF 17 - supernatant from 1 volume methanol

PF 18 - supernatant from 3 volumes methanol

PF 19 - supernatant from 6 volumes methanol

PF 20 - supernatant from 9 volumes methanol

PF 21 - precipitate from Sephadex fraction in 1 volume methanol

PF 22 - precipitate from Sephadex fraction in 3 volumes methanol

PF 23 - precipitate from Sephadex fraction in 6 volumes methanol

PF 24 - precipitate from Sephadex fraction in 9 volumes methanol.

PF 25 - supernatant from Sephadex fraction in 1 volume methanol

PF 26 - supernatant from Sephadex fraction in 3 volumes methanol

PF 27 - supernatant from Sephadex fraction in 6 volumes methanol

PF 28 - supernatant from Sephadex fraction in 9 volumes methanol

CHLOROFORM EXTRACTION of SUPERNATANT FLUIDS from PSEUDOMONAS CULTURES.

Equal volumes of PF 1 or PF 2 and dry chloroform (Analar) were shaken vigorously by hand and the two layers allowed to settle in a separating funnel. The chloroform and aqueous layers were collected in separate containers. The aqueous layer was further extracted with an equal volume of chloroform and the two layers separated as before. The chloroform fractions were pooled.

a) Chloroform soluble material

The chloroform was evaporated off in vacuo and the sediment which remained was weighed. The sediment (1 mg from 1 ml of original Pseudomonas fraction) was resuspended in Hendry's phosphate buffer, pH 7.3 (solution III, see page 90) and its

effect on mitochondrial respiration was measured manometrically.

Fractions

PF 29 - chloroform-soluble fraction from PF 1

PF 30 - chloroform-soluble fraction from PF 2

b) Aqueous soluble material

The aqueous fraction was heated *in vacuo* to evaporate off any chloroform which still remained. The effect of this fraction on mitochondrial respiration was measured manometrically.

Fractions

PF 31 - chloroform-insoluble fraction from PF 1

PF 32 - chloroform-insoluble fraction from PF 2

FRACTIONATION of CHLOROFORM EXTRACTS on an ALUMINA COLUMN

Anhydrous alumina (Shandon, γ - Al_2O_3), was mixed with dry chloroform (Analar) to produce a slurry. This was poured into a glass column (2 x 23 cms). A concentrated chloroform extract (2 ml) of *Pseudomonas* culture fluid, PF 2, was placed on the column and the fractions were eluted with chloroform. Three fractions of three different colours were obtained:-

1) yellow, 2) pale green, and 3) blue. All the fractions of the same colour were pooled, the chloroform was evaporated off and the dried sediment resuspended in 2 ml sucrose phosphate buffer, pH 7.3 (solution III, see page 97). The effect of the various fractions on the respiration of mouse liver mitochondria was tested manometrically. In addition, the solubilities of the various fractions in a number of solvents was tested.

The ultraviolet spectra of the Pseudomonas fraction (PF 2) and the column fractions were obtained using the Unicam SP 800 spectrophotometer.

Fractions

PF 33 - yellow
PF 34 - pale green
PF 35 - blue

} fractions from an alumina column.

SEPHADEX GEL FILTRATION of SUPERNATANT FLUID from PSEUDOMONAS CULTURES.

Sephadex is a modified dextran obtained by fermentation of sugar. The linear macromolecules of dextran are cross linked to produce Sephadex, giving a three-dimensional network of polysaccharide chains. These cross linked dextran gels were obtained from

Pharmacia Limited, Uppsala, Sweden; their properties are shown in Table 14.

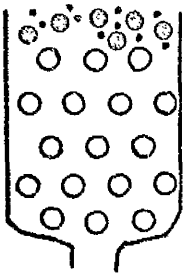
Gel filtration with Sephadex is a chromatographic separation resulting from restricted molecular diffusion through a column or bed of cross linked granules. The liquid imbibed by the gel particles is available as solvent to solute molecules of different sizes to a degree dependent on the porosity of the granules. (Figure 16).

a) Preparation of column: 1) Sephadex G 10; G 15; G 25

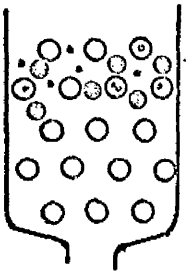
The dry Sephadex powder (20 gm) was suspended in excess 0.05 M Hendry's phosphate buffer, pH 6.45 (Solution I, prepared by adding 65 ml NaH_2PO_4 (26.0 gm/litre solution) to 35 ml Na_2HPO_4 (18.0gm/litre solution)), and stirred. The Sephadex was left to settle out and the buffer was decanted. This procedure was repeated three times to remove the "fines". The gel was mixed with 150 - 200 ml buffer to form a slurry. The slurries were left for several hours at 20° to swell. When complete swelling had occurred, 150 ml of the slurry was poured slowly into a 23 cm column, taking care that no air bubbles were trapped in the mixture. When the column was evenly packed buffer was added and the horizontal upper surface of the gel was covered with a close fitting

TYPE	Approximate Exclusion Limit (mw)	Water regain (g H_2O/g dry gel)	Wet density (g/cm^3)	Particle size (microns)	Bed volume/ ml/g dry gel
Sephadex G - 25	5,000	2.5 ± 0.2	1.13		5
Fine				20-80	
Coarse				100-300	
Sephadex G - 50	10,000	5.0 ± 0.3	1.07		10
Fine				20-80	
Coarse				100-300	
Sephadex G - 75	50,000	7.5 ± 0.5	1.05	40-120	12-15
Sephadex G - 100	100,000	10.0 ± 1.0	1.04	40-120	15-20
Sephadex G - 200	200,000	20.0 ± 2.0	1.02	40-120	30-40

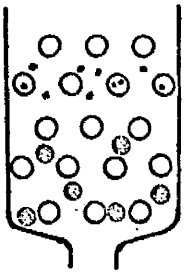
TABLE 14: Different Sephadex gels and their properties.



A solution of low- and high-molecular-weight substances introduced onto the surface of a Sephadex column. The solutes are represented by small and large solid circles, respectively. Open circles represent Sephadex particles.



As the solution travels down the column, small molecules are retarded on entering the pores of the Sephadex gel, while molecules of high molecular weight are confined to the liquid medium exterior to the gel proper.



Elution causes the complete separation of the two solutes. The small molecules, having a longer path to travel (within the gel), are still held in the column when the large ones are eluted. The low-molecular-weight solute is subsequently washed out with more solvent.

Figure 16 The principles of Sephadex gel filtration

filter paper. The buffer was run through the column for 24 hours to equilibrate the gel.

2) Sephadex G 75. In this case, 20 gm of dry Sephadex powder was added to either excess 0.03M Hendry's phosphate buffer, pH 6.45 or to excess 0.05M Hendry's phosphate buffer, pH 6.45. (Solution I). The "fines" were removed as previously described, and the washed Sephadex was mixed with 400 ml. buffer to form a slurry. This slurry was left for 24 hours at 20° to allow the gel to swell. The slurry was poured slowly into a 23 cm column as before.

b) Gel filtration: Immediately prior to charging the column, the excess buffer was pipetted off to within $\frac{1}{4}$inch of the filter paper. The column continued to run until this buffer soaked into the filter paper. The sample (14.5 ml of Pseudomonas fraction PF 1) was added to the equilibrated column and once it had soaked into the filter paper, more buffer was added from a reservoir.

3 ml fractions were collected on an Ultrorac II fraction collector. A series of brown-coloured fractions were eluted first, closely followed by yellow-brown to colourless fractions and a series of blue-coloured fractions from G 10, G 15 and G 25 Sephadex. In G 75 Sephadex, a series of colourless fractions followed by brown-coloured fractions and green fractions was obtained. The effect of these fractions on the

respiration of mitochondria was tested manometrically. The elution pattern of these fractions was read at 280nm.

Fractions

PF 36 - brown coloured fractions (from G 10, G 15 and G 25)

PF 37 - yellow-brown to colourless fractions

PF 38 - blue coloured fractions

PF 39 - colourless fractions (from G 75)

PF 40 - brown coloured fractions

PF 41 - green coloured fractions

THE SYNTHESIS OF PYOCYANIN AND ITS DERIVATIVES

1. Biologically synthesised pyocyanin and its derivatives

Crystallisation of Pyocyanin

The isolation of pyocyanin in the crystalline form was carried out using a modification of the method of Wrede and Strack (1924). 100 mls of Pseudomonas fraction (PF 2) was extracted with 50 ml of dried chloroform (Analar) by shaking overnight at 4°. The chloroform layer was separated from the mixture and evaporated to dryness. The dried sediment was resuspended in 5 ml dried chloroform and 10 - 15 ml dried petroleum spirit were added dropwise until dark blue, needle-like crystals appeared. When no more crystals came out of the chloroform solution, the apple-green solvent layer was gently decanted. The dark blue crystalline mass was dissolved in distilled

water and freeze-dried; a mass of fine crystals were obtained.

Preparation of 1-hydroxyphenazine from pyocyanin:- An alkaline solution of the pyocyanin (obtained above) was allowed to stand overnight; the colour gradually changed from blue to red-violet. On acidification with IN HCl the colour changed to yellow and a fluffy precipitate was obtained. After freeze-drying a large fluffy pinkish-brown precipitate was obtained - the α-oxyphenazine of Schoental (1941).

The effect of crystalline pyocyanin and 1 - hydroxyphenazine (obtained above) on the respiration of mouse liver mitochondria was compared with that of samples of crystalline pyocyanin and 1-hydroxyphenazine kindly provided by Dr R. Schoental.

2. Chemically synthesised pyocyanin and its derivatives

Pyrogallol monomethyl ether, 1 - methoxyphenazine, 1 - hydroxyphenazine and pyocyanin were chemically synthesised by a modification of the method of Wrede and Strack (1929) described by Surrey (1946) (Figure 17).

Preparation of Pyrogallol 1 - Monomethyl ether.

The apparatus consisted of a 1 litre three-necked flask fitted with a gas inlet tube extending about 3 cm into the flask and connected to the flask through a

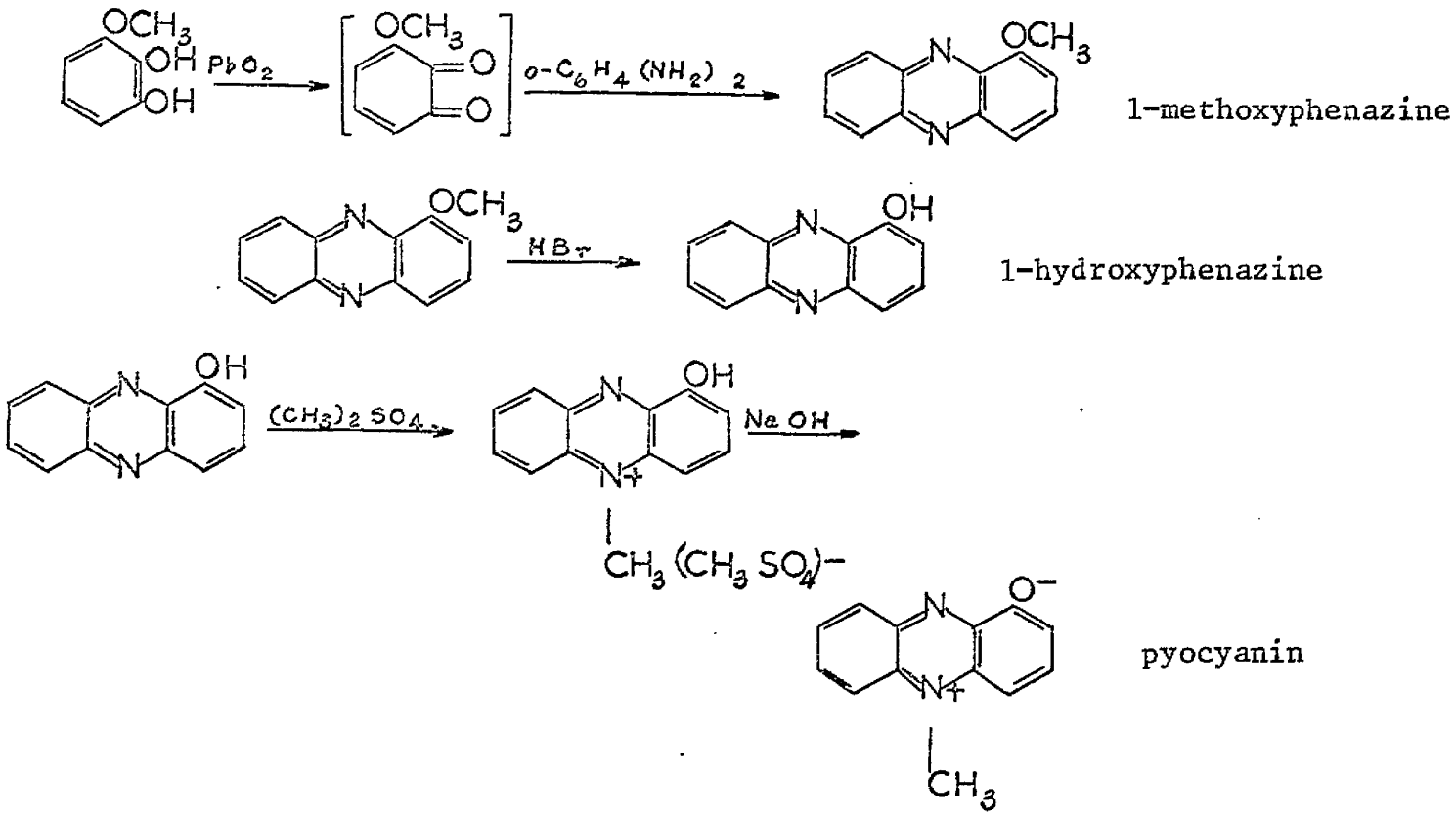


Figure 17 The chemical synthesis of pyocyanin and its derivatives

bubbler, a thermometer extending to the bottom, and a reflux condenser connected at the upper end with an exit tube leading to the hood. The reaction was carried out in an atmosphere of nitrogen. 2N sodium hydroxide (200 ml) was placed in the flask; 60.8 gm of crushed 2 - hydroxy - 3 methoxy-benzaldehyde (-o- vanillin) was slowly added to the alkali in the flask. The mixture was stirred with a magnetic stirrer until almost all the solid had dissolved.

6% hydrogen peroxide (284 ml) was added in 25 ml amounts over a period of 1 hour; the temperature was held between 40° and 50°. The temperature increased to about 45° and a dark solution was formed after the addition of the first portion of hydrogen peroxide; the temperature was allowed to fall to 40° before the addition of the next portion of hydrogen peroxide.

After the addition of all the hydrogen peroxide, the reaction mixture was cooled to 20° (room temperature) and saturated with sodium chloride. The reaction mixture was extracted with 100 ml portion of ether; a total volume of 700 ml ether was used. The combined ether extracts were dried over sodium sulphate for 48hr.

Ether was removed in vacuo and pyrogallol monomethyl ether was collected by heating the residue vigorously under reduced pressure. According to Surrey (1946) the pyrogallol monomethyl ether distilled over at 136 - 138° under 22 mm Hg pressure but in practice this did not occur. A light yellow oil was obtained which solidified on standing at room temperature for 24 hr; approximately 40 gm of pyrogallol monomethyl ether were obtained.

Preparation of 1-methoxyphenazine

Pyrogallol monomethyl ether (10 gm) was dissolved in 3 l. of dry benzene in a flask and 200 gm powdered lead dioxide was added. It was imperative that the lead dioxide was a fresh, black preparation. The mixture was shaken, by hand, for 20 min. and filtered through an 11 cm. Buchner funnel to remove a reddish-brown solid. The filter cake was washed with 400 ml benzene. A solution of 9 gm o-phenylenediamine in 80 ml glacial acetic acid and 200 ml benzene was immediately added to the filtrate; a mechanical stirrer was used. The dark brown solution was left at room temperature for 1½ hr and subsequently divided into two portions. Each portion was washed three times with water, twice with 5% NaOH solution and finally twice with water, 100 ml portions being used each time. The washed

benzene solutions were shaken with 50 gm anhydrous sodium carbonate and 5 gm Norit and filtered through an 11 cm. Buchner funnel. The filtrate was mixed with 60 gm activated alumina; the mixture was shaken until a filtered sample was light yellow in colour. The alumina was removed by filtration through a coarse folded filter paper and the cake of alumina was washed until the filtrate was colourless. The benzene was removed from the combined filtrates and washings by vacuum distillation. The residual yellow solid was dissolved in 10 ml hot pyridine. Distilled water was added to the point of incipient precipitation and the mixture was left at 4°. Light yellow crystals were filtered on a 7 cm Buchner funnel washed with water and air dried. The yield after 24 hr was 1.69 gm but this amount progressively increased with time so that after 3 days the total yield was 3.0 gm.

Preparation of 1-hydroxyphenazine.

A solution of 2 gm 1-methoxyphenazine in 125 ml of 55% hydrobromic acid was placed in a 250 ml round-bottomed flask fitted with a reflux condenser. The flask was immersed in an oil bath and the solution was heated at 110 - 120° for 5 hr; any gases evolved were

absorbed in a water trap. After five hours the dark brown solution was cooled to room temperature, diluted with 125 ml distilled water and almost neutralised with 100 ml of 35% NaOH followed by an additional volume of 10% NaOH. The reaction mixture was faintly acid to litmus; at this point there was also a change in colour from dark brown to bright yellow. The mixture was extracted with 40 ml portions of ether until the ether extracts were colourless; a total volume of 600 ml ether was used. The combined ether extracts were extracted with 25 ml portions of 10% NaOH solution. The purple sodium salt separated during this extraction and adhered to the wall of the separating funnel. It was dissolved in water and added to the NaOH extracts. This extraction was continued until no more purple sodium salt was removed from the ether extracts. Water was added to the NaOH extracts until all salts were in solution. The extracts were made acid to litmus with acetic acid; accompanied by a change in colour from purple to bright yellow. The acidified extracts were re-extracted five times with 50 ml portions of ether. The ether extracts were dried over anhydrous sodium sulphate and the ether was removed by vacuum distillation. The residue was dissolved in 10 ml hot ethanol and water was added to the point of

incipient precipitation. Norit (0.5 gm) was added and the hot solution was filtered. The filtrate was cooled in ice water and the bright yellow solid was filtered on a 7 cm Buchner funnel, washed with water and dried at 100°. The yield of 1-hydroxyphenazine was ∿ 1.0 gm.

Preparation of pyocyanin

1-hydroxyphenazine (0.5 gm) was dissolved in 10 ml methyl sulphate (0.1 mole) and placed in a 250 ml flask fitted with a calcium chloride drying tube and heated at 100° in an oil bath for 10 min. The solution was cooled to room temperature and 75 ml dry ether was added. The dark brown solid which formed was filtered on a 7 cm Buchner funnel and washed with 150 ml of dry ether. The dry methosulphate was dissolved in 30 ml water and made alkaline by the addition of 10% NaOH. The dark ruby coloured solution was extracted exhaustively with successive 15 ml portions of chloroform until no more blue substance was removed in the extract; this procedure was continued for 36 hr and 5 litres of chloroform were used. The combined chloroform solutions were extracted three times with 20 ml portions of 5% HCl. The combined red coloured, acid extracts were made alkaline with 10% NaOH;

accompanied by a colour change. The alkaline solution was extracted exhaustively with 25 ml amounts of chloroform until no more blue substance was removed; 2 litres of chloroform were used. The combined chloroform solutions were dried over anhydrous sodium sulphate. The dried extracts were decanted and the chloroform was removed by vacuum distillation.
The blue crystalline residue was dissolved in 5ml water at 60° and cooled in an ice-bath, when a mass of dark blue crystals were formed. This product was filtered on a 7 cm Buchner funnel and dried in the dark in a vacuum desiccator over calcium chloride. The yield was 386 mg of dark blue needles.

Fractions

PF 42 - biological pyocyanin

PF 43 - biological 1-hydroxyphenazine

PF 44 - Schoental biological pyocyanin

PF 45 - Schoental biological 1-hydroxyphenazine

PF 46 - chemical pyrogallol monomethyl ether

PF 47 - chemical 1-methoxyphenazine

PF 48 - chemical 1-hydroxyphenazine

PF 49 - chemical pyocyanin

TREATMENT of FRACTIONS OBTAINED FROM PSEUDOMONAS AERUGINOSA

Those fractions which showed an inhibitory effect on mitochondrial respiration were subjected to the following treatments in order to determine their properties.

a) Heat Treatment:- Before heating, the pH of the fraction was adjusted from 8.5 - 8.8 to 7.3. Two methods of heating were used :-

i. heating at 100° - the fraction PF 2 or PF 30 (5 mls) was centrifuged at 4,000 rpm for 10 minutes and the supernatant fluid was transferred to a test tube which was placed in a boiling water bath held at 100° for 30 minutes.

ii. heating at 121° - the fraction PF 1or PF 29 was centrifuged as described above and the supernatant fluid was autoclaved at 15 lbs/in^2 for 15 minutes. The heated samples were diluted in Hendry's phosphate buffer, pH 7.3 (solution II) prepared by adding 20 ml NaH_2PO_4 (26.0 gm/litre solution) to 80 ml Na_2HPO_4 (18.0 gm/litre solution) for use in manometric experiments.

b) Treatment with Trypsin:- The fraction (PF 2 or PF 30) or control fluid (1 ml) was incubated at 37° for

3 hours with 1 ml of Trypsin (1 mg/ml solution, B.D.H.). The trypsinised fractions were diluted in Hendry's phosphate buffer, pH 7.3 for use in manometric experiments.

c) Treatment with Proteinase:- The fraction (PF 2 or PF 30) or control fluid (1 ml) was incubated at 55° for 18 hours with 1 ml of crystalline Proteinase (1 mg/ml solution, Koch-Light). The fraction plus proteinase was approximately pH 8.8. Note that the optimum activity of crystalline proteinase was 55° and fractions were treated without proteinase at 55° to ensure that they were not inactivated at this temperature. The treated fractions were diluted for use in manometric experiments using Hendry's phosphate buffer pH 7.3 (Solution II, see page 90).

d) Digestion with pepsin:- The fraction (PF 2 or PF 30) or control fluid (1 ml) was incubated at 37° for 3 hours with 1 ml aqueous solution of pepsin (1 mg/ml solution, ex porcine mucosa, twice crystallised).

Further controls, incubated at 37°C for 3 hours were: 1) supernatant fraction + distilled water; 2) supernatant fraction + Hendry's phosphate buffer pH 7.3; 3) control fluid + Hendry's phosphate buffer pH 7.3. These extra controls were included since addition of pepsin or distilled water to the supernatant

fraction or control fluid resulted in a drop of pH to 2.0; addition of Hendry's phosphate buffer pH 7.3 to the supernatant fraction or control fluid gave a pH of 7.3 - 8.

The treated fractions were diluted for use in manometric experiments using Hendry's phosphate buffer, pH 7.3, solution II.

e) Acid hydrolysis:- 1 ml 12N hydrochloric acid (Analar) or 1 ml Hendry's phosphate buffer, pH 7.3 was added to 1 ml of fraction (PF 2, PF 30 or PF 2 trypsinised) or 1 ml of control fluid in an ice bath. This procedure enabled an acid hydrolysis to be carried out with 6NHCL. The ampoules were sealed and placed in an oven at 105° for 18 hours. After incubation the HCl was evaporated off and the sediment was resuspended in 1.0 ml distilled water. The pH was adjusted, where necessary, to pH 7.0 - 8.0. These fractions were used in manometric experiments.

f) Dialysis of fractions. The supernatant fluid PF 2 was dialysed (2.5 mls) in Visking tubing against distilled water or 0.03 M Hendry's phosphate buffer, pH 6.45 ($^1/_5$ solution I, Page 80), at 4° for 48 hours. The dialysed fractions and dialysate were used in manometric experiments.

PAPER CHROMATOGRAPHY OF PSEUDOMONAS FRACTIONS

Two dimensional amino-acid chromatography was carried out using acid hydrolysates of

1) crude Pseudomonas supernatant fluid fraction - PF 2
2) chloroform extract of crude supernatant fraction - PF 30
3) chloroform extract of trypsinised supernatant fraction PF 30 trypsinised.
4) control fluid - PF 2C

After acid hydrolysis (page 92), HCl was evaporated off on a boiling water bath and the residues were resuspended in 1 ml distilled water.

25µl of a fraction or a standard amino-acid mixture (Shandon Scientific Co. Ltd., London CH 42) was spotted on to each paper using a micro pipette.

Two ascending systems were used :-

1. Butanol acetic

Butanol: acetic acid: water

(130: 30: 50 v/v)

2. Phenol ammonia

Distilled water (125 ml) was added to 500 gm phenol to produce a saturated phenol solution. In use 200 ml saturated phenol solution were mixed with 2 ml ammonia (Smith 1960).

Each solvent was run for 18 hours and the solvent evaporated off in a fume cupboard (note that evaporation of phenol required 24 hours in the fume cupboard). After the papers were dried, the spots were developed in a solution of ninhydrin in acetone (0.01% ninhydrin in 95% acetone) by drawing the papers through a trough containing these reagents. The papers were then dried by placing in a hot oven (105°) for 2 - 3 minutes. The spots were preserved by dipping the papers in a weak solution of copper nitrite or cobalt nitrite.

HAEMOLYTIC PROPERTIES of PSEUDOMONAS FRACTIONS

The haemolytic properties of Pseudomonas fraction PF 2 were determined by titrating against red blood corpuscles from rabbits, mice, sheep, horse and human beings.

Serial doubling dilutions from $\frac{1}{2}$ - 1/2000 of crude Pseudomonas fraction were carried out in 0.5 ml amounts of diluent (75 ml saline, 24 ml nutrient broth, 1 ml 1% merthiolate.)

0.5 ml of 2% red blood cell solution was added to each dilution and after mixing, the tubes were incubated at 37° for 1 hour. Controls were set up with diluent and red blood cells.

The dilution causing 50% haemolysis was accepted

as the end point of the test; this was the haemolytic titre of the fraction.

ANTIBIOTIC ACTIVITY of PSEUDOMONAS FRACTIONS

The antibiotic activity of various fractions was tested, namely: 1) PF 2C

2) PF 2 NCTC 6750

3) PF 30

4) PF 32

5) PF 2 NCTC 8545

on the following organisms:

Staphylococcus aureus	NCTC 4135
Escherichia coli	NCTC 8196
Streptococcal species	HE 7
Bacillus species	77 MD 0559

Two methods were used to determine the antibacterial effect:-

Method A. The organism to be tested was grown overnight on a nutrient-agar slope and this growth was emulsfied in a few drops of distilled water and added to 20 ml molten non-nutrient agar at 56°; this was mixed quickly and an agar plate was poured. Five basins (5mm in diameter) were cut out of the agar and each basin was filled with one of the five fractions to be tested. The plate was incubated overnight at 37°C. After overnight incubation a small piece of agar

was cut out of each plate and placed in nutrient broth and incubated at 37° overnight to check that the organisms were still viable. A second control was set up by plating out the four organisms on non-nutrient agar and incubating at 37°.

Method B. A trough was cut out of an uninoculated nutrient agar plate using a sterile scalpel. Double strength agar (1 ml) was added at 56° to 1 ml of the fraction to be tested. This was mixed thoroughly and poured into the trough and allowed to set. A single streak of each of the test organisms was plated at right angles to the trough and incubated overnight at 37°. These plates were set up in duplicate; the second set was left at 4° overnight to allow the toxin to diffuse and inoculation of the test organisms was carried out next day. The duplicate set of plates was incubated at 37° overnight.

THE EFFECT OF PSEUDOMONAS FRACTIONS, PYOCYANIN AND ITS DERIVATIVES ON RESPIRATORY SYSTEMS.

There are a considerable number of established methods used to measure the respiratory activity of tissue, cell and enzyme systems, based upon an alteration in pressure when oxygen is taken up and carbon dioxide is liberated. Probably one of the best known of these is Warburg manometry. The Warburg apparatus was used for the measurement of oxygen uptake by mitochondria or tissue culture cells. Pseudomonas fractions were added to respiring mitochondria or tissue culture cells and their effects on respiration noted. The activity of these fractions at various sites in the electron transport chain was investigated (Figure 18).

A. THE EFFECT OF PSEUDOMONAS FRACTIONS, PYOCYANIN AND ITS DERIVATIVES ON MITOCHONDRIAL RESPIRATION.

1) Preparation of liver or kidney mitochondrial suspensions.

Reagents:- Hendry's sucrose phosphate buffer, pH 7.3 Solution III. This was prepared by adding 8.5 gm sucrose to a mixture of 100 ml distilled water and 20 ml Solution II (see page 90), i.e. 0.25 M sucrose

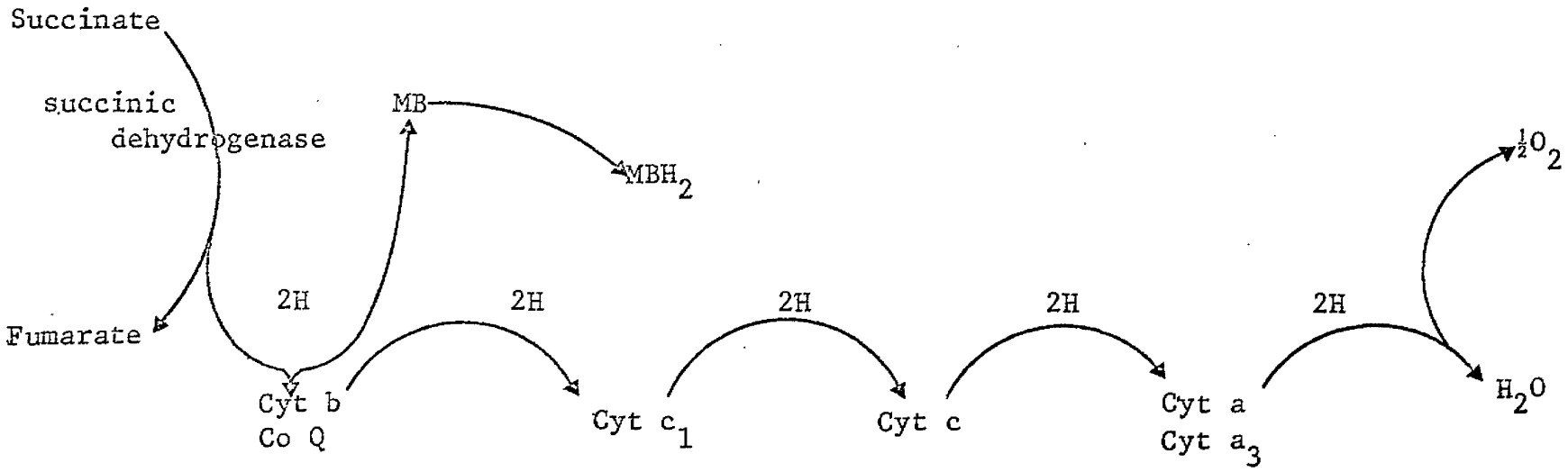


Figure 18 Working model of the respiratory chain in mitochondria

containing 0.023M phosphate buffer, pH 7.3 (Hendry, 1948).

The majority of manometry experiments were carried out using mouse liver mitochondria. However, some experiments were done using mouse kidney mitochondria. The method of preparation and manometric assay were similar in both cases.

Fresh mitochondria were prepared for each experiment using a modification of Schneider's method (1948). Three Porton white mice (35 - 45 gm) were killed by cervical dislocation and exsanguinated by slitting the throat. The liver or kidneys were removed from each mouse and placed in 10 ml solution III. Connective tissue was gently removed with forceps and the liver tissue was minced. The minced tissue was carefully homogenised in a tissue grinder (Baird and Tatlock Ltd.) by applying ten strokes of the glass plunger, N.B. Vigorous homogenisation causes the mitochondria to become swollen and in this state they are unsuitable for respiration experiments. The homogenate in 50 ml solution III was centrifuged at 2000 rpm for 10 mins. at 4^{o} in an MSE super minor centrifuge after which the supernatant fluid was retained and the sediment was discarded. The supernatant fluid was centrifuged at 8,500 rpm for 10 mins. at 4^{o} in an MSE 18 high speed

centrifuge. The sediment was retained and washed in 20 ml solution III and recentrifuged at 8,500 rpm for 10 mins at 4°. Finally the sediment was resuspended in 4 ml solution III and stored for 1 hour at 4°. For a Warburg manometry experiment, this mitochondrial suspension was diluted 1:3 in solution III immediately before use.

2. Absorption of Pseudomonas fractions with mitochondria.

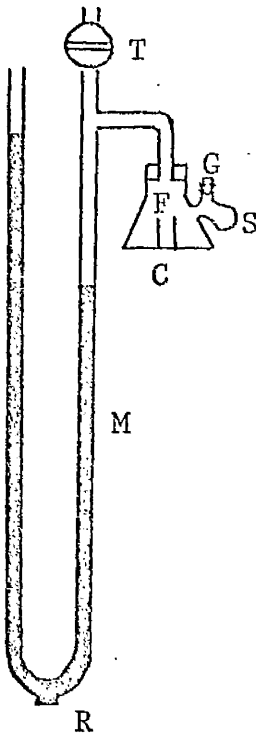
1 ml freshly prepared mitochondria were incubated at 37° for 1 hour with 1 ml undiluted raw toxin (PF 2) or 1 ml undiluted control fluid (PF 2C).

The mitochondria were spun at 8,500 rpm for 10 minutes at 4° and the supernatant fluid diluted 1:3 for use in a Warburg manometry experiment (final dilution of absorbed Pseudomonas fraction was 1:36).

3. The measurement of mitochondrial respiration using the Warburg manometric technique.

The apparatus consisted of a flask and manometer (Figure 19) both of which were calibrated before being used in experiments, in order to determine the flask constant.

Calibration with mercury: A permanent reference mark was scratched on the side arm of the manometer above the ground glass joint; the manometer was held at an angle of 30° with the side arm uppermost, by



k = (Vg)C + (Δ k) (ml fluid) where

k = flask constant

Vg = (sum of the flask volume and manometer volume) - (volume of fluid used in flask)

C = a constant dependent on temperature

Δk = change in constant with change in volume of the flask

F = flask

S = side-arm

G = side-arm stopper

C = centre well

M = manometer

R = fluid reservoir

T = three-way stopcock

Figure 19 The Warburg constant volume respirometer, Umbreit, Burris and Stauffer (1959)

clamping it to a ringstand. A short rubber tube and screwclamp were fitted to the gas inlet tube of the manometer, and filled with mercury. The stopcock of the manometer was left open. The amount of mercury in the tube was adjusted by means of the screw clamp, and tilting of the manometer, till the mercury just filled the space between the reference point (150 mm) and the reference scratch mark. The stopcock was closed and the mercury allowed to run into a vessel of known weight; the vessel with the mercury was weighed. The manometer was clamped upright; a Warburg flask was weighed, filled with mercury, taking care that no air bubbles were trapped in the mercury, and attached to the dry manometer joint. The mercury rose up the manometer by capillary action and the amount of mercury in the flask was adjusted till it just reached the reference scratch mark, when the joint was firm. The flask filled with this amount of mercury was weighed.

The volume of the Warburg flask to the scratch mark was calculated by dividing the weight of mercury by its density; the volume of the manometer from the scratch mark to 150 mm was calculated in the same way. The sum of the flask volume and the

manometer volume minus the liquid volume to be used in the flask is the Vg to be substituted in the equation to determine the flask constant.

The flask constant was calculated as follows:-

$k = (Vg)C + (\Delta k)(\text{ml fluid})$ where

k = flask constant

Vg = (Sum of the flask volume and manometer volume) - (volume of fluid used in flask).

C = a constant dependent on temperature

Δk = change in constant with change in volume of the flask.

The results were calculated by observing the changes in the level of the manometer fluid of the reaction flask manometer and of the thermobarometer at given time intervals. The uptake in mm. over any given time interval was obtained by subtracting each reading from the one following it; any necessary corrections were made for thermobarometer changes.

Each of these values in mm. for various time intervals was multiplied by the flask constant to give the µl oxygen taken up in each time interval.

Reagents i) <u>manometer fluid</u> - Krebs manometer fluid of known density was used in the manometers -

Anhydrous sodium bromide	44.0 gm
Stergene detergent	1.0 gm
Evans blue dye	0.3 gm
distilled water	1000 ml

Density was 1.033 at 20°.

(ii) Buffer - used was Hendry's sucrose phosphate buffer, pH 7.3, i.e. solution III (see page 97).

(iii) Substrates were -

a) 0.2M sodium succinate (B.D.H.)

1.08gm/20 ml solution III

b) p. phenylene diamine (ppH/di; Koch-Light)

0.0812gm/5 ml solution III

c) Glucose (Analar)

1% v/v in solution III

(iv) Cytochrome C (crystalline, Koch-Light)

0.0072 gm/3ml solution III

(v) Ubiquinone (Q_{10} ex beef heart mitochondrion:Koch-Light)

10mg/ml ethanol, stock solution.

A 1:10 dilution of the stock solution was prepared in solution III for experiments.

(vi) Methylene blue dye - 0.001 M solution

This solution was used in Thunberg tubes.

(vii) 20% NaOH - 0.2 ml NaOH was placed in the centre well of a Warburg flask to absorb the carbon dioxide produced on respiration of intact cells. This ensured that the recorded pressure changes were entirely due to oxygen absorption.

Standard Warburg assay:- four flasks were used, each containing solution III, mitochondria and succinate in the main well; *Pseudomonas* fractions or control fluid were contained in the side arm. A thermobarometer was included containing 3 ml solution III. This assay is outlined in Table 15. The flasks and attached manometers were immersed in a water bath at constant temperature, 37°, and between readings, the system was shaken to promote a rapid gas exchange between the fluid and gas phases. At time zero, the fluid in the manometers was set at 150 mm in the arm attached to the flask and the reading in the open arm recorded before closing the stopcock; the TB was set at 140 mm and a reading recorded. Readings thereafter were taken at 15 min intervals and the fractions in the side arm could be tipped at any given time (usually 15 minutes after zero time). The total volume was always 3 ml; this was obtained by adding an appropriate amount of solution III. Readings were taken from 0 to 75 - 90 minutes.

		Total oxygen uptake				TB
	Flasks	1	2	3	4	5
Main well:	solution III (ml)	1.7	1.7	1.7	1.7	3.0
	mitochondria	0.5	0.5	0.5	0.5	-
	succinate	0.3	0.3	0.3	0.3	-
Side arm:	control fluid	0.5	0.5	-	-	-
	Pseudomonas fraction	-	-	0.5	0.5	-

TABLE 15: Standard method for measurement of oxygen uptake by mouse liver mitochondria in the Warburg apparatus.

I. THE EFFECT OF PSEUDOMONAS FRACTIONS ON MITOCHONDRIAL RESPIRATION

a) The effect of preincubating mitochondria with Pseudomonas fractions.

In this experiment, the Pseudomonas fractions (PF 1, PF 2, PF 1 heated or PF 2 heated) or control fluid were placed in the main well with the mitochondria and the succinate was placed in the side arm. The fractions were preincubated with the mitochondria for 15 or 30 minutes before closing the flasks; the succinate was tipped immediately after the zero reading. This method is outlined in Table 16.

b) The effect of adding Pseudomonas fractions to mitochondria immediately after equilibration.

This experiment was done using the standard method outlined in Table 15, with the exception that the Pseudomonas fractions PF 1 or PF 2 or control fluids were tipped immediately after the zero reading.

c) The effect of adding Pseudomonas fractions to actively respiring mitochondria.

This method was the same as the standard method (see Table 15). The Pseudomonas

		Total oxygen uptake				TB
	Flasks	1	2	3	4	5
Main well:	solution III (ml)	1.7	1.7	1.7	1.7	3.0
	mitochondria	0.5	0.5	0.5	0.5	-
	Pseudomonas fraction	-	-	0.5	0.5	-
	control fluid	0.5	0.5	-	-	-
Side arm:	succinate	0.3	0.3	0.3	0.3	-

TABLE 16: Method for measurement of oxygen uptake by mouse liver mitochondria in the Warburg apparatus with preincubation of control fluid or Pseudomonas fraction.

fractions PF 1 or PF 2 or control fluid were added to actively respiring mitochondria 15 or 30 mins. after zero time.

d) The effect of heated Pseudomonas fractions on mitochondria. The Pseudomonas fractions PF 2 or PF 30 heated at 100° and 121° were tested for their activity on respiring mitochondria according to the methods outlined in Tables 15 and 16.

e) The effect of trypsinised Pseudomonas fractions on mitochondria. These fractions were tested for their activity on respiring mitochondria according to the standard method outlined in Table 15.

f) The effect of proteinase-treated Pseudomonas fractions on mitochondria. These fractions were tested for their activity on respiring mitochondria according to the standard method outlined in Table 15.

g) The effect of pepsin-treated Pseudomonas fractions on mitochondria. These fractions were tested for their activity on respiring mitochondria according to the standard method outlined in Table 15.

h) The effect of acid-hydrolysed Pseudomonas fractions on mitochondria. These fractions were tested for their activity on respiring mitochondria according to the standard method outlined in Table 15.

i) The effect of dialysed Pseudomonas fractions and dialysate on mitochondria. These fractions were tested for their activity on mitochondria according to the standard method outlined in Table 15.

j) The effect of absorbed Pseudomonas fractions on mitochondria. The Pseudomonas fractions absorbed with mitochondria were tested for their activity on respiring mitochondria according to the standard method outlined in Table 15.

k) The effect of methanol-precipitated Pseudomonas fractions on mitochondria. These fractions were tested for their activity on mitochondria according to the standard method outlined in Table 15.

l) The effect of chloroform extracted Pseudomonas fractions on mitochondria. Both chloroform and aqueous extracts were tested for their activity on respiring mitochondria according

to the standard method outlined in Table 15.

m) The effect of Pseudomonas fractions from an alumina column on mitochondria. The various fractions obtained from this column (PF 33, PF 34, PF 35) were tested for their activity on respiring mitochondria according to the standard method outlined in Table 15.

n) The effect of Pseudomonas fractions from various Sephadex columns on mitochondria. The fractions obtained from Sephadex columns (PF 36, PF 37, PF 38, PF 39, PF 40 and PF 41) were tested for their activity on mitochondria according to the standard method outlined in Table 15.

o) The effect of biologically synthesised Pseudomonas fractions on mitochondria. Both pyocyanin and 1 - hydroxyphenazine at various concentrations were tested for their activity on respiring mitochondria according to the standard method outlined in Table 15. Concentrations varied from 0.08 mg/ml (final) to 0.25 mg/ml (final).

As already mentioned (Page 55) intact mitochondria have a permeability barrier to phenazonium compounds; this can be partly reduced by addition of calcium ions to the mitochondrion. 0.225mg Ca CO_3 in 1.7 ml

solution III (page 97) along with the mitochondria and succinate was placed in the main well of a Warburg flask - pyocyanin was placed in the side arm and added to respiring mitochondria (See Table 17).

In d) to n) above where the Pseudomonas fractions were undergoing various treatments, the final concentration of the fraction during a Warburg assay was always the same as the final concentration of untreated Pseudomonas fraction and control fluid.

II. THE EFFECT OF CHEMICALLY SYNTHESISED DERIVATIVES OF PYOCYANIN ON MITOCHONDRIAL RESPIRATION

Chemically synthesised derivatives of pyocyanin were tested for their activity on respiring mitochondria according to the standard method outline in Table 15. Their action on respiring mitochondria was compared with biological preparations of pyocyanin and 1-hydroxyphenazine.

		Total oxygen uptake								TB
	Flasks	1	2	3	4	5	6	7	8	9
Main well:	solution III (ml)	1.7	1.7	1.7	1.7	-	-	-	-	3.0
	0.225mg $CaCO_3$ in solution III	-	-	-	-	1.7	1.7	1.7	1.7	-
	mitochondria	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-
	succinate	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	-
Side arm:	control fluid	0.5	0.5	-	-	0.5	0.5	-	-	-
	0.25mg crystalline pyocyanin in distilled water	-	-	0.5	0.5	-	-	0.5	0.5	-

TABLE 17: The effect of adding calcium ions and crystalline pyocyanin, biologically prepared, on oxygen uptake by mouse liver mitochondria.

III. THE SITE OF ACTION OF PSEUDOMONAS FRACTIONS IN THE ELECTRON TRANSPORT SYSTEM

i) Succinic dehydrogenase - the activity of the Pseudomonas fractions (PF 2 or PF 2 heated or PF 30) at the site of succinic dehydrogenase was measured in Thunberg tubes under anaerobic conditions. Thunberg tubes were used because experimental difficulties were encountered in Warburg manometric measurements of the activity at this site namely, cyanide was required to inhibit the reactions to the right of succinic dehydrogenase in the electron transport chain. (Figure 18).

The Thunberg tubes were set up containing 3 ml reaction mixture, as outlined in Table 18, and evacuated for 5 minutes on an Edwards High vacuum pump (model 25C 50) with constant shaking to remove air bubbles. After evacuation, the side arms were twisted through 90° and the sealed tubes were placed in a 37° water bath for 30 minutes. After equilibration the contents of the side arm were tipped into the main well and the tubes were examined at

		Test	Control
Main well:	solution III (ml)	1.4	1.4
	mitochondria	0.5	0.5
	Pseudomonas fraction	0.5	-
	control fluid	-	0.5
Side arm:	succinate	0.3	0.3
	0.001M methylene blue dye	0.3	0.3

TABLE 18: The effect of Pseudomonas fractions at the succinic dehydrogenase site using Thunberg tubes.

2 - 3 minute intervals for the production of leuco-methylene blue. The time required for 90% reduction of the dye was taken as a measure of dehydrogenase activity.

ii) Cytochrome oxidase - the activity of the Pseudomonas fractions (PF 2, PF 2 heated, PF 30 or PF 36) at the site of cytochrome oxidase in the electron transport system was measured manometrically in the Warburg apparatus according to the method outlined in Table 19, using paraphenylenediamine as substrate (Slater, 1949); mouse liver mitochondria oxidize this substrate which enters at the site of cytochrome C (Figure 18). To further investigate activity at this site, cytochrome C was added to mitochondria, treated with Pseudomonas fractions, respiring on sodium succinate as outlined in Table 20.

iii) Ubiquinone (CoQ) - the activity of Pseudomonas fractions (PF 2, PF 2 heated or PF 30) at the site of CoQ in the electron transport system was measured manometrically by -

		Total oxygen uptake								TB
	Flasks	1	2	3	4	5	6	7	8	9
Main well:	solution III (ml)	1.7	1.7	1.7	1.7	1.7	1.7	1.7	1.7	3.0
	mitochondria	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-
	control fluid	0.5	0.5	-	-	0.5	0.5	-	-	-
	Pseudomonas fraction	-	-	0.5	0.5	-	-	0.5	0.5	-
Side arm:	succinate	0.3	0.3	0.3	0.3	-	-	-	-	-
	ppH/di	-	-	-	-	0.3	0.3	0.3	0.3	-

TABLE 19: The measurement of Cytochrome oxidase in the Warburg apparatus using succinate and paraphenylenediamine as substrate.

Flasks	Total oxygen uptake				TB
	1	2	3	4	5
Main well: solution III (ml)	1.4	1.4	1.4	1.4	3.0
mitochondria	0.5	0.5	0.5	0.5	-
succinate	0.3	0.3	0.3	0.3	-
side arm (1): control fluid	0.5	0.5	-	-	-
Pseudomonas fraction	-	-	0.5	0.5	-
side arm (2): Cytochrome C	0.3	0.3	0.3	0.3	-

TABLE 20: The effect on oxygen uptake of adding Cytochrome C to mouse liver mitochondria treated with Pseudomonas fractions, respiring on succinate.

a) addition of CoQ to mitochondria treated with fractions as outlined in Table 21, and

b) protecting mitochondria with excess CoQ and subsequent exposure of these mitochondria to a Pseudomonas fraction in the Warburg apparatus as outlined in Table 22.

In a) above the addition of CoQ during succinate oxidation resulted in an immediate upset of the gaseous equilibrium within the manometer due to the release of an ethanolic vapour. To counteract this, the manometer was opened to the atmosphere in both limbs and the fluid level was reset to 150 mm and allowed to equilibrate before closing. Further readings were taken in the normal way.

B. THE EFFECT OF PSEUDOMONAS FRACTIONS ON TISSUE CULTURE CELLS.

As stated previously any experiments carried out with suspensions of mitochondria, of necessity involves the study of the mitochondrion in the isolated state. Tissue cells simulate, to some extent, the permeability barrier which often exists in the animal body. Oxygen

		Total oxygen uptake								TB
	Flasks	1	2	3	4	5	6	7	8	9
Main well:	solution III (ml)	1.4	1.4	1.4	1.4	1.7	1.7	1.7	1.7	3.0
	mitochondria	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-
	succinate	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	-
Side arm (1):	control fluid	0.5	0.5	-	-	0.5	0.5	-	-	-
	Pseudomonas fraction	-	-	0.5	0.5	-	-	0.5	0.5	-
Side arm (2):	0.3mg CoQ in ethanol + solution III (final concentration 0.1mg/ml)	0.3	0.3	0.3	0.3	-	-	-	-	-

TABLE 21: The effect on oxygen uptake of adding ubiquinone (CoQ) to mouse liver mitochondria treated with Pseudomonas fractions.

		Total oxygen uptake								TB
Flasks		1	2	3	4	5	6	7	8	9
Main well:	solution III (ml)	1.4	1.4	1.4	1.4	1.7	1.7	1.7	1.7	3.0
	mitochondria	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	-
	succinate	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	-
	0.3mg CoQ in ethanol plus solution III	0.3	0.3	0.3	0.3	-	-	-	-	-
Side arm:	control fluid	0.5	0.5	-	-	0.5	0.5	-	-	-
	Pseudomonas fraction	-	-	0.5	0.5	-	-	0.5	0.5	-

TABLE 22: The effect on oxygen uptake of preincubating mouse liver mitochondria with ubiquinone (CoQ) prior to treatment with Pseudomonas fractions.

uptake by tissue culture cells was measured in the Warburg apparatus.

1) Preparation of tissue cultures for use in Warburg manometry experiments. Two cells lines were used

i) L "S" cells, derived from subcutaneous mouse liver; this formed a smooth suspension.

ii) BHK - strain C 13 - derived from baby hamster kidney tissue.

Media or Reagents a) These cells were grown in Eagle's complete medium i.e. ETC (Eagle 1959)

ETC

Eagle's salts, vitamins and amino-acids	80%
Calf serum	10%
Trpytose phosphate (Difco)	10%

The composition of Eagle's salts, vitamins and amino-acids is given in Tables 23 - 25.

b) Trypsin-Versene 1 volume of Trypsin (0.25% Difco trypsin in Tris saline) was added to 4 volumes of Versene (Diaminoethanetetra-acetic acid, disodium salt, BDH) immediately before use.

For large scale experiments these cells

Eagle's salts	To make 1000 ml
NaCl	6.4 gm
KCl	0.4 gm
$CaCl_2$	0.2 gm
$MgSO_4 . 7H_2O$	0.2 gm
$NaH_2PO_4 . 2H_2O$	0.14gm
Dextrose	4.5 gm
Ferric nitrate	0.0001 gm
L Glutamine	0.292 gm
Penicillin	100,000 units
Streptomycin	0.1 gm
Antimycotic 0.02%	1.0 ml
Phenol Red 1%	1.5 ml
Dissolve in about 500 ml of distilled water	
For Eagle's medium,	
add: $NaHCO_3$	2.75 gm
Amino acids (concentrated)	50 ml
Vitamins (concentrated)	4 ml
Distilled water to	1000 ml

Flush with CO_2 till orange
Sterilise by millipore filtration, using G.S. membrane (0.22μ)
Bottle in 80 ml amounts and store at 4°.
Check for bacterial contamination and plating efficiency.

TABLE 23: The composition of Eagle's medium, before addition of calf serum

Amino-acids (concentrated)	To make 1000 ml
L. Arginine, mono Hcl	0.84 gm
L. Cystine (dissolve in 5 ml $^{N}/_{1}$ NaOH)	0.48 gm
L. Histidine, mono HCl	0.384 gm
L. Isoleucine	1.048 gm
L. Leucine	1.048 gm
L. Lysine, mono HCl	1.462 gm
L. Phenylalanine	0.66 gm
L. Threonine	0.952 gm
L. Tryptophan	0.16 gm
L. Tyrosine	0.724 gm
L. Valine	0.936 gm
L. Methionine	0.3 gm
Inositol	0.07 gm
Phenol Red 1%	0.04 ml
Distilled water up to	1000 ml
Heat to about 56° (but not above 60°)	
Dispense in 100 ml amounts and store at -20°	

TABLE 24: The composition of Eagle's amino-acids.

VITAMINS (CONCENTRATED)	TO MAKE 100 ml
Choline Chloride	0.05 gm
Folic acid (dissolve in 5 ml $^{N}/_{1}$ NaOH)	0.05 gm
Nicotinamide	0.05 gm
D.L. Pantothenic acid, Ca salt	0.05 gm
Pyridoxal HCl	0.05 gm
Thiamine HCl (Aneurine)	0.05 gm
Riboflavin	0.005gm

Dissolve in distilled water and make up to 100 ml.

Bottle in 8 ml amounts; store at -20°

TABLE 25: The composition of Eagle's vitamins.

were grown in burler bottles gassed with pure CO_2. ETC (200 mls) was placed in a burler bottle and seeded with either approximately 20×10^6 cells of the growth from two Pyrex baby bottles. The burler bottles were slowly rotated to ensure contact of the fluid with the sides of the glass bottle. This was essential in the case of cell lines which produced a monolayer. After 3 days incubation at 37° the tissue culture cells were either harvested and used in manometric experiments or further propagated.

To harvest the cells, two methods were used -

a) L "S" cells (a cell suspension). The contents of each burler was transferred aseptically to sterile glass bottles and centrifuged at 1000 rpm for 5 minutes. The sediment was resuspended in fresh suspending fluid which could be either i) complete Eagle's medium, or ii) Eagle's medium containing no glucose, i.e. a substrate was added later during the course of a Warburg experiment.

b) BHK cells (a cell monolayer). The medium was gently decanted off; the monolayer was washed with 25 ml Trypsin-Versene solution and the washings were carefully removed.

25 ml Trypsin-Versene solution were added and the bottle was quickly rotated by hand to remove the cells. The cell suspension was centrifuged at 1000 rpm for 5 minutes and the sediment was resuspended in fresh suspending fluid as for L "S" cells.

After counting the cells in a haemocytometer, they were used for Warburg manometry or for seeding fresh tissue cultures.

2) Maintenance of cell lines. To maintain the cell lines, growth in a Pyrex baby bottle was sufficient. In this case, 1 x 10^6 cells were seeded into 10 ml ETC in each baby bottle. The baby bottles were gassed with a mixture of 95% oxygen and 5% carbon dioxide for about 15 seconds and subsequently incubated at 37° for 3 days. The temperature was not allowed to deviate beyond the range 35.5 - 37.5°.

Harvesting of the cells from these baby bottles was similar to the method used with burlers.

I. THE EFFECT OF PSEUDOMONAS FRACTIONS ON THE RESPIRATION OF TISSUE CULTURE CELLS; AND COMPARISON WITH THE EFFECT OF STAPHYLOCOCCAL PRODUCTS ON TISSUE CULTURE CELLS.

a) The effect on respiration of varying the number of tissue culture cells per flask.

Preliminary experiments were carried out to determine the optimal number of BHK or L "S" cells per Warburg flask. To measure the respiration of these cells in the Warburg manometer, after harvesting they were suspended in complete Eagle's medium. The cells were counted and varying numbers from 17×10^6 to 80×10^6 in 2.8 ml ETC were added to Warburg flasks, as outlined in Table 26. The glucose in the ETC is utilised as a substrate by these cells.

b) The effects of adding i) Pseudomonas fraction, ii) Wood 46, and iii) SOF to tissue culture cells.

For these experiments the cells were suspended in the following medium :-

160 ml Eagle's salts + amino-acids
0.64 ml Vitamins
16 ml calf-serum

		Total oxygen uptake						TB
Flasks		1	2	3	4	5	6	7
Main well:	2.8ml Eagle's medium plus varying numbers of cells	17×10^6	25×10^6	34×10^6	47×10^6	62×10^6	80×10^6	
	distilled water (ml)	-	-	-	-	-	-	3.0
Centre well:	NaOH (ml)	0.2	0.2	0.2	0.2	0.2	0.2	-

TABLE 26: The effect of varying tissue culture cell numbers on cell respiration, using the Warburg apparatus.

No substrate was included in this medium, until respiration was being measured. Oxidizable carbohydrate as substrate, in the form of Glucose (1% v/v) or 0.2m sodium succinate was contained in the main well in the case of Pseudomonas fractions, PF 2 or PF 30 (Table 27) which required no preincubation period as did Wood 46 and SOF; or was tipped at zero time (Table 28; preincubation of Wood 46 and SOF in main well.) The rate of oxidation of these substrates was measured at 37° using NaOH to absorb any carbon dioxide liberated. The speed of shaking the Warburg flasks was varied from 70 - 100 cycles/minute.

	Flasks	Total oxygen uptake 1	2	3	4	5	6	7	8	TB 9
Main well:	cells in suspending fluid* (ml)	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	-
	distilled water	-	-	-	-	-	-	-	-	3.0
	succinate	0.5	0.5	-	-	0.5	0.5	-	-	-
	glucose	-	-	0.5	0.5	-	-	0.5	0.5	-
Side arm:	control fluid	0.5	0.5	-	-	-	-	0.5	0.5	
	Pseudomonas fraction	-	-	0.5	0.5	0.5	0.5	-	-	
Centre well:	NaOH (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	-

* suspending fluid was Eagle's vitamins, salts and calf serum

TABLE 27: The effect of Pseudomonas fractions on the respiration of tissue culture cells

	Total oxygen uptake												TB
Flasks	1	2	3	4	5	6	7	8	9	10	11	12	13
Main well: cells in suspending fluid* (ml)	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	-
distilled water	-	-	-	-	-	-	-	-	-	-	-	-	3.0
control fluid	0.5	0.5	0.5	0.5	-	-	-	-	-	-	-	-	-
Wood 46	-	-	-	-	0.5	0.5	0.5	0.5	-	-	-	-	-
SOF	-	-	-	-	-	-	-	-	0.5	0.5	0.5	0.5	-
Side arm: succinate (ml)	0.5	0.5	-	-	0.5	0.5	-	-	0.5	0.5	-	-	-
glucose	-	-	0.5	0.5	-	-	0.5	0.5	-	-	0.5	0.5	-
Centre well: NaOH (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	-

* suspending fluid was Eagle's vitamins salts and calf serum

TABLE 28: The effect of Wood 46 and SOF on the respiration of tissue culture cells.

POLAROGRAPHY

The biological oxygen monitor (YS 1 model 53) provides a means of measuring oxygen uptake by biological systems. This instrument produces in 5 - 20 mins. similar O_2 uptake curves to those obtained in 1 - 2 hours using Warburg apparatus. The instrument contains a polarographic electrode known as the oxygen probe which is responsible for the extremely sensitive results obtained. Figure 20 shows the probe, plunger and sample chamber. A thin membrane stretched over the end of the probe is permeable to gases and allows them to enter the interior of the probe. When a suitable polarising voltage is applied across the cell, oxygen will react at the cathode causing a current to flow through the cell; the amount of current which flows is proportional to the amount of oxygen to which the membrane is exposed. The membrane diffusion is directly proportional to pressure and the oxygen-cell current relationship is stoichiometric, thus a linear relationship exists between external oxygen pressure and cell current.

The effect of i) crude Pseudomonas fraction, and control fluid, ii) biologically synthesised pyocyanin and 1-hydroxyphenazine, and iii) chemically synthesised

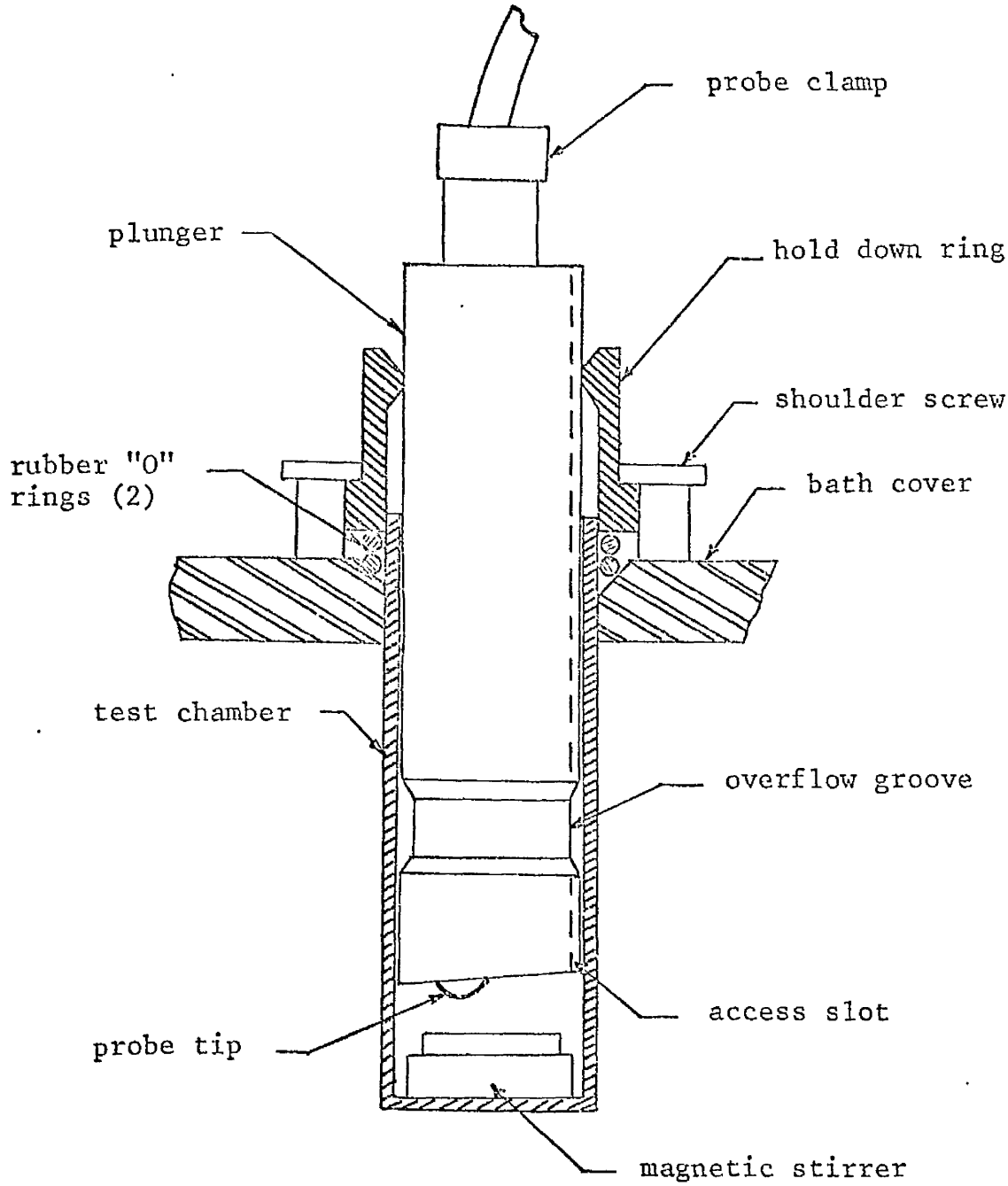


Figure 20 The probe, plunger and sample chamber of the biological oxygen monitor

pyocyanin and its derivatives on the activity of respiring mitochondria was measured in the polarograph. The total volume in all tests was 3 ml.

The effect of adding CoQ to mitochondria, before and after treatment with these fractions, was also measured in the polarograph (Table 29).

Reagents - i) Buffer used was Hendry's sucrose phosphate (solution III).

ii) Substrate was 0.2 m sodium succinate

iii) Mitochondria were used undiluted.

PF 2 and PF 2C were diluted $^{1}/_{6}$ for use in these experiments while all other fractions were used from a stock solution of each containing 0.5 mg/ml.

To determine rate of oxygen use: the meter reading at the start and finish of a given time interval was noted e.g.:-

Start 92%

Finish 66%

Time interval 5 minutes.

92 - 66 = 26% of the oxygen was used up in 5 minutes.

It was shown by calibration of the apparatus that the buffer (Solution III) used, had the same O_2 content as Ringer's solution. For 3.0

Experiment	Buffer (ml)	Fraction (ml)	Name of fraction
1	2.5	0.1 PF 2	Supernatant fluid from
2	2.4	0.2	K.W. & R (medium A) sloppy agar
3	2.3	0.3	
4	2.5	0.1 PF 2C	Supernatant fluid from
5	2.4	0.2	uninoculated K W & R
6	2.3	0.3	(medium A) sloppy agar
7	2.5	0.1 PF 43	biological 1-hydroxy-
8	2.45	0.15	phenazine
9	2.4	0.2	
10	2.5	0.1 PF 48	chemically synthesised
11	2.45	0.15	1-hydroxyphenazine
12	2.4	0.2	
13	2.5	0.1 PF 47	chemically synthesised
14	2.4	0.2	1-methoxyphenazine
15	2.3	0.3	
16	2.5	0.1 PF 46	chemically synthesised
17	2.4	0.2	pyrogallol monomethyl-ether
18	2.3	0.3	
19	2.5	0.1 PF 49	chemically synthesised
20	2.4	0.2	pyocyanin
21	2.3	0.3	
22	2.5	0.1 PF 42 or PF 44	biologically synthesised
23	2.4	0.2	pyocyanin
24	2.3	0.3	
25	2.2	1) 0.1 PF 43 or PF 48 2) 0.3 CoQ	
26	2.1	1) 0.3 CoQ 2) 0.2 PF 43 or PF 48	
27	2.3	1) 0.2 PF 43 or PF 48 2) 0.1 CoQ	
28	2.35	1) 0.15 PF 43 or PF 48 2) 0.1 CoQ	

In all experiments the buffer was solution III, page 97; the substrate was 0.3 ml 0.2M sodium succinate and 0.1 ml undiluted mouse liver mitochondria were used.

TABLE 29: The effect of Pseudomonas fraction, pyocyanin and its derivatives on the uptake of oxygen by mouse liver mitochondria, measured in the polarograph.

millilitres of Ringer's solution at 37°

5.0 x 3 = 15 microlitres of O_2 when saturated with air. Hence the sample consumed 15 x 26% = 3.9 microlitres of O_2 in 5 minutes.

IN VIVO EXPERIMENTS USING PSEUDOMONAS FRACTIONS

Preliminary experiments were carried out using Porton white mice and albino guinea-pigs.

1. The pathogenic effect of various Pseudomonas spp. on mice.

A stock culture of each of the following -

Pseudomonas aeruginosa NCTC 6750

Pseudomonas diminuta NCTC 8545

Pseudomonas spp. MSU 5306 Western Infirmary, Glasgow,

containing 40 x 10^8 organisms/ml was made from a 24 hour broth culture. 0.5 ml of each stock culture was injected i/p into six mice which were observed hourly. At post mortem swabs were taken of the heart, liver and kidneys: these were plated out on nutrient agar plates.

2. The effect on mice of a supernatant fluid from a 24 hour broth culture.

The culture was centrifuged at 10,000 rpm. for 10 min. at 4° and 0.5 ml of the supernatant fluid or uninoculated nutrient broth was injected i/p into six mice; similarly 0.5ml of the supernatant fluid or uninoculated nutrient broth was injected i/v into six mice. The mice were observed over a period of three weeks.

3. The effect of PF 2 and PF 30 on mice.

0.5 ml of Pseudomonas fraction or control fluid was injected i/p or i/v into six mice; similarly

0.5 ml of Pseudomonas fraction or control fluid was injected sub-cutaneously into the shaved skin of the belly of six mice. The mice were observed over a three week period. The Pseudomonas fractions were sterilised for in vivo experiments by passing them through a Millipore filter, 0.45 nm porosity. These sterilised fractions were tested for their effect on mouse liver mitochondrial respiration.

4. The effect of pyocyanin and 1-hydroxyphenazine (biological preparation) on mice.

0.5 ml of sample containing 0.25 mg of chemical was injected into six mice either i/p or i/v; similarly 0.5 ml was injected subcutaneously into six mice. The mice were observed over a three week period.

5. The effect on mitochondria from mice injected with Pseudomonas aeruginosa.

Nine mice were injected i/p with 1×10^8 organisms of Pseudomonas aeruginosa NCTC 6750. Mitochondria were prepared from the livers of

these mice at 2, 4 and 17 hours after injection. The respiration of these mitochondria, and the effect of a Pseudomonas fraction PF 2 on them, was measured manometrically. (See Table 15).

6) Testing of urine samples from mice injected with Pseudomonas aeruginosa.

Six mice were injected i/p with 0.5 ml of a suspension of Pseudomonas aeruginosa containing 1.3×10^8 organisms. Urine samples were tested with Hema-Combistix at 2 hours, 5 hours and 24 hours after injection to determine any pH change in the urine, or changes in output of protein, carbohydrate or blood. Urine samples from uninoculated mice were used as controls.

7) The effect of PF 2 and PF 30 on guinea pigs.

The guinea pigs were injected i/p with 1 ml PF 2 or PF 30; similarly 1 ml was injected intramuscularly. The guinea pigs were observed over a period of 4 weeks.

These Pseudomonas fractions, PF 2 and PF 30, were sterilised for in vivo experiments by passing them through a millipore filter, 0.45nm porosity. These sterilised fractions were tested for their effect on mouse liver mitochondrial respiration.

8. The effect on mitochondria from mice injected with pyocyanin or 1-hydroxyphenazine (chemical preparations).

Pyocyanin (0.2 ml containing 6.6mg), 1-hydroxyphenazine (0.2 ml containing 2 mg) or diluent was injected either i/p or i/v into six mice. In addition, 0.1 ml 1-hydroxyphenazine (containing 1 mg) was injected i/v into mice. Suspensions of mitochondria were prepared from animals which either died or were sacrificed; respiration of these mitochondria was measured in the polarograph. The experiments were set up as outlined in Table 29, but, in this case, 2.5 ml solution III, page 97, 0.3 ml 0.2 M sodium succinate and 0.2 ml undiluted mitochondria were used throughout.

9. The effects of adding pyocyanin or 1-hydroxyphenazine to macrophages obtained from an albino guinea-pig.

A guinea-pig was injected i/p with 20ml sterile paraffin oil and left for 4 days. The animal was killed and the peritoneal cavity perfused with 200 ml sterile saline. The peritoneal fluid was aspirated, pooled and centrifuged at 1000 rpm for 5 min at 4^{o}; the sediment was resuspended in 1.5ml

of Solution III, page 97. The respiration of the macrophages was measured, utilising 0.2 M sodium succinate, in the polarograph. The effect of pyocyanin or 1-hydroxyphenazine on respiration was tested by a) adding either sample to respiring macrophages and b) preincubation of either sample with macrophages for 1 hour at 37° after which Solution III and substrate were added.

The experiments were set up as outlined in Table 29, but in this case, 2.3 ml Solution III, 0.3 ml 0.2 M sodium succinate, 0.25 ml macrophages and 0.15 ml pyocyanin or 1-hydroxyphenazine (0.5mg per ml stock solution of either sample) were used throughout.

R E S U L T S

1. Oxidase test

The 6 organisms used, namely Pseudomonas aeruginosa, NCTC 6750; Pseudomonas diminuta, NCTC 8545; Pseudomonas aeruginosa, MSU 14388, Stobhill Hospital), Pseudomonas spp.(MSU 5306, Western Infirmary), Pseudomonas hydrophila NCTC 7810 and Pseudomonas spp. NCTC 7452 (achromogenic) were all oxidase positive; i.e. the reagent (1% tetramethylparaphenylene diamine) turned blue within 15 secs.

2. To test for the breakdown of glucose using Hugh and Leifson's medium

Three tubes of Hugh and Leifson's medium were stab inoculated with each of the 6 organisms to be tested; one was incubated aerobically at 37°, one incubated anaerobically at 37° and the surface of the third tube was covered with a layer of sterile paraffin oil and aerobically incubated at 37°.

The 6 organisms utilised glucose oxidatively, i.e. there was a colour change from blue to yellow in the tube which had been incubated aerobically at 37°. There was no utilisation of glucose in either of the other two tubes.

3. Growth at 42° in nutrient broth

The 6 organisms were each inoculated into 2 nutrient broth tubes (4 x ½), one to be incubated at 37° and the other at 42°, for 24 hours. The following grew at both 37° and 42° :-

Pseudomonas aeruginosa, NCTC 6750

Pseudomonas diminuta, NCTC 8545

Pseudomonas aeruginosa (MSU 14388, Stobhill Hospital)

Pseudomonas spp. (MSU 5306, Western Infirmary)

The following grew only at 37°:-

Pseudomonas hydrophila, NCTC 7810

Pseudomonas spp. NCTC 7452 (achromogenic)

4. Preparation of fractions from Pseudomonas cultures:

I. i) Sloppy agar standard medium (incubation at 37° for 24, 48, or 72 hours), see Page 70.

The supernatant fluid was obtained by centrifugation of the sloppy agar cultures after incubation for 24, 48 or 72 hours.

As shown in Table 30, three of the six organisms produced a diffusible pigment. In the case of Pseudomonas aeruginosa, NCTC 6750, and Pseudomonas aeruginosa, MSU 14388, this pigment was a dark blue green after 48 or 72 hours

Organism	Diffusible pigment	Inhibition of O_2 uptake %
Pseudomonas aeruginosa, NCTC 6750	blue - green	90 - 100
Pseudomonas aeruginosa, MSU 14388	blue - green	90 - 100
Pseudomonas spp. MSU 5306	red - brown	90 - 100
Pseudomonas diminuta, NCTC 8545	No pigment	-
Pseudomonas hydrophila, NCTC 7810	No pigment	-
Pseudomonas spp. NCTC 7452 (achromogenic)	No pigment	-

TABLE 30: Pigment production by various Pseudomonas species.

incubation. Pseudomonas spp. (MSU 5306) produced a red-brown pigment. The other three species produced no pigment even after 72 hours incubation. After 24 hours incubation, there was little pigment production by any of the six organisms.

ii) Sloppy agar King, Ward and Raney A (for pyocyanin, incubation at 37° for 24 or 48 hours).
The supernatant fluid was obtained by centrifugation of a sloppy agar culture of Pseudomonas aeruginosa, NCTC 6750 after incubation at 24 or 48 hours; at 24 hours the supernatant fluid was a deep blue-green in colour and at 48 hours, the colour was the same.

iii) Sloppy agar King, Ward and Raney B (for fluorescein; incubation at 37° for 24 or 48 hours).
The supernatant fluid was obtained by centrifugation of a sloppy agar culture of Pseudomonas aeruginosa, NCTC 6750 after incubation at 24 or 48 hours. After 24 hours the colour of the supernatant fluid was pale yellow-green while at 48 hours the colour had deepened to a deeper green.

II. Static liquid culture.

Nutrient broth, King, Ward and Raney liquid media A and B were inoculated with Pseudomonas aeruginosa, NCTC 6750 and incubated for 18, 52 76 and 120 hours. There was no evidence of pigment productions in any of these liquid cultures.

III. Shaking liquid culture.

This method was the same as II except that the cultures were shaken for 24 or 48 hours. After 24 hours, the nutrient broth culture fluid was faintly brown while at 48 hours the colour had deepened. After 24 hours, the K.W. & R. medium A culture fluid was pale green while after 48 hours the colour was more pronounced. After 24 hours, the K.W. & R. medium B culture fluid was bright green which intensified after 48 hours incubation.

5. Methanol precipitation of PF 1 and PF 36.

When equal volumes of Pseudomonas fraction and methanol were left at -6° overnight, there was no precipitation although the fluid appeared to be faintly cloudy.

When Pseudomonas fraction: methanol was 1:3 or 1:6 (v/v) a heavy precipitate was formed. This was collected by centrifugation at 10,000 rpm for 10 mins at 4^{o}. When Pseudomonas fraction: methanol was 1:9 (v/v) a fine precipitate was formed. This was collected by centrifugation at 10,000 rpm for 10 mins at 4^{o}.

6. Chloroform extraction of PF 1 and PF 2

Extraction of crude Pseudomonas fraction with Analar chloroform resulted in a yield of 1 mg dried chloroform extract per 1 ml original Pseudomonas fraction. The chloroform layer was deep blue in colour while the aqueous portion was brown in colour.

7. Fractionation of chloroform extracts of PF 2 on an alumina column.

2 ml concentrated chloroform extract was placed on an alumina column and eluted with chloroform. Three fractions were obtained -

i) yellow PF 33

ii) pale green PF 34

iii) blue PF 35

All the fractions of the same colour were pooled, the chloroform evaporated off and the dried sediment resuspended in the following solvents - chloroform, petroleum ether, acetone, ethanol, ethyl-acetate, benzene and distilled water. The solubility of each of the fractions in these solvents is shown in Figure 21.

It will be noticed that, in general, these fractions could not be completely separated on the basis of their solubilities in organic solvents. The most striking solubility difference was observed when the three fractions were dissolved in acetone; the solubility of the green fraction was > the blue > the yellow.

The ultraviolet spectra of these fractions was compared with that of PF 2 using the Unicam Sp 800 spectrophotometer. The yellow fraction showed a peak at 275 nm. The green fraction showed peaks at 253 nm and 370 nm. The blue fraction showed a peak at 330 nm. The parent Pseudomonas fraction, PF 2, showed peaks at 255 nm and 330 nm (Figure 22).

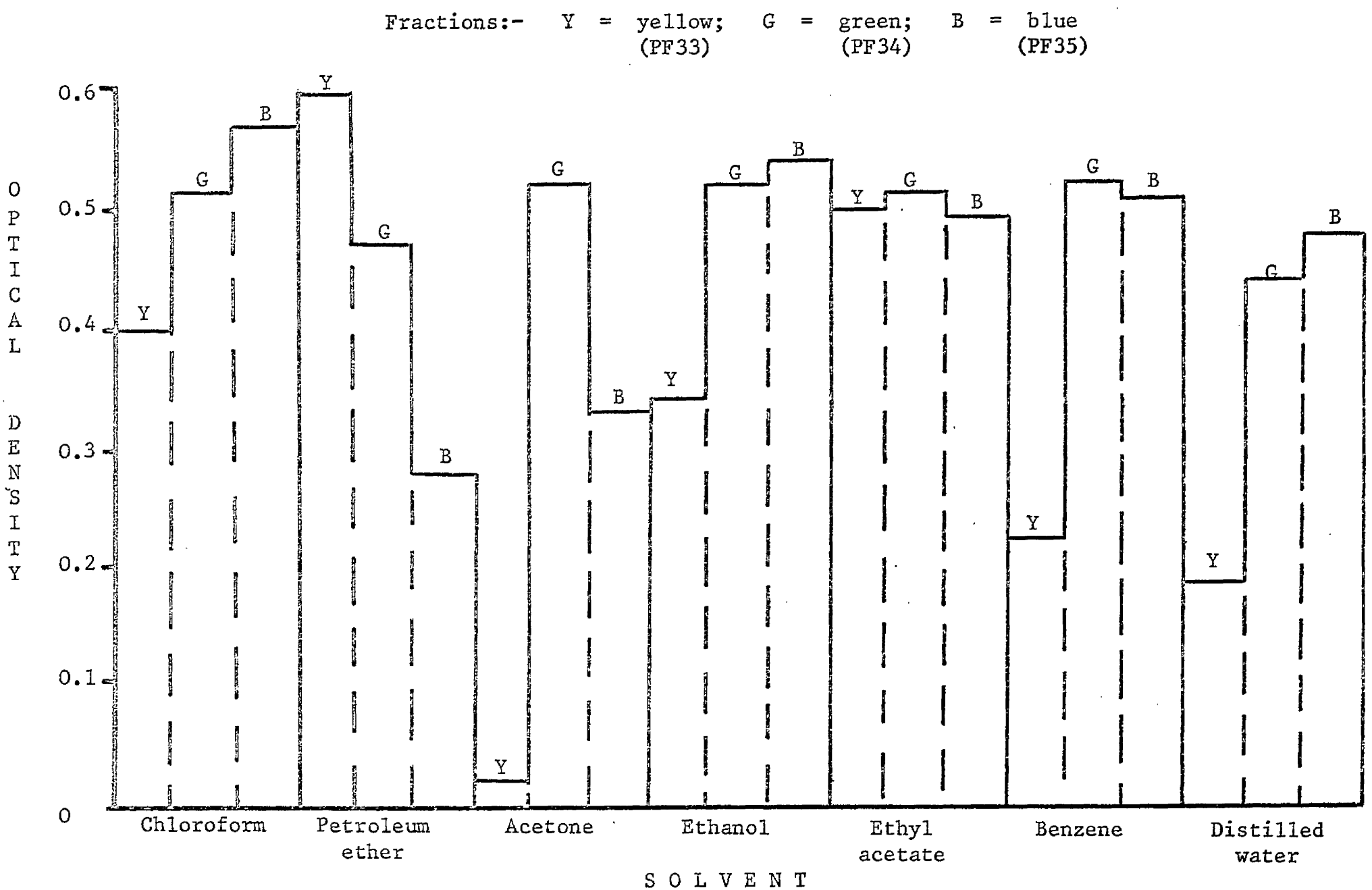


Figure 21 The solubility of Pseudomonas fractions (ex alumina) in various solvents

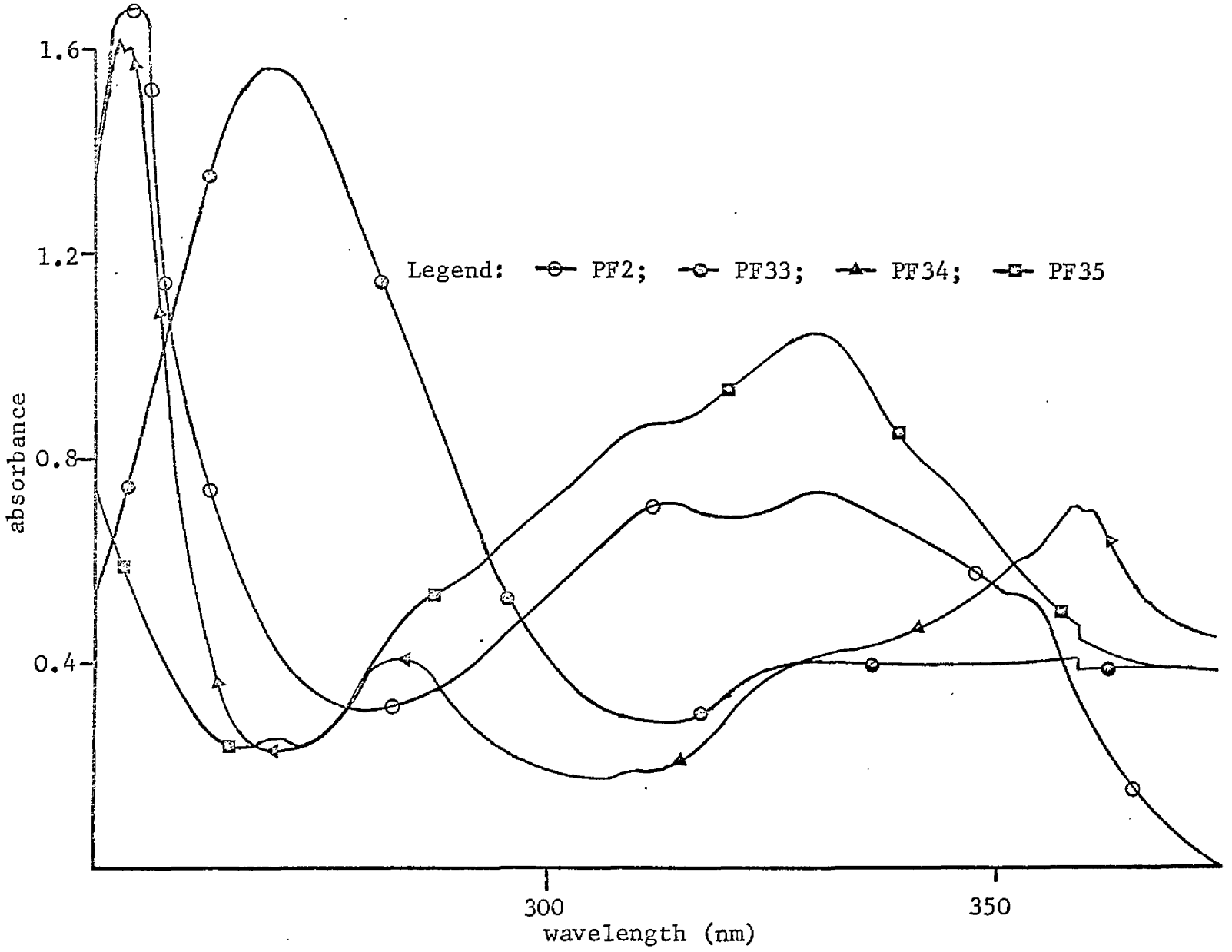


Figure 22 The ultraviolet spectra of fractions from an alumina column

8. Sephadex gel filtration of supernatant fluids from Pseudomonas cultures.

PF 1 was filtered through columns of G 10, G 15, G 25 and G 75 Sephadex. Three sets of fractions were obtained -

i) brown PF 36

ii) yellow-brown to colourless PF 37

iii) blue PF 38

from G 10, G 15, and G 25 Sephadex columns. The elution pattern of these fractions from G 10, G 15 and G 25 Sephadex was very similar (Figure 23). Although the elution patterns showed that these coloured fractions were not separated within distinct peaks, the activity on mitochondria determined manometrically (see page 147) coincided with the colour pattern.

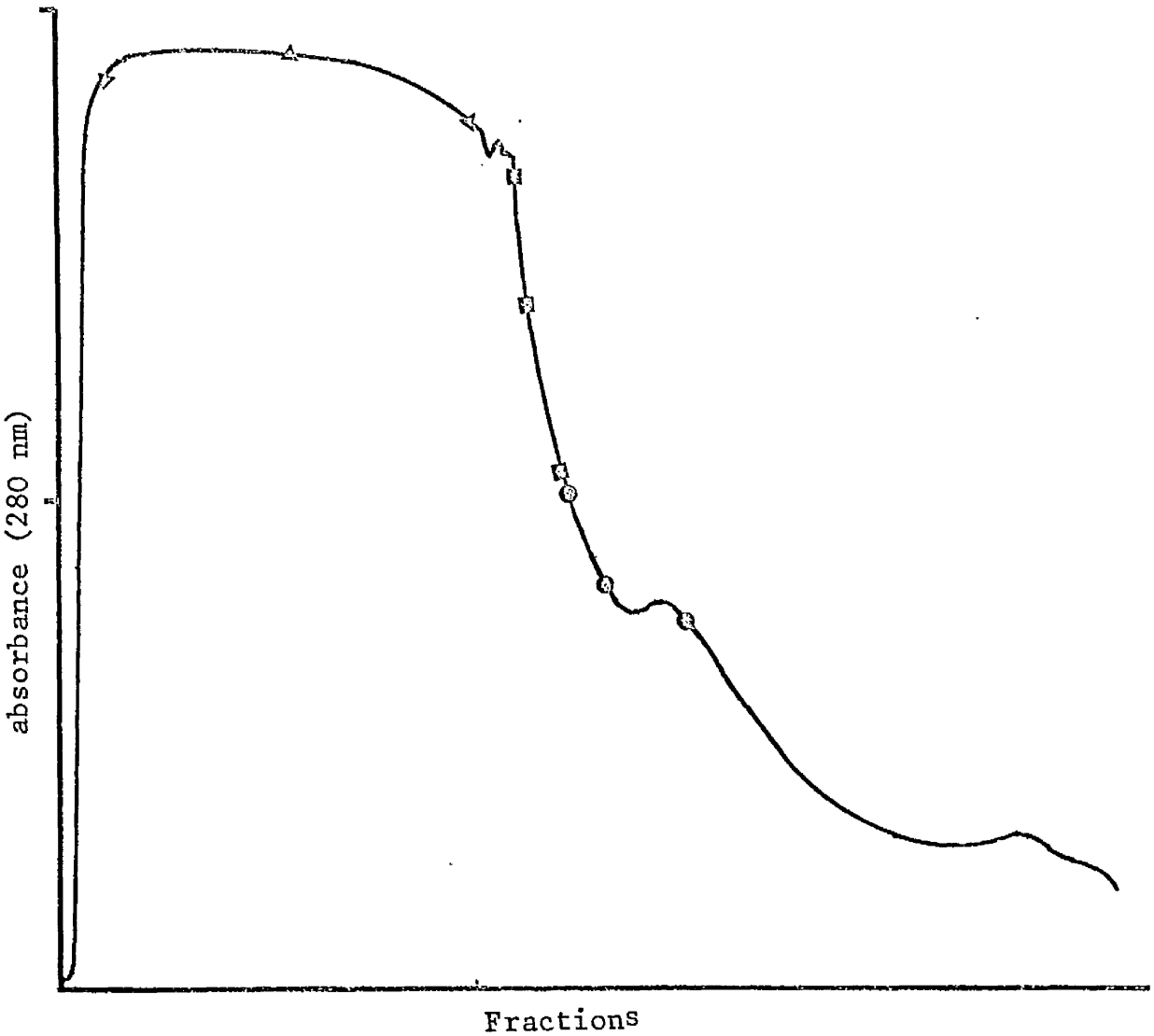
Three sets of fractions -

i) colourless PF 39

ii) brown PF 40

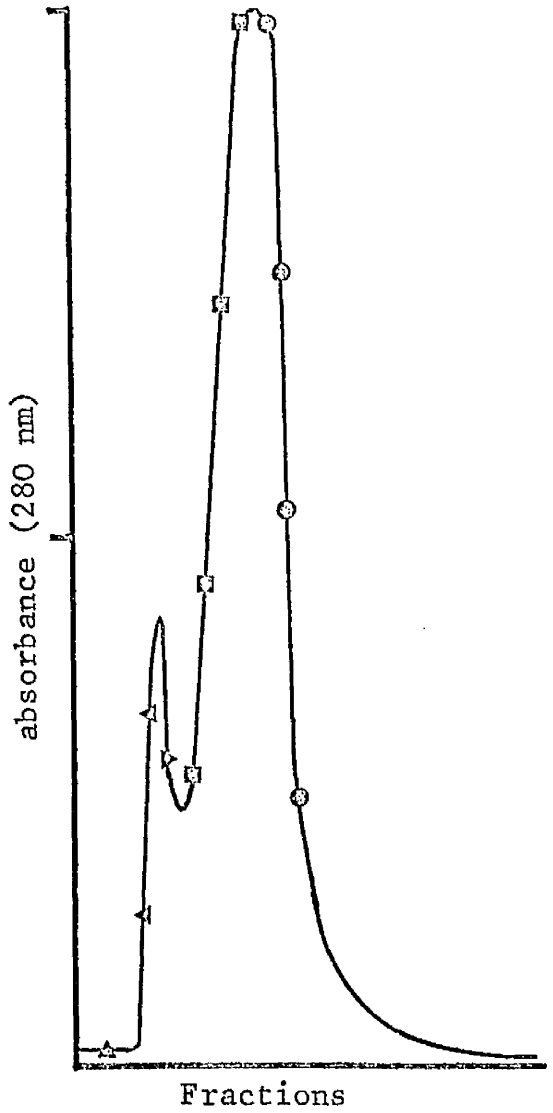
iii) green PF 41

were obtained from the G 75 Sephadex column. The elution pattern of these fractions is shown in Figure 24. A definite separation into a major and minor peak was obtained. However, the use of 0.05 M Hendry's phosphate buffer, pH 6.45



Legend: —△— brown (PF36); —□— colourless (PF37); —○— blue (PF38)

Figure 23 The elution pattern of fractions from G10, G15 or G25 Sephadex columns



Legend: colourless (PF39)
brown (PF40)
green (PF41)

Figure 24 The elution pattern of fractions from a G75 Sephadex column

(i.e. $\frac{1}{3}$ solution I) and 0.03 M Hendry's phosphate buffer, pH 6.45 (i.e. $^{1}/_{5}$ solution I) resulted in a slight difference in elution of the two fractions. With 0.05 M Hendry's phosphate buffer, pH 6.45 the elution fractions were as follows:

1 - 33 ml colourless

34 - 48 ml brown

49 - 63 ml green

whereas with 0.03 M Hendry's phosphate buffer, pH 6.45, the elution was as follows:

Tubes 1 - 39 ml colourless

40 - 57 ml brown

58 - 75 ml green

It is interesting that the activity on mitochondria, see page 147 did not coincide with the colour pattern of the fractions.

9. <u>The synthesis of pyocyanin and its derivatives.</u>

i) Biologically synthesised pyocyanin and its derivatives.

The ultraviolet spectra of pyocyanin, 1 - hydroxyphenazine and similar samples from Dr R. Schoental were obtained in the Unicam SP 800 ultraviolet spectrophotometer using methanol as the reference solvent.

a) Pyocyanin showed a peak at 320 nm.

b) Pyocyanin (Schoental) showed a peak at 320 nm.

c) 1-hydroxyphenazine showed a peak at 297 nm.

d) 1-hydroxyphenazine (Schoental) showed a peak at 295 nm, as shown in Figure 25.

ii) Chemically synthesised pyocyanin and its derivatives.

The ultraviolet spectra of pyocyanin, 1-hydroxyphenazine, 1-methoxyphenazine and pyrogallol-monomethylether were obtained in the Unicam SP 800 ultraviolet spectrophotometer using methanol as reference

a) Pyocyanin showed a peak at 320 nm.

b) 1-hydroxyphenazine showed a peak at 263 nm.

c) 1-methoxyphenazine showed a peak at 260 nm.

d) pyrogallol monomethylether showed a peak at 268 nm, as shown in Figures 26 and 27.

10. Dialysis of Pseudomonas fractions.

After dialysis of PF 2 at 4° for 48 hours with either distilled water or 0.03 M Hendry's phosphate buffer, pH 6.45, the Pseudomonas fraction was brown but the fluid outside the dialysis sac was green. There was no change in the volume of the sample during dialysis.

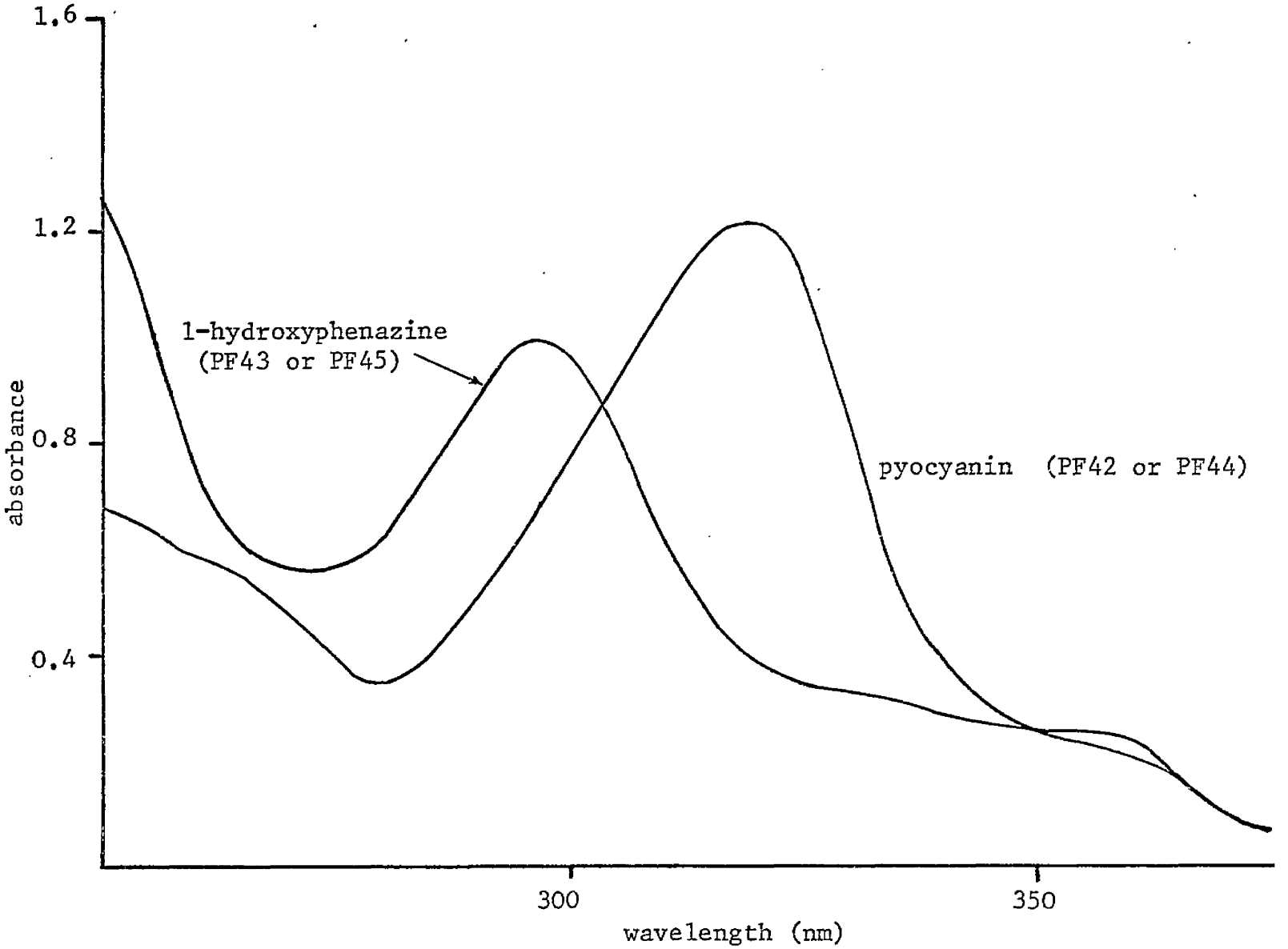


Figure 25 The ultraviolet spectra of a biological preparation of 1-hydroxyphenazine and pyocyanin

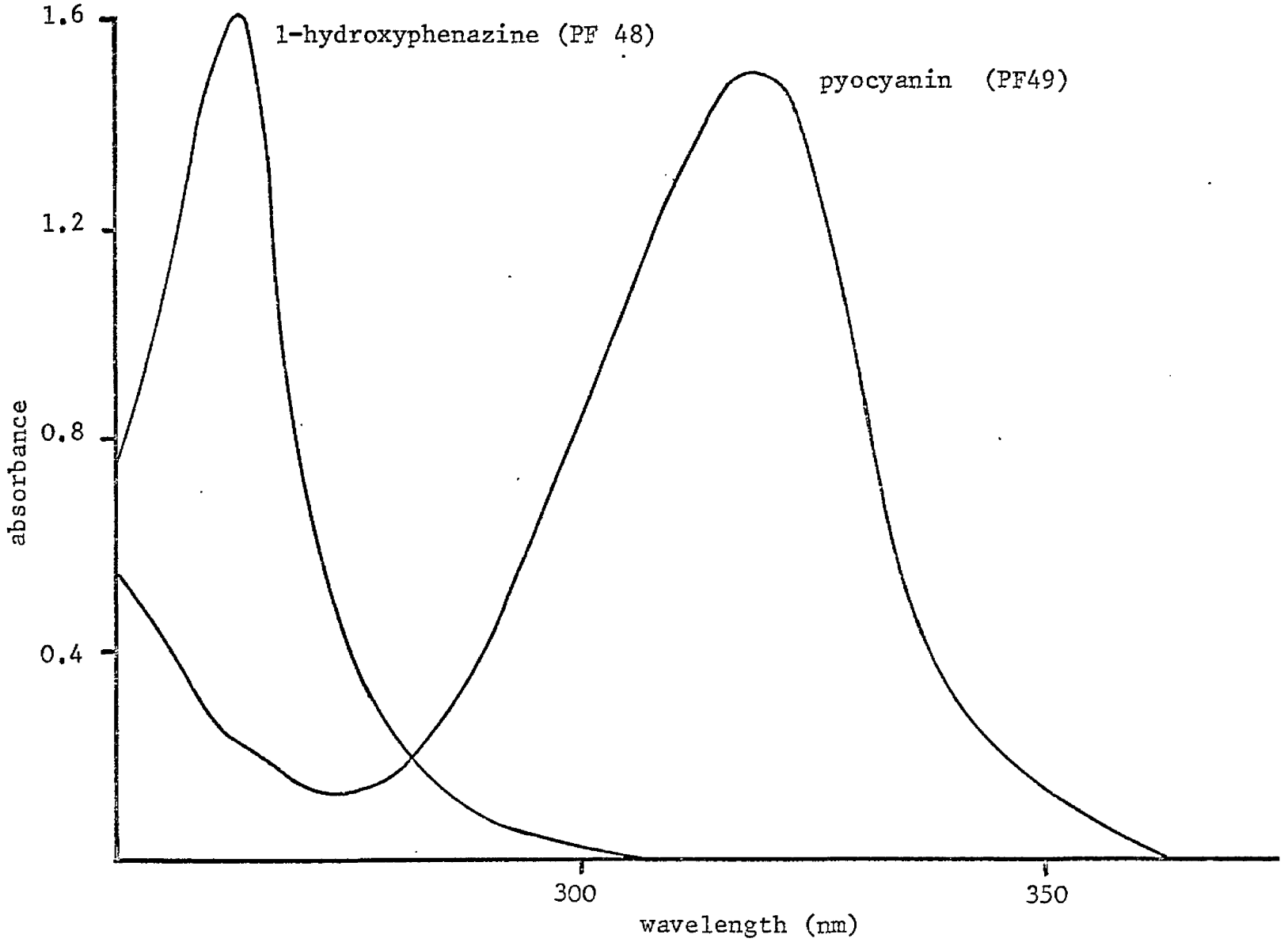


Figure 26 The ultraviolet spectra of a chemical preparation of 1-hydroxyphenazine and pyocyanin

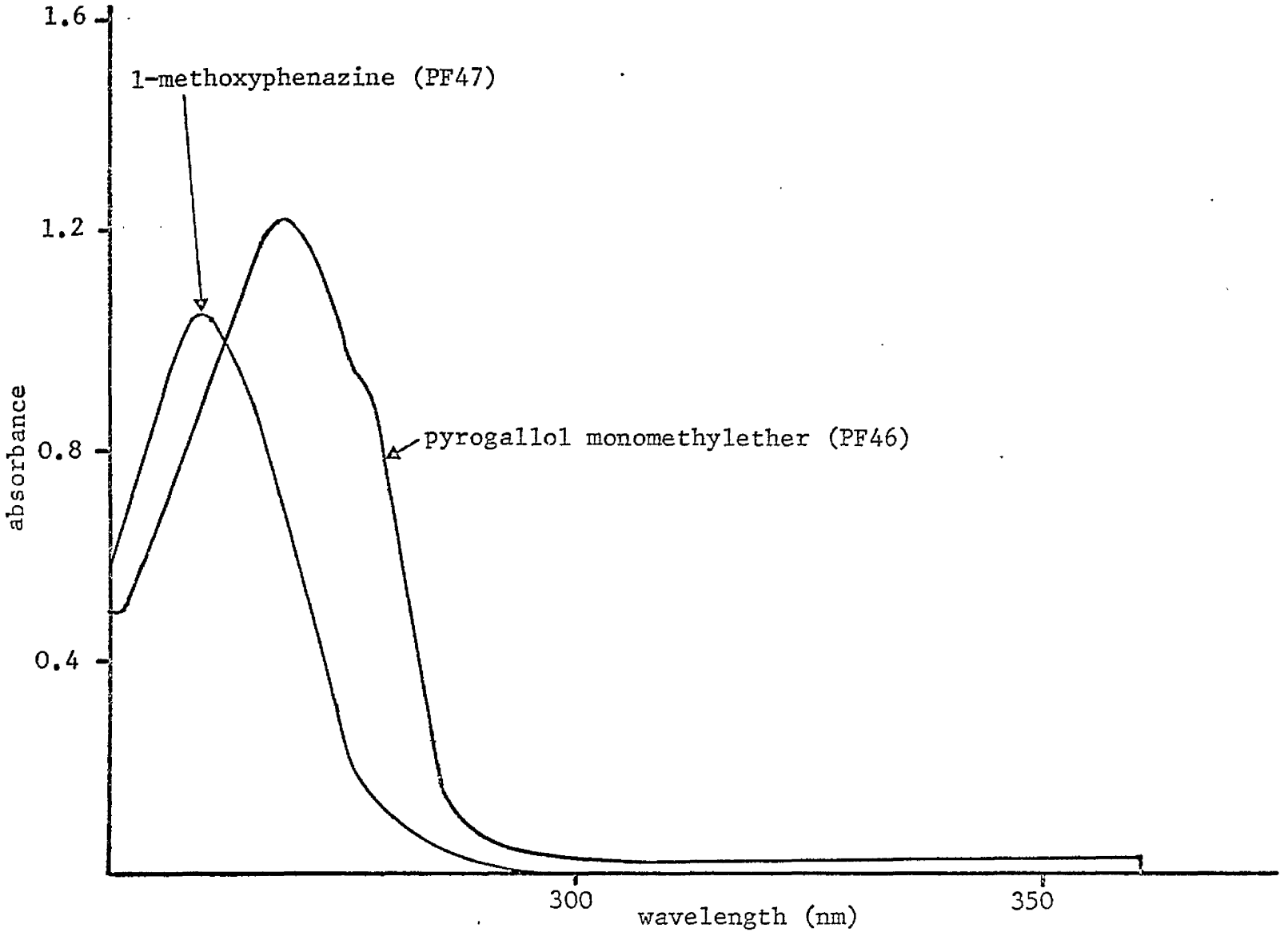


Figure 27 The ultraviolet spectra of a chemical preparation of 1-methoxyphenazine and pyrogallol monomethylether

11. Paper Chromatography of Pseudomonas fractions.

Two dimensional amino-acid chromatography of acid hydrolysates of PF 2, PF 30, PF 30 (trypsinised) and PF 2C was carried out. Both PF 2 and PF 2C showed the following amino-acid spots, aspartic acid, glutamic acid, serine, glycine, alanine, lysine, valine, leucine and iso-leucine; these were probably derived from culture fluid. PF 30 and PF 30 trypsinised showed no traces of amino acids.

12. Haemolytic properties of Pseudomonas fractions

The haemolytic activity of PF 2 on rabbit, mouse, sheep, horse and human red blood corpuscles was tested. The haemolytic titres were low. With sheep red blood corpuscles no lysis was seen, with horse red blood corpuscles the titre varied from $\frac{1}{4}$ or $1/8$; with rabbit, mouse and human red blood corpuscles the titre was $1/32$ for all three.

13. Antibiotic activity of Pseudomonas fractions.

Five fractions - PF 2C, PF 2 NCTC 6750, PF 30, PF 32 and PF 2 NCTC 8545 - were tested for their antibiotic activity on the following organisms:

Staphylococcus aureus, NCTC 4135

Escherichia coli, NCTC 8196

Streptococcal spp. HE 7

Bacillus spp. 77MD0559

Method A.

Basins were cut in a non-nutrient agar plate pre-seeded with one of the organisms under test. Each of the basins was filled with one of the fractions. Staphylococcus aureus was inhibited by both PF 2 and PF 32 (Plate 1); Escherichia coli was not inhibited by any of the 5 fractions; the Streptococcal spp. HE7 showed less inhibition by both PF 2 and PF 32 (Plate 2); the Bacillus spp. 77MD0559 was inhibited only by PF 2.

Method B

A trough was cut in a nutrient agar plate and filled with a mixture of 1 ml double strength agar and 1 ml of the fraction to be tested; see page 96. One set of plates was streak inoculated with each of the four organisms and PF 2 inhibited the growth of Staphylococcus aureus almost completely whereas the other three organisms were less inhibited, Plate 3.

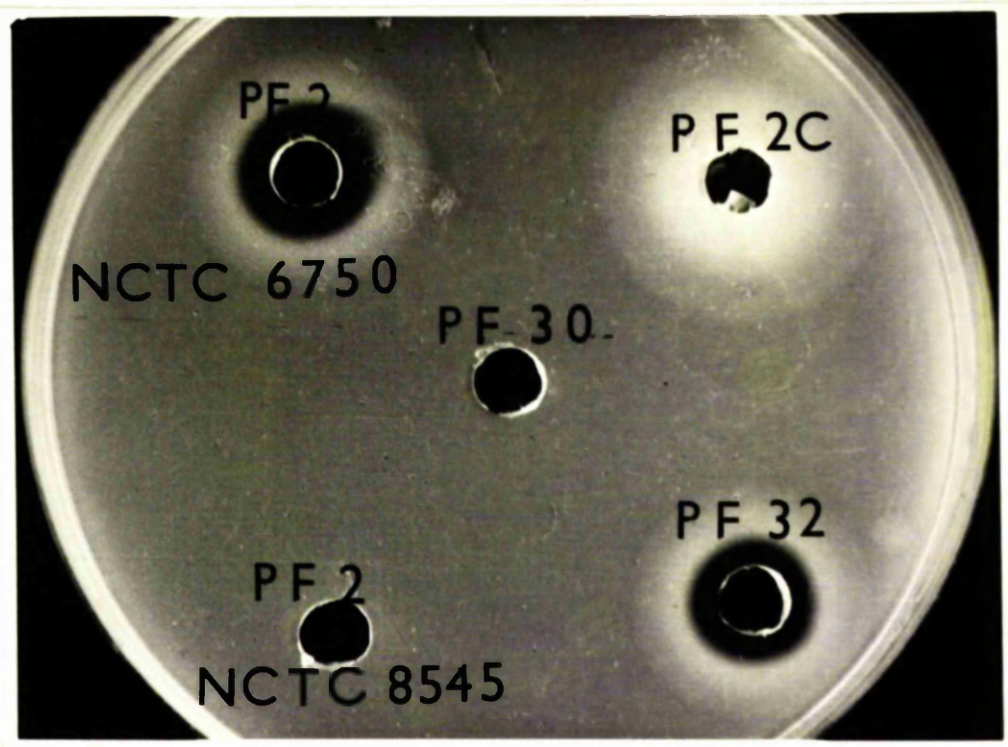


Plate 1. Note zones of inhibition with fractions PF 2 and PF 32. The antibiotic activity of Pseudomonas fractions on Staphylococcus aureus, NCTC 4135.

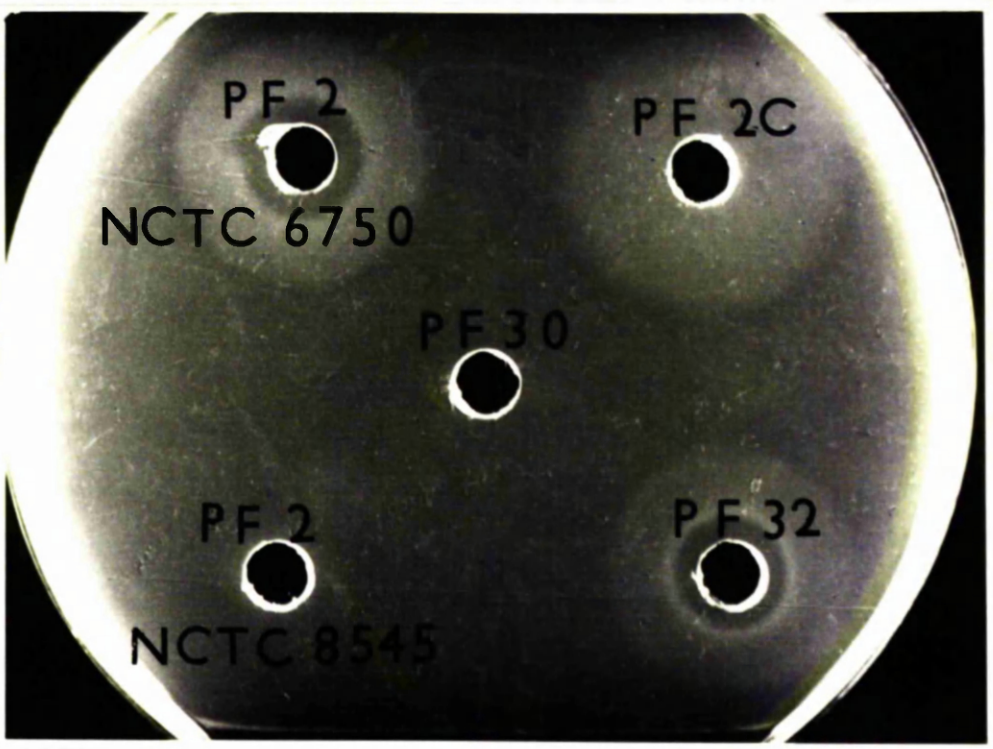


Plate 2. Note zones of inhibition with fractions PF 2 and PF 32. The antibiotic activity of Pseudomonas fractions on Streptococcal species, HE 7.

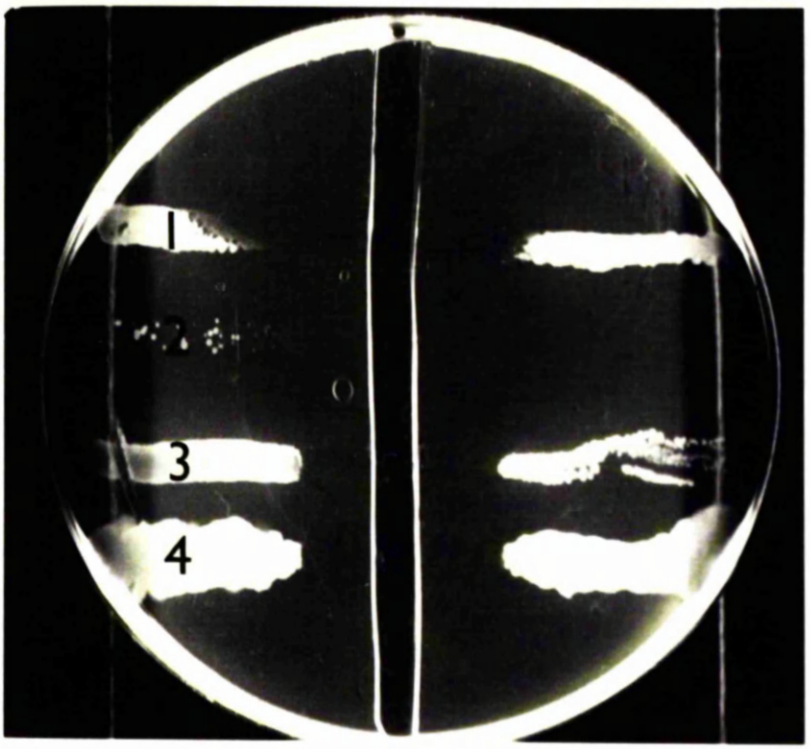


Plate 3. The antibiotic activity of PF 2 on 1) Escherichia coli, 2) Staphylococcus aureus, 3) Streptococcal spp. HE 77, and 4) Bacillus spp. 77HD0559.

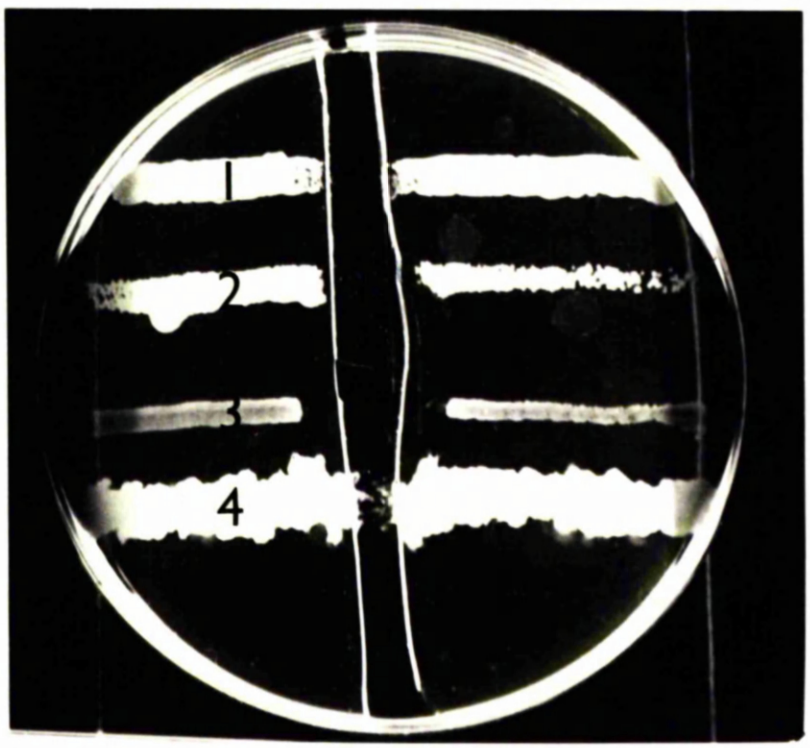


Plate 4. The antibiotic activity of PF 32 on 1) Escherichia coli, 2) Staphylococcus aureus, 3) Streptococcal spp. HE 7 and 4) Bacillus spp. 77HD0559.

PF 32 inhibited the growth of the streptococcal species and to a very slight extent the growth of Staphylococcus aureus, Plate 4.

A second set of plates was left overnight, to allow diffusion of the Pseudomonas fractions and streaked with each of the four organisms together with Pseudomonas aeruginosa and another Pseudomonas spp. PF 2 was strongly inhibitory to all the organisms except the two Pseudomonas spp. (Plate 5).

PF 32 was still only inhibitory to the Streptococcal spp. HE7, and slightly inhibitory to Staphylococcus aureus (Plate 6).

The other fractions PF 2C, PF 30 and PF 2 (NCTC 8545) showed no inhibitory action.

14. The effect of Pseudomonas fractions, pyocyanin, and its derivatives on mitochondrial respiration using the Warburg manometric technique.

The activity of Pseudomonas fractions was measured by comparing the oxygen uptake of treated and untreated mitochondria. An impairment of 90 - 100% was regarded as significant; all assays were carried out in duplicate and only those experiments where pairs closely corresponded

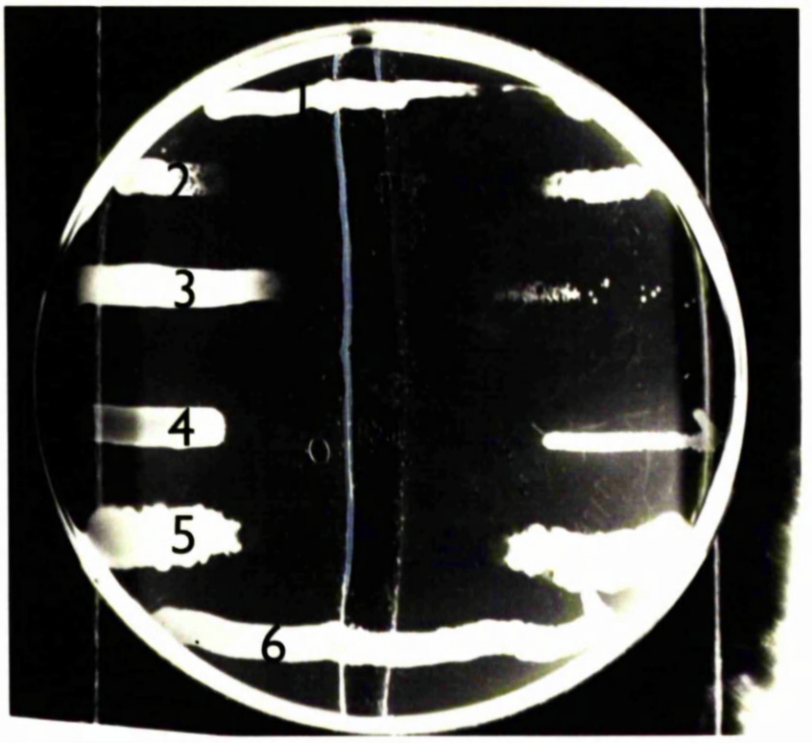


Plate 5. The antibiotic activity of PF 2 (diffused overnight) on 1) Pseudomonas spp., 2) Escherichia coli, 3) Staphylococcus aureus, 4) Streptococcus spp. HE 7, 5) Bacillus spp. 77MD0559, and 6) Pseudomonas aeruginosa.

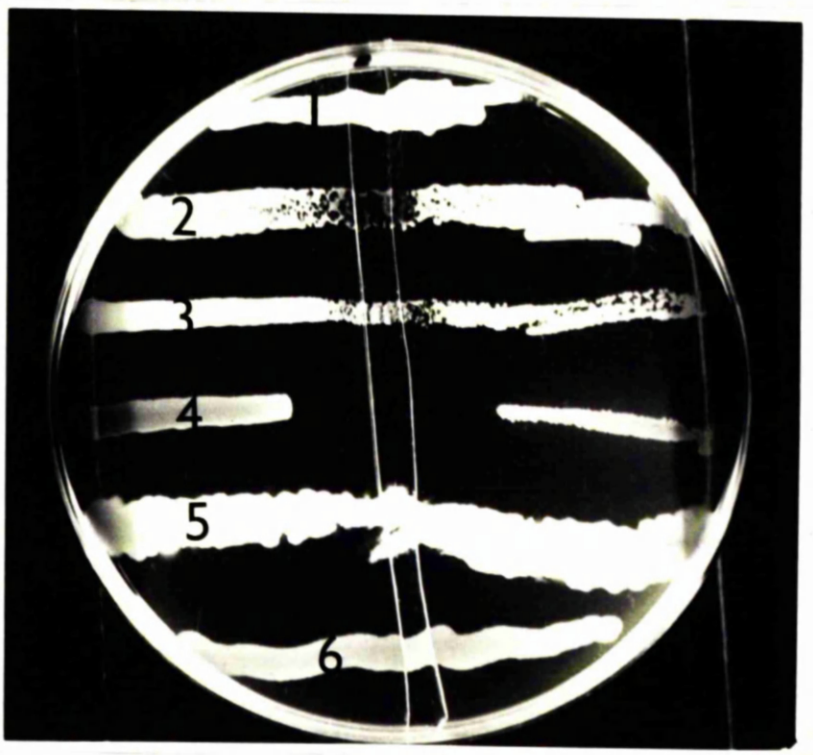


Plate 6. The antibiotic activity of PF 32 (diffused overnight) on 1) Pseudomonas spp., 2) Escherichia coli, 3) Staphylococcus aureus, 4) Streptococcus spp. HE 7, 5) Bacillus spp. 77MD0559, and 6) Pseudomonas aeruginosa.

(up to 5% variation) were accepted as valid.

1) The effect of different methods of production of Pseudomonas fractions on their activity towards mitochondrial respiration.

Four methods of production using three different media were tested. (See pages 70 - 74).

i) Sloppy agar cultures

24 hours incubation at 37°. As shown in Figure 28, fractions PF 1 and PF 2 caused strong inhibition of O_2 uptake. Before the Pseudomonas fractions were tipped into the main well of the Warburg flask there was no difference in the uptake of O_2 between test and control. After tipping, the uptake of O_2 in the control was 328 µl after 90 mins. whereas the uptake in the presence of a) PF 1 was 92 µl, b) PF 2 was 96 µl and c) PF 3 was 218 µl.

48 hour incubation at 37°

Fractions PF 1, PF 2 and PF 3 caused strong inhibition of O_2 uptake. The uptake of O_2 in the control after 90 mins. was 325 µl and in

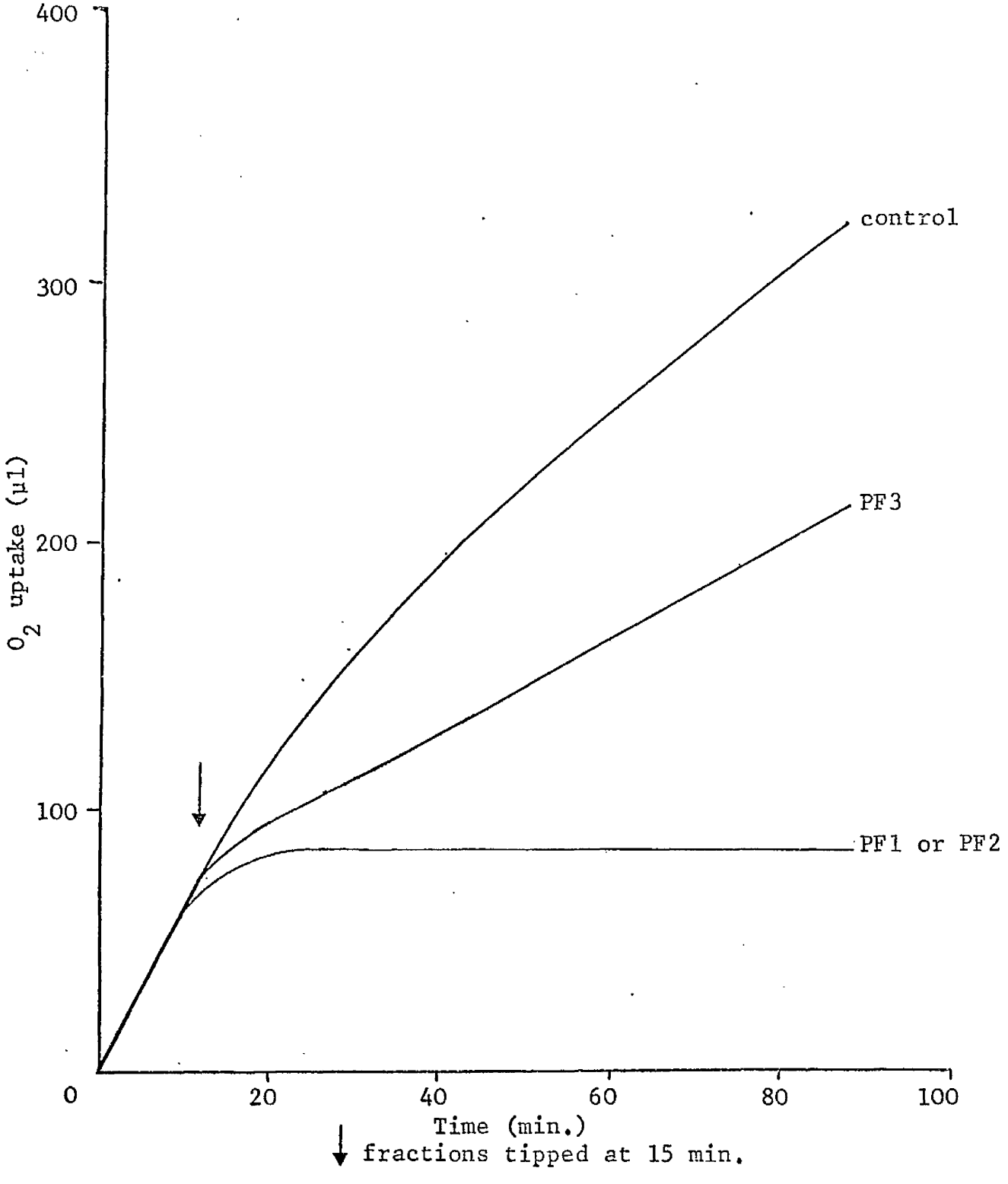


Figure 28 The effect of sloppy agar culture supernatant fluids from Pseudomonas aeruginosa (24 hours at 37°) on the respiration of mouse liver mitochondria

the tests containing PF 1, PF 2 or PF 3 was 118 µl, 112 µl and 110 µl respectively.

ii) Static liquid cultures

Fractions PF 4, PF 5 and PF 6 caused no inhibition of mitochondrial respiration; after 75 min. the O_2 uptake was similar in both the test and control flasks.

iii) Shaking liquid cultures.

24 hours incubation at 37°

Fractions PF 7, PF 8 and PF 9 caused slight inhibition of mitochondrial respiration; after 75 min. the O_2 uptake in the control flasks was 291 µl whereas in PF 7, PF 8 and PF 9, it was 221 µl, 276 µl and 245 µl respectively.

48 hours incubation at 37°

Fractions PF 7 and PF 8 caused an increased inhibition in the region of 50% when prepared from 48 hour old cultures; the O_2 uptake in the control flasks was 326 µl whereas in PF 7, PF 8 and PF 9, it was 156 µl, 152 µl and 247 µl respectively, after 90 min.

iv) Disintegrates of Pseudomonas organisms.

None of the supernatant fluids (PF 10, PF 11 and PF 12) caused any significant inhibition of mitochondrial respiration. The O_2 uptake was similar in both the test and control flasks after 90 min.

2) The effect of Pseudomonas fractions on mitochondrial respiration.

As shown in Figure 29, there was no difference observed when a) mitochondria were preincubated with the Pseudomonas fraction, b) the fraction was added immediately after equilibration, and c) the fraction was added to actively respiring mitochondria; in all cases, the inhibition of O_2 uptake was greater than 90%. In addition it was found that the inhibitory effect of the Pseudomonas fraction could be diluted out; the O_2 uptake by a $^1/_{48}$ dilution was 10 µl and by a $^1/_{360}$ dilution was 300 µl after 80 min. d) when Pseudomonas fractions PF 2 and PF 30 were tested it was found that there was no difference between the inhibition caused by heated or unheated fractions, Figure 30.

e) The effect of trypsinised Pseudomonas fractions on mitochondria.

Trypsinisation (3 hours at 37°) did not affect the inhibitory activity of PF 2 or PF 30; the O_2 uptake in the control flask after 80 min. was 400 µl, in flasks containing untreated PF 2 or PF 30 it was 5 µl and in flasks containing treated PF 2 or PF 30 it was 9 µl.

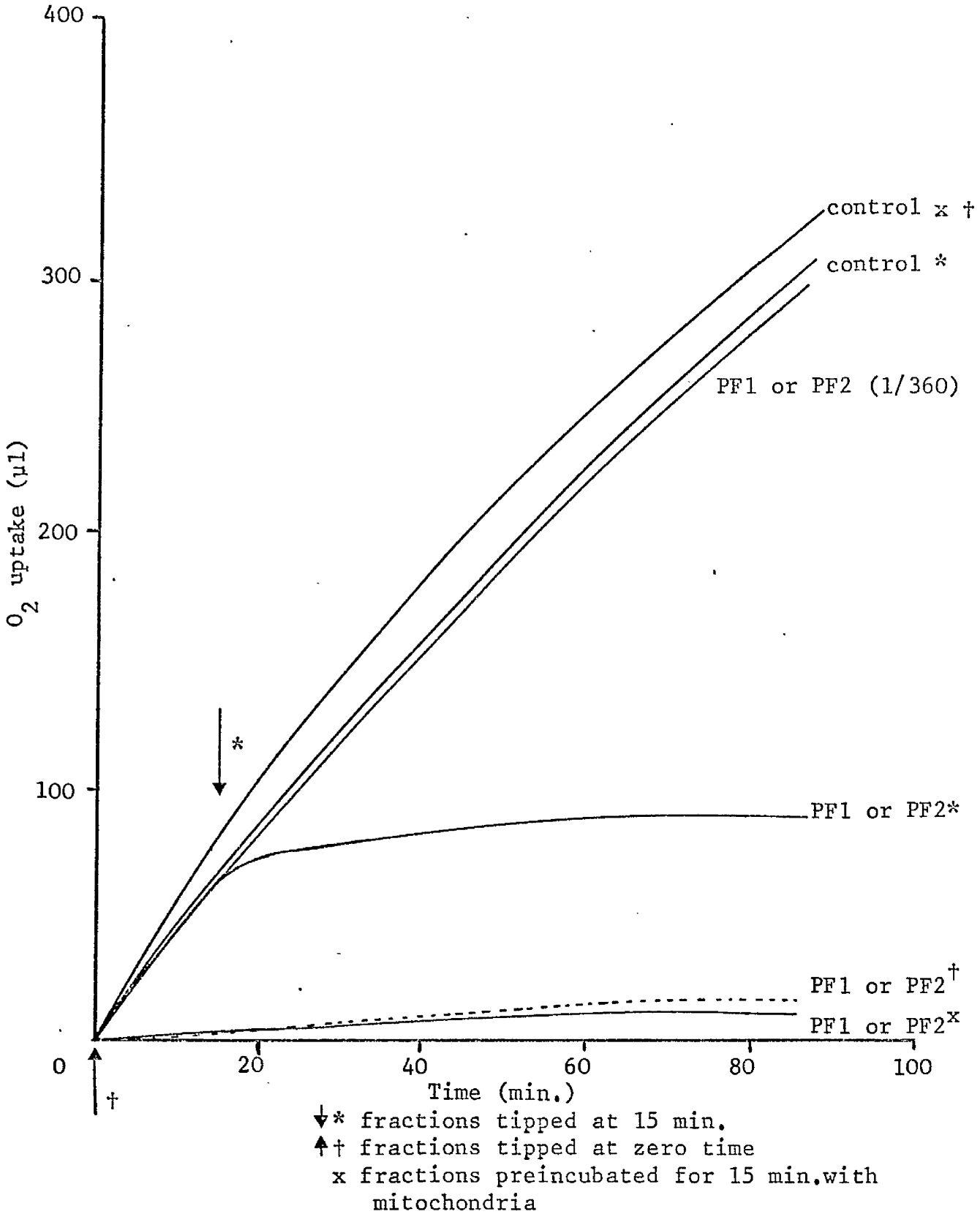


Figure 29 The effect of adding Pseudomonas fractions to mouse liver mitochondria at various times and the effect of diluting Pseudomonas fractions

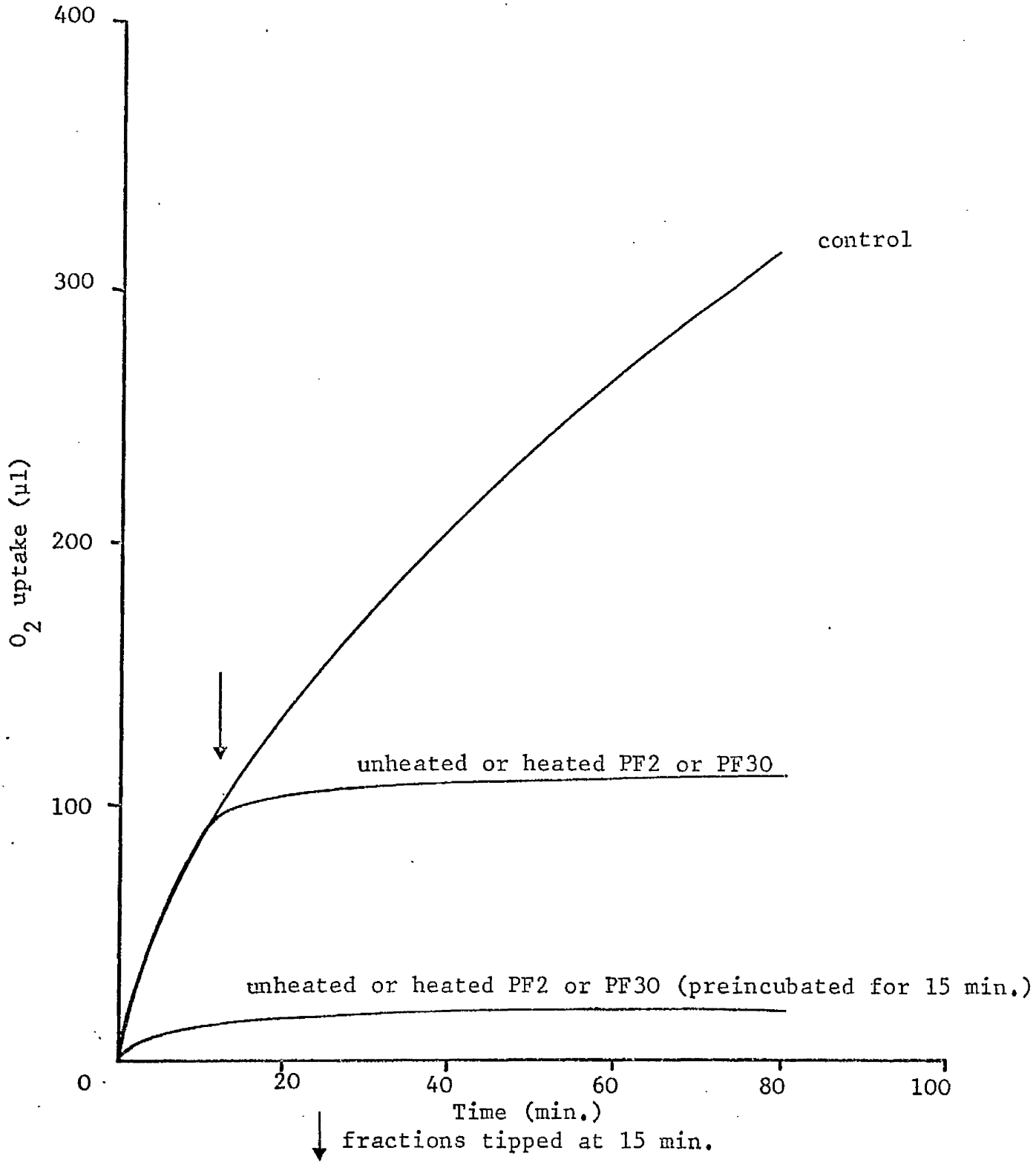


Figure 30 The effect of heated and unheated Pseudomonas fractions on the respiration of mouse liver mitochondria

f) The effect of proteinase-treated Pseudomonas fractions on mitochondria.

The fractions, treated with proteinase for 18 hours at 55°, were tipped at zero time. It was found that this treatment slightly reduced the inhibitory activity of the fractions without affecting the corresponding control fluids (Figure 31). This result provides further evidence for the elucidation of the chemical nature of the active principle and will be discussed later.

g) The effect of pepsin treated Pseudomonas fractions on mitochondria.

The fractions digested with pepsin for 3 hours at 37° were tipped 15 min. after zero time. The O_2 uptake in the control flasks was 372 µl after 90 min.; two additional controls were included in this experiment -

i) pepsin-digested PF 20, in which the O_2 uptake after 90 min. was 350 µl and

ii) PF 2 or PF 30, incubated at 37° for 3 hours at pH 2, (i.e. in distilled water) in which the O_2 uptake after 90 min. was 202 µl (Figure 32). These controls were included since addition of pepsin or distilled water

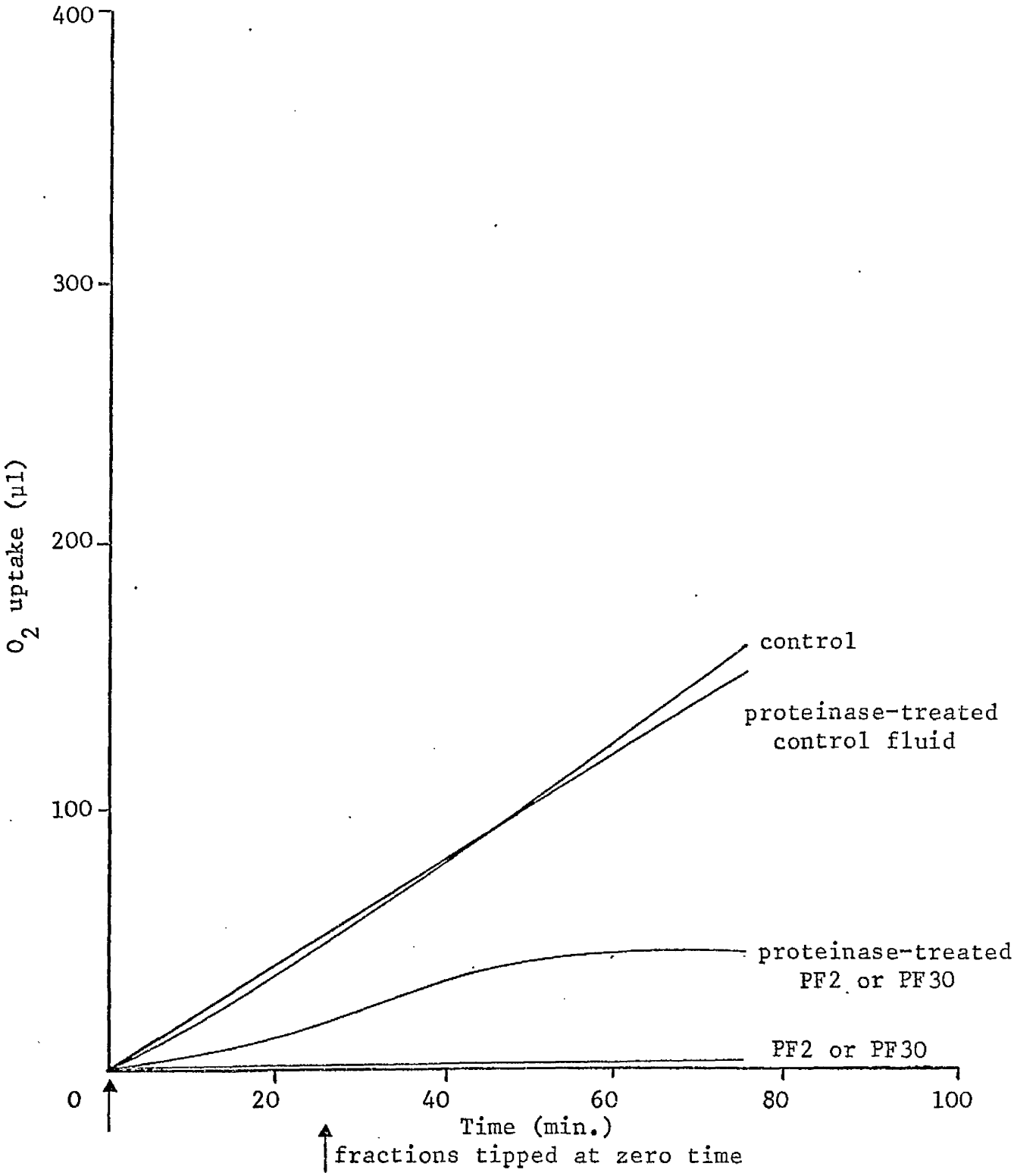


Figure 31 The effect of proteinase-treated Pseudomonas fractions on the respiration of mouse liver mitochondria

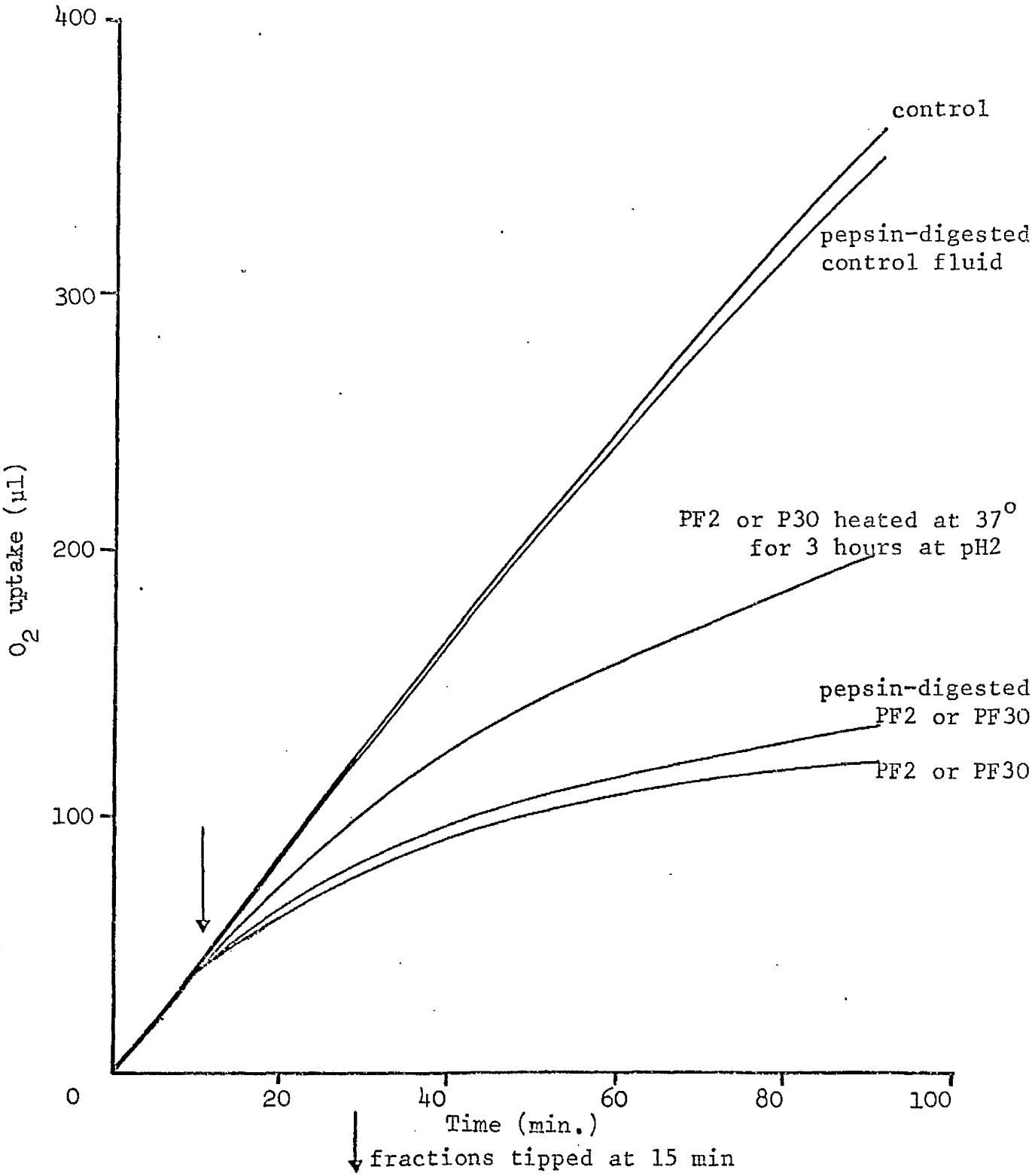


Figure 32 The effect of pepsin-digested Pseudomonas fractions on the respiration of mouse liver mitochondria

to supernatant fractions or control fluid resulted in a drop of pH as follows:-

	pH
1 ml pepsin + 1 ml PF 2 or PF 30	2
1 ml pepsin + 1 ml PF 20	2
1 ml distilled water + 1 ml PF 2 or PF 30	2
1 ml buffer + 1 ml PF 2 or PF 30	7 - 8
1 ml buffer + 1 ml PF 20	7 - 8

The O_2 uptake in flasks containing PF 2 or PF 30 was 120 µl after 90 min., in flasks containing pepsin-treated PF 2 or PF 30 was 140 µl after 90 min. It was concluded that the pepsin treated fractions showed similar activity to untreated fractions.

h) Acid hydrolysis of Pseudomonas fractions.

Fractions PF 2, PF 30 and trypsinised PF 2 were treated with 6N HCl for 18 hours at 105°. This treatment removed most of the inhibitory activity of these fractions on mitochondrial respiration. The O_2 uptake in the untreated control flasks was 319 µl after 80 min., in the treated control flasks was 311 µl after 80 min., in the flasks containing treated fractions it was 290 µl, in the flasks containing untreated fractions it was 131 µl. The values

for additional controls included in this experiment were i) flask containing control fluid heated at 105° for 18 hours, 264 µl, and ii) flasks containing Pseudomonas fractions, heated at 105° for 18 hours, 255 µl. The heat treatment by itself affected both the control fluid and the inhibitory action of the Pseudomonas fractions, although not to the same extent as found with acid treated preparations. An important point to note here, is that the control fluids and fractions were heated at 105° for 18 hours in Hendry's sucrose phosphate buffer, pH 7.3 (solution III, page 97). These results are shown in Figure 33.

i) The effect of dialysed Pseudomonas fractions and dialysate on mitochondria.

The fractions were dialysed against distilled water or solution I (page 80) for 48 hours. It was found that dialysed PF 2 possessed no inhibitory activity on mitochondrial respiration; the O_2 uptake in the control flasks was 200 µl after 80 min. and in the flasks containing the dialysed fraction or dialysing solution it was 180 µl. In addition, recombination of the dialysed PF 2 and the dialysing solution had little effect; the O_2 uptake was 168µl. The uptake in the flask containing non-dialysed PF2 was 6µl in 80min, (Figure 34).

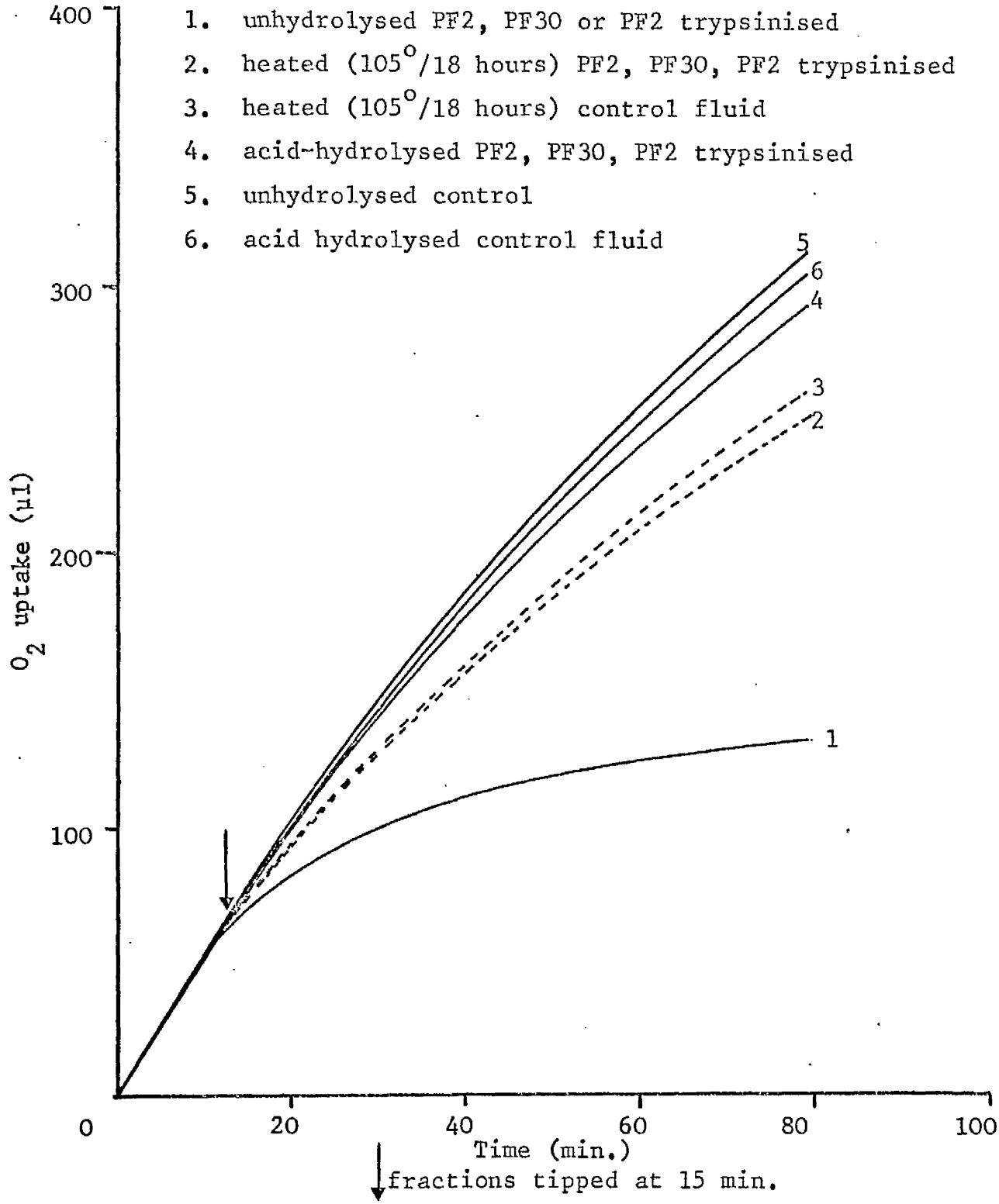


Figure 33 The effect of acid-hydrolysed Pseudomonas fractions on the respiration of mouse liver mitochondria

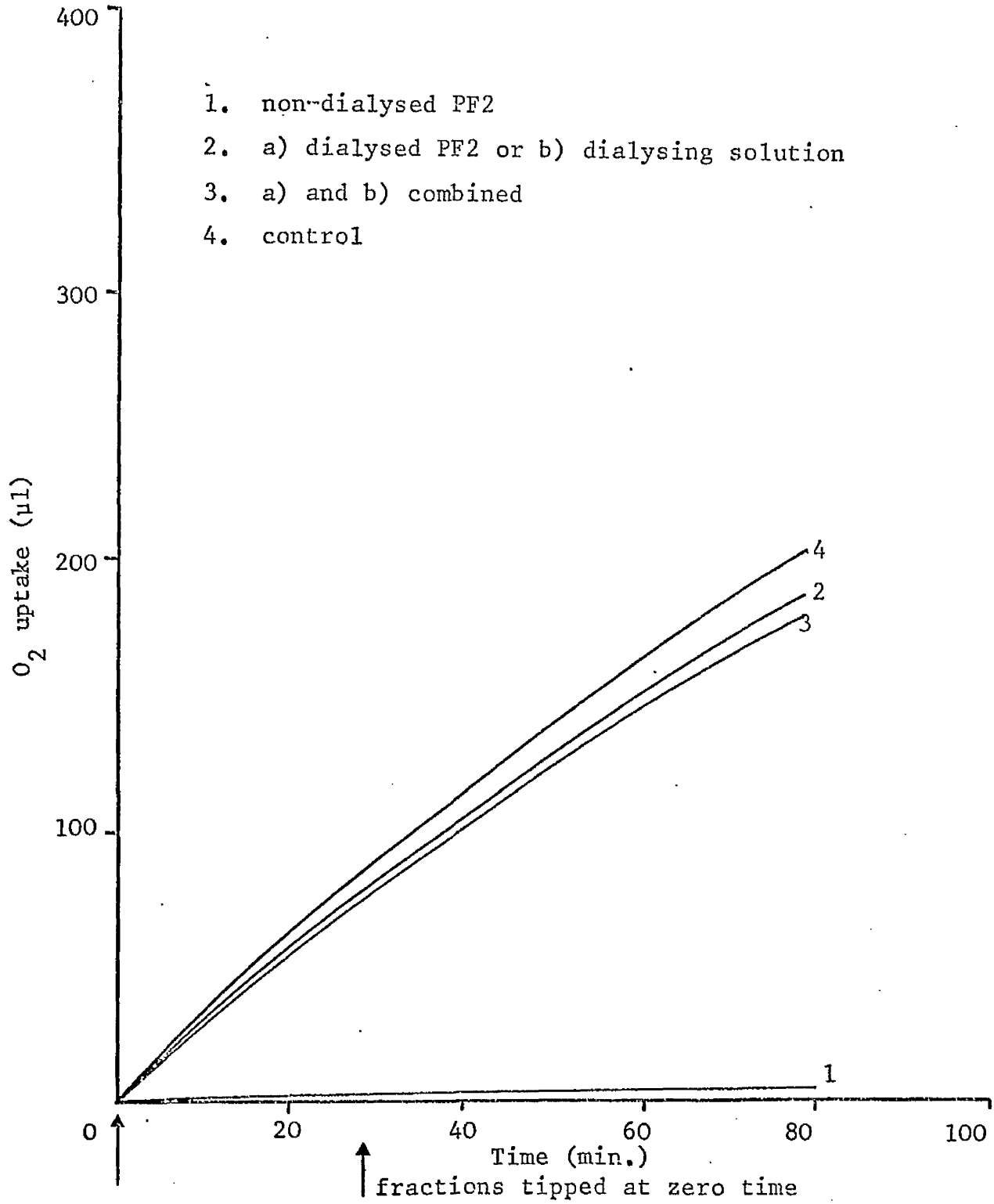


Figure 34 The effect of dialysed Pseudomonas fraction and dialysing solution on the respiration of mouse liver mitochondria

j) The effect of absorbed Pseudomonas fractions on mitochondria.

The experiment was set up as outlined in Table 15, using Pseudomonas fractions absorbed with mouse liver mitochondria. As shown in Figure 35, absorption of PF 2 with mouse liver mitochondria reduced its inhibitory action on mouse liver mitochondrial respiration when tipped on to respiring mitochondria 15 min. after zero time. The O_2 uptake by mitochondria treated with PF 2 was 117 µl in 85 min., and by mitochondria treated with absorbed PF 2 was 200 µl in 85 min.; in the control flask the uptake was 367 µl in 85 min.

k) The effect of methanol-precipitated Pseudomonas fractions on mitochondria. (precipitation of PF 2 and PF 36 using 1, 3, 6 and 9 volumes of methanol respectively, page 75.) The experiment was set up as outlined in Table 15 using the precipitates and supernatant fluids (i.e. PF 13-28) from methanol precipitation of PF 2 and PF 36. None of these fractions inhibited O_2 uptake by mouse liver mitochondria.

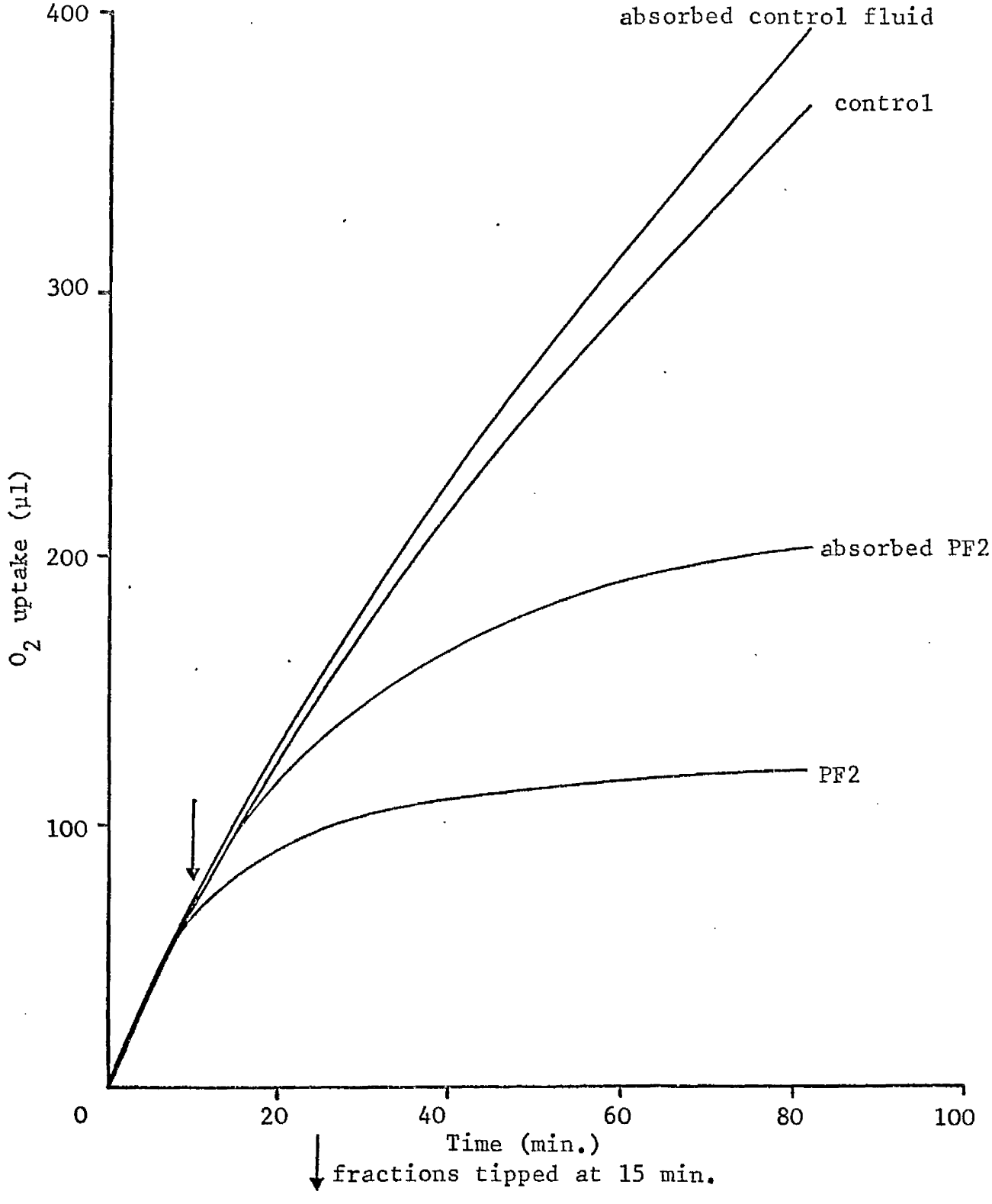


Figure 35 The effect of Pseudomonas fractions, absorbed with mitochondria, on the respiration of mouse liver mitochondria

1) The effect of chloroform extracted Pseudomonas fractions on mitochondria.

Equal volumes of fraction and chloroform were shaken together and allowed to settle out in a separating funnel. The experiment was set up as outlined in Table 15; the Pseudomonas fractions (PF 1, PF 2, PF 29, PF 30, PF 31, PF 32) were tipped on to respiring mitochondria 15 min. after zero time. The chloroform extract of Pseudomonas fractions, PF 1 or PF 2, showed an inhibitory effect on the uptake of oxygen by mouse liver mitochondria, Figure 36. The O_2 uptake by mitochondria treated with non-chloroform extracted PF 1 or PF 2 was 87 µl in 90 min., by mitochondria treated with the chloroform extracts PF 29 or PF 30 was 107 µl in 90 min. After chloroform extraction of PF 1 and PF 2 it was found that the inhibitory effect was lost; the O_2 uptake by mitochondria treated with PF 31 or PF 32 was 274 µl in 90 min. which was similar to the result obtained in the control flasks.

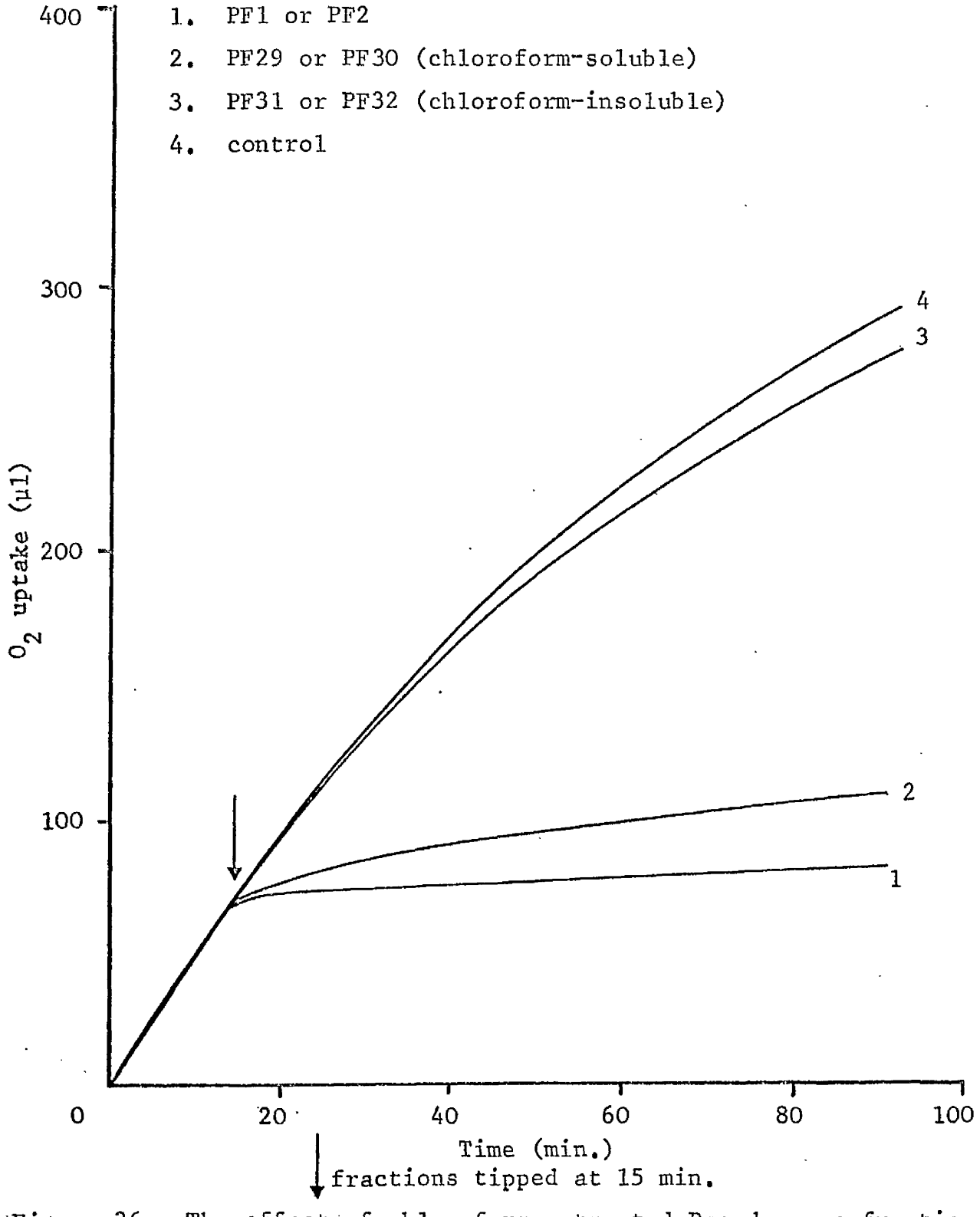


Figure 36 The effect of chloroform extracted Pseudomonas fractions on the respiration of mouse liver mitochondria

m) The effect of Pseudomonas fractions, from an alumina column, on mitochondria.

The experiment was set up as outlined in Table 15. Three fractions were obtained from the alumina column, yellow (PF 33), pale green (PF 34) and blue (PF 35) and the effect of each of these on respiring mitochondria was observed. As shown in Figure 37, most of the inhibitory factor was eluted in PF 33; however, there was still some inhibitory activity in PF 34 and PF 35. The O_2 uptake by mitochondria treated with PF 33, PF 34 or PF 35 was 135 µl, 183 µl and 191 µl respectively compared with the control value of 239 µl in 80 min.; the O_2 uptake by PF 2 was 96 µl in 80 min.

n) The effect of Pseudomonas fractions from Sephadex columns on mitochondria.

The experiment was set up as outlined in Table 15. Three series of coloured fractions were obtained from G 10, G 15 and G 25 Sephadex columns; brown (PF 36), yellow-brown to

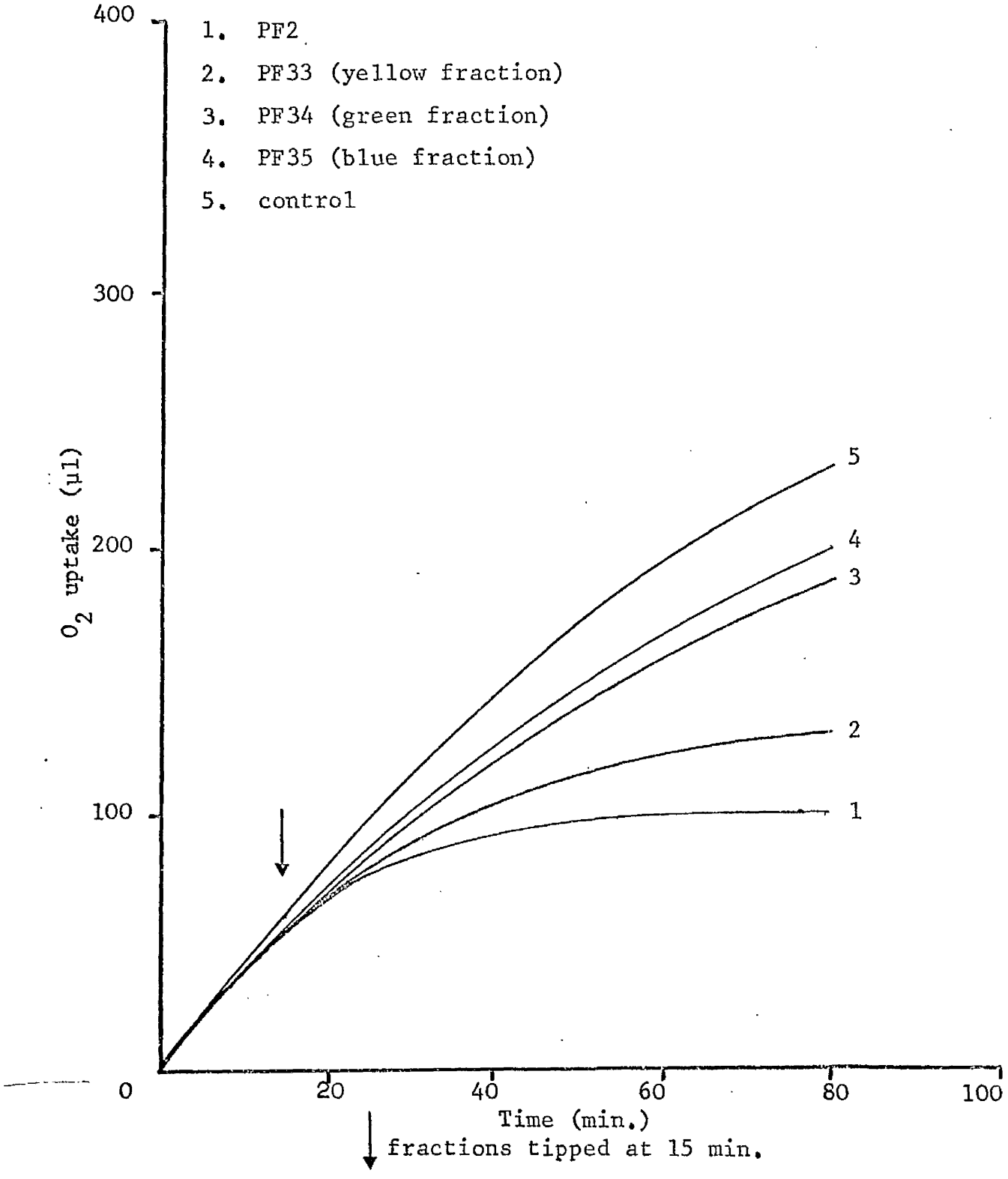


Figure 37 The effect of Pseudomonas fractions from an alumina column on the respiration of mouse liver mitochondria

colourless (PF 37), blue (PF 38) and their effect on the O_2 uptake by mouse liver mitochondria was observed. The fractions were added to the mitochondria immediately after the zero reading.

As shown in Figure 38, PF 36 (from Sephadex G 10, G 15 and G 25) showed an inhibitory effect on the uptake of oxygen by mouse liver mitochondria, whereas PF 37 showed no inhibitory effect and PF 38 showed an insignificant inhibitory effect.

Three sets of fractions were obtained from Sephadex G 75 columns; colourless fractions corresponding to the minor peak (PF 39), brown (PF 40) and green (PF 41) corresponding to the major peak.

An inhibitory effect on the uptake of O_2 by mouse liver mitochondria was found with PF 41 and to a lesser extent with PF 39 and PF 40, as shown in Figure 39.

o) i) <u>The effect of biologically synthesised fractions on mitochondria.</u>

The experiment was set up as outlined in Table 15 using pyocyanin (PF 42) and 1 - hydroxyphenazine (PF 43) which were

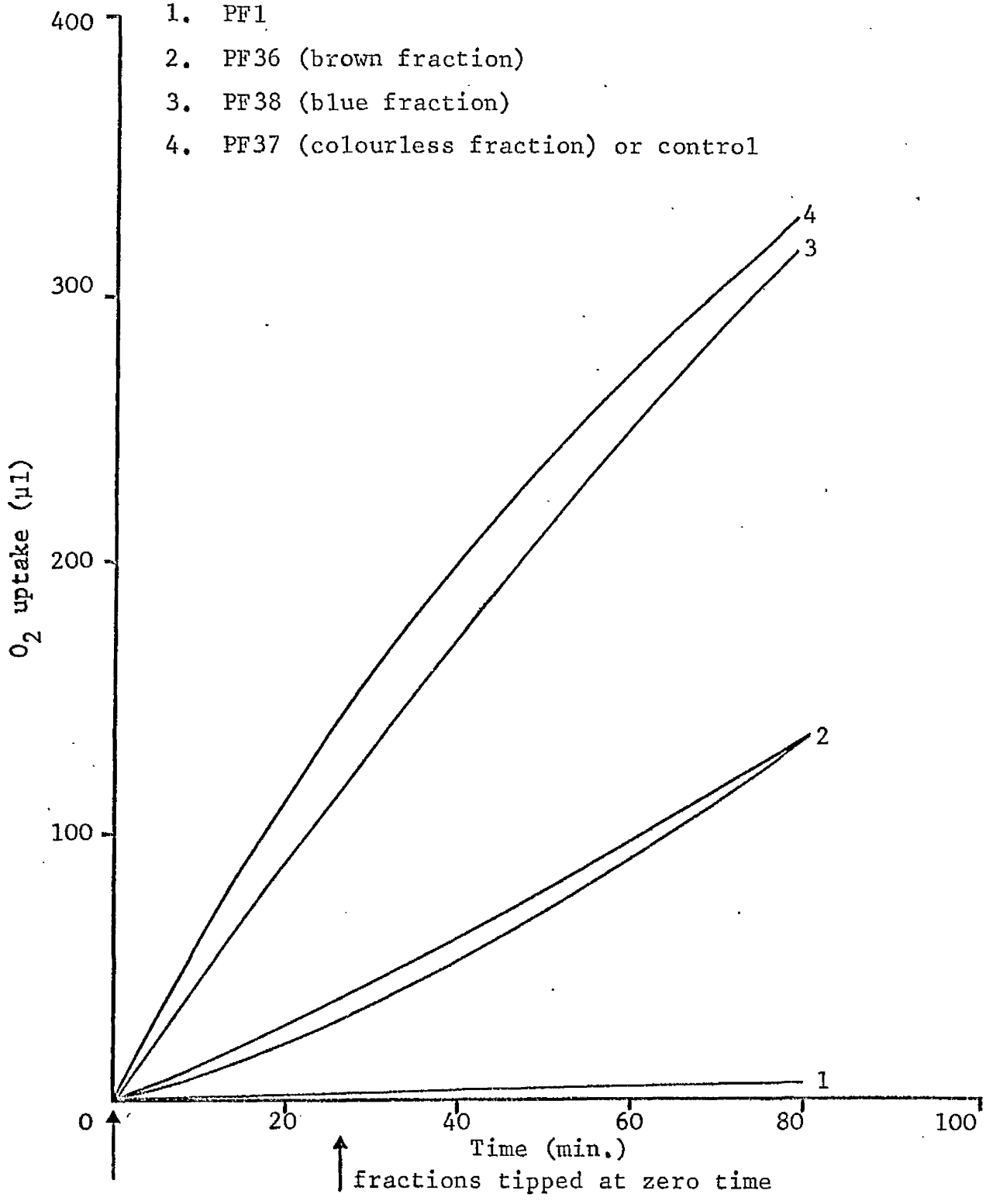


Figure 38 The effect of Pseudomonas fractions from Sephadex columns - G10, G15 and G25 - on the respiration of mouse liver mitochondria

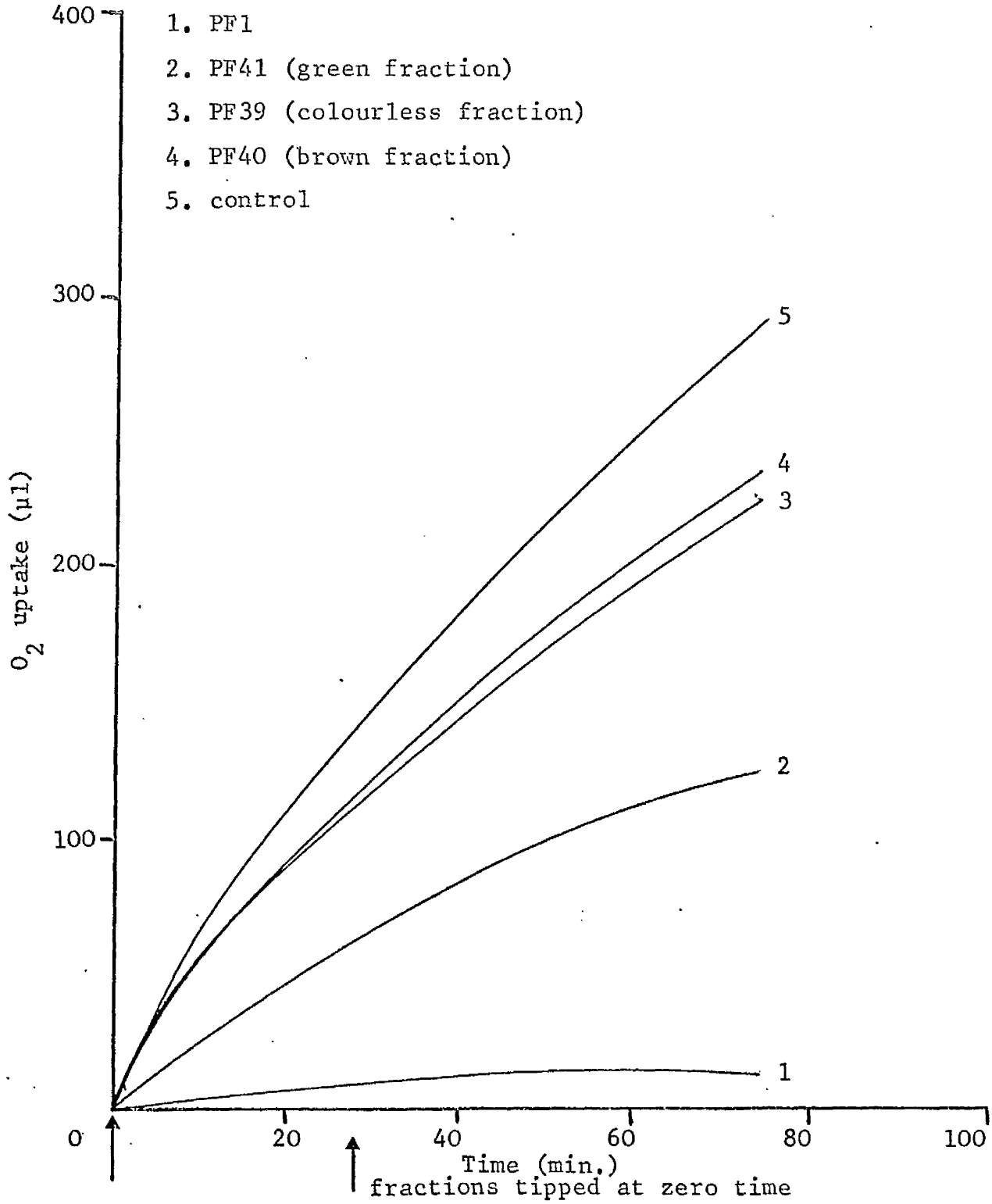


Figure 39 The effect of Pseudomonas fractions from a G75 Sephadex column on the respiration of mouse liver mitochondria

tipped on to respiring mitochondria 15 min. after zero time. In addition, biological preparations of pyocyanin (PF 44) and 1 - hydroxyphenazine (PF 45) from Dr R. Schoental were tested for a comparison.

As shown in Figure 40, both PF 43 and PF 45 showed an inhibitory effect on the uptake of oxygen by mouse liver mitochondria in final concentrations of 0.08 mg/ml, 0.16 mg/ml and 0.25 mg/ml reaction mixture. The O_2 uptake by mitochondria treated with PF 43 and PF 45 was 81 µl in 75 min. regardless of which concentration was used. The O_2 uptake by mitochondria treated with PF 2 was 104 µl in 75 min.; by mitochondria in the control flasks was 357 µl in 75 min.

The fractions PF 42 and PF 44 showed a lesser inhibitory effect on the uptake of oxygen by mouse liver mitochondria but, in this case, the inhibition was concentration dependent; with PF 42 or PF 44, inhibition with 0.25 mg/ml reaction mixture > 0.16 mg/ml > 0.08 mg/ml.

Legend for Figure 40 overleaf

1. PF2
2. PF43 or PF45 (i.e. biological 1-hydroxyphenazine in concentrations of 0.08 mg/ml, 0.16 mg/ml, 0.25 mg/ml.)
3. PF42 or PF44 (i.e. biological pyocyanin concentration 0.25 mg/ml.)
4. PF42 or PF44 (concentration 0.16 mg/ml.)
5. PF42 or PF44 (concentration 0.08 mg/ml.)
6. control

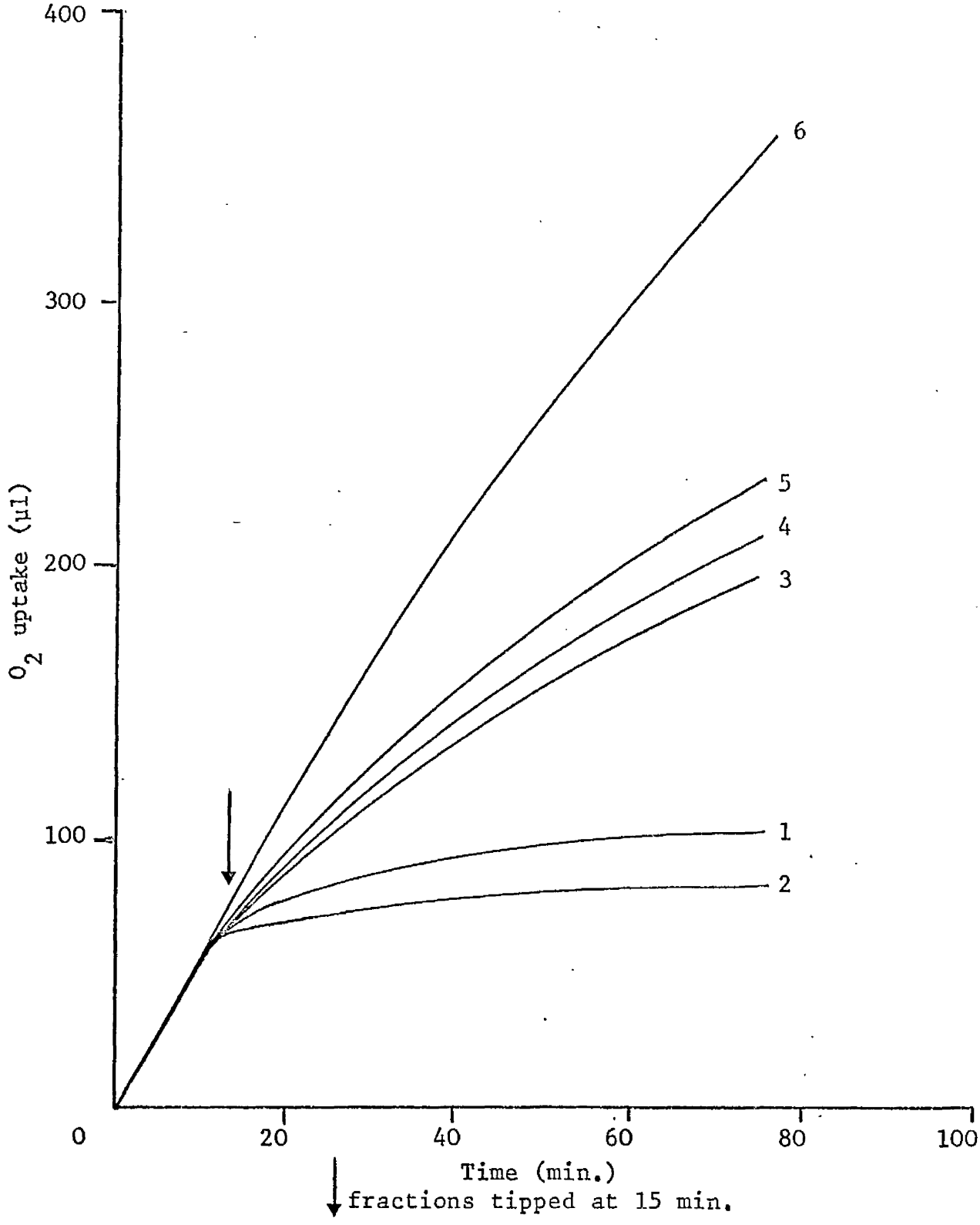


Figure 40 The effect of biological preparations of pyocyanin and 1-hydroxyphenazine on the respiration of mouse liver mitochondria

ii) The effect of adding calcium ions and pyocyanin (PF 42) to mouse liver mitochondria.

Calcium carbonate (0.225 mg in 1.7 ml solution III) together with the mitochondria and succinate was placed in a Warburg flask to which was added, 15 min. after zero time, the biological preparation of pyocyanin, in a final concentration of 0.08 mg/ml. The addition of calcium ions did not affect the inhibitory activity of pyocyanin (PF 42) on oxygen uptake by mouse liver mitochondria.

3) The effect of chemically synthesised derivatives of pyocyanin on mouse liver mitochondrial respiration.

Preliminary experiments were carried out to test the effect of chemically synthesised derivatives of pyocyanin on mouse liver mitochondria, using the Warburg apparatus. Other experiments to test the effect of these chemically synthesised derivatives of pyocyanin were carried out using the oxygen electrode (see page 158). The chemicals tested were pyrogallol - monomethylether, PF 46;

1 - methoxyphenazine, PF 47; and

1 - hydroxyphenazine, PF 48. The experiment was set up as outlined in Table 15, and the fractions were tipped on to respiring mouse liver mitochondria 15 min. after zero time. The results obtained with PF 46, PF 47 and PF 48 in a final concentration of 0.25 mg/ml closely resembled the result obtained when PF 2 was added to respiring mouse liver mitochondria, Figure 41. Similar results were obtained with these fractions using final concentrations of 0.08 mg/ml and 0.16 mg/ml reaction mixture.

4) The site of action of Pseudomonas fractions in the electron transport system

i) succinic dehydrogenase site

This was measured in Thunberg tubes; the time required for 90% reduction of the dye was taken as a measure of dehydrogenase activity.

In the control tube (PF 2C) 90% reduction of methylene blue was observed in 15 min.; after this time, the tubes containing PF 2, PF 2 heated and PF 30 showed 80-90% reduction of methylene blue.

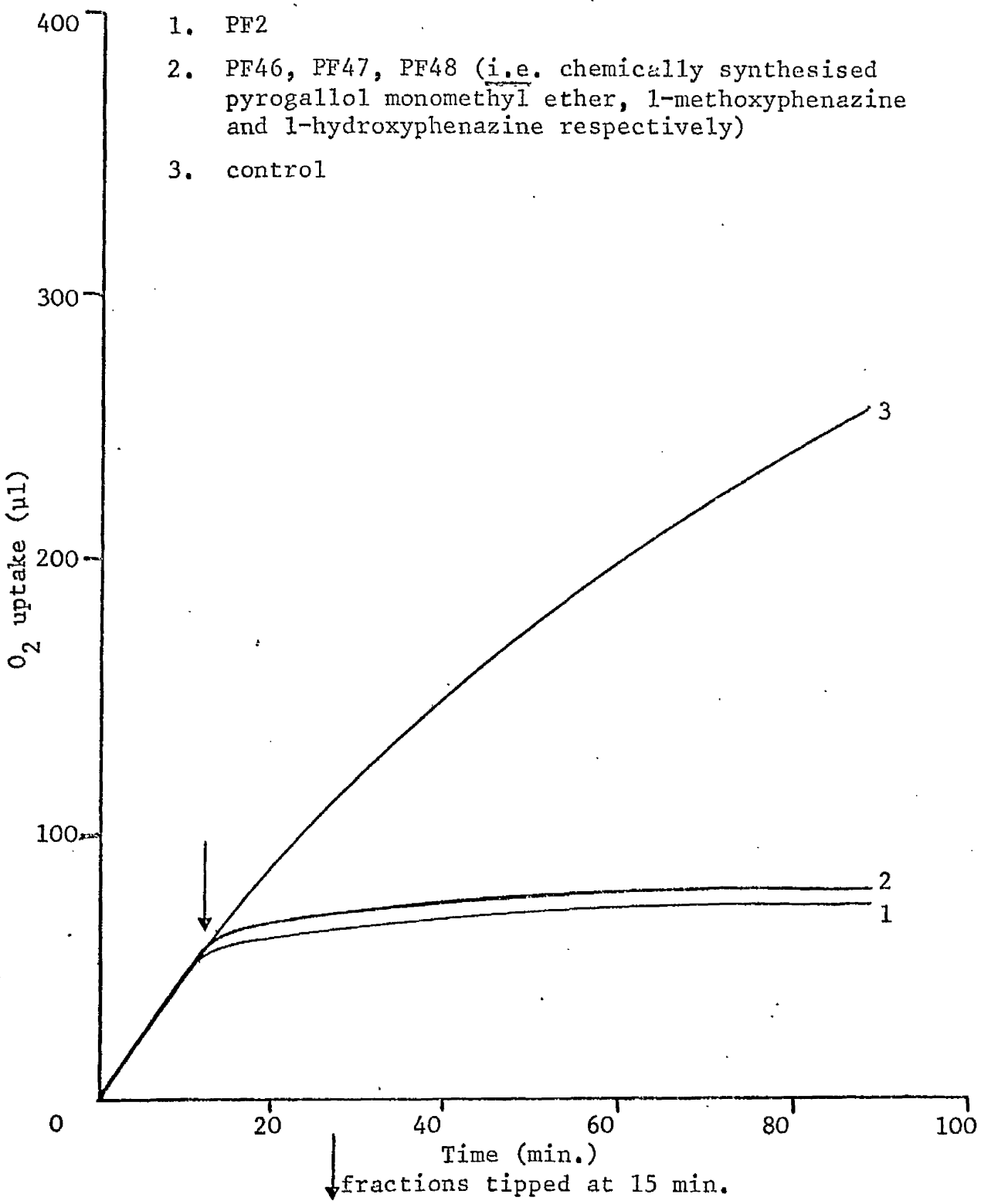


Figure 41 The effect of chemically synthesised derivatives of pyocyanin on the respiration of mouse liver mitochondria

ii) cytochrome oxidase site

This was measured in the Warburg apparatus using succinate or paraphenylenediamine as substrate (see Table 19). The fractions PF 2, PF 2 heated, PF 30 and PF 36 were preincubated with mouse liver mitochondria and a) sodium succinate, or b) paraphenylenediamine was tipped at zero time.

As shown in Figure 42, mitochondria utilising succinate were inhibited by PF 2, PF 2 heated, PF 30 and, to a lesser extent, by PF 36. The O_2 uptake by mitochondria treated with PF 2, PF 2 heated or PF 30 was 5 - 10 µl in 75 min., by mitochondria treated with PF 36 was 105 µl in 75 min.; the O_2 uptake in the control flasks was 230 µl in 75 min.

Mitochondria utilising paraphenylenediamine, which enters at the site of cytochrome C, were not inhibited by any of these fractions, although the presence of the paraphenylenediamine slowed down slightly the oxygen uptake by mouse liver mitochondria. The O_2 uptake by mitochondria treated with PF 2, PF 2 heated and PF 36 was 190 µl in 75 min.; and by mitochondria in the control flasks was 198 µl in 75 min.

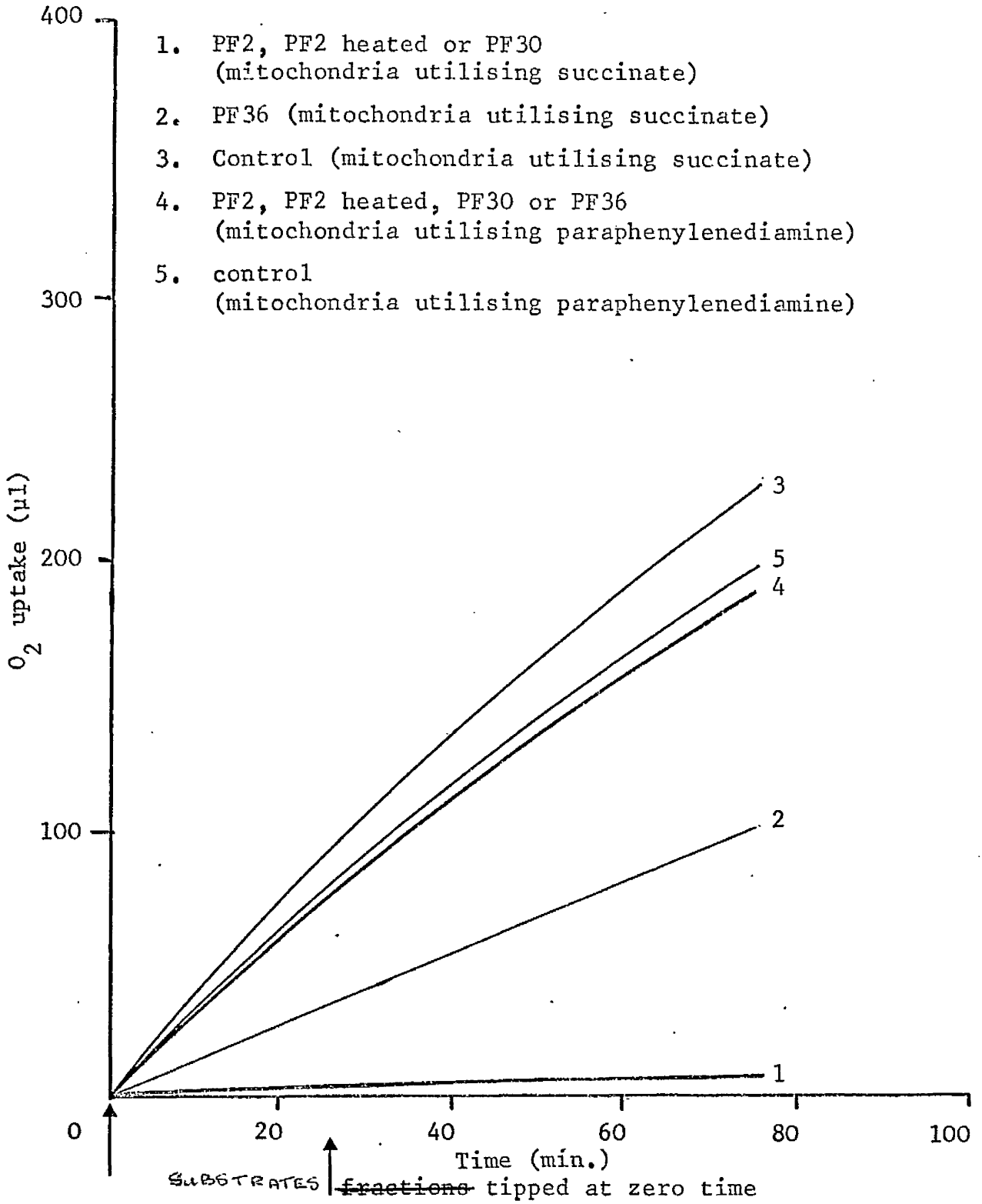


Figure 42 The effect of Pseudomonas fractions at the site of cytochrome oxidase in the electron transport chain

The addition of cytochrome C to mitochondria treated with PF 2, PF 2 heated, PF 30 and PF 36, respiring on succinate did not affect the inhibitory activity of the fractions. The results were similar to those shown in Figure 42.

iii) Ubiquinone (CoQ) site.

This was measured in the Warburg apparatus using succinate as substrate. The experiments were set up as outlined in a) Table 21, CoQ was added to mitochondria treated with Pseudomonas, and b) Table 22, protecting mitochondria with excess CoQ and subsequently exposing these mitochondria to Pseudomonas fractions.

a) As shown in Figure 43, PF 2, PF 2 heated, and PF 30 inhibited the uptake of oxygen when tipped on to respiring mitochondria 15 min. after zero time. After 60 min. CoQ (final concentration 0.1 mg/ml reaction mixture) was tipped from a second side arm in one set of flasks. The uptake of oxygen was restored to a level comparable with the uptake in control flasks; this restoration lasted for 20 min. After 40 min., i.e. before the addition of CoQ, the O_2 uptake

Legend for Figure 43 overleaf

1. PF2, PF2 heated or PF30
2. PF2, PF2 heated + ubiquinone
3. PF30 + ubiquinone
4. control or control + ubiquinone

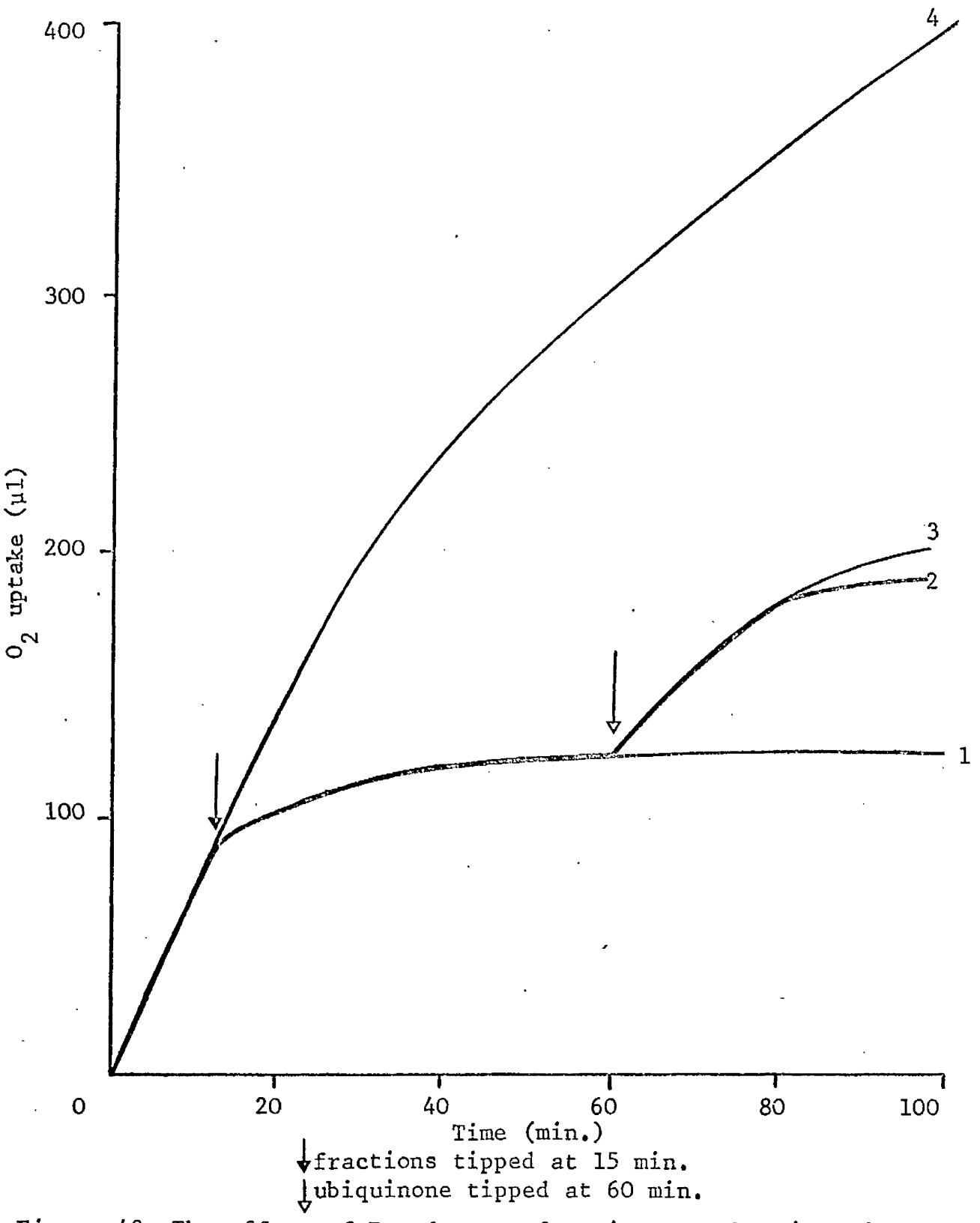


Figure 43 The effect of Pseudomonas fractions at the site of ubiquinone in the electron transport chain

in flasks containing PF 2, PF 2 heated, or PF 30, added after 15 min., was 120 µl and after 100 min. it was 124 µl; in the control flasks, the O_2 uptake was 242 µl after 40 min. and 404µl after 100 min.

After 100 min., i.e. after the addition of CoQ in a separate series of flasks, the O_2 uptake was 188 µl, 188 µl and 202 µl in flasks containing PF 2, PF 2 heated and PF 30 respectively; in the control flasks it was 404 µl.

Attempts to protect mitochondria by adding excess CoQ yielded a poor result. The O_2 uptake by mitochondria treated with PF 2, PF 2 heated or PF 30, added at zero time, was 11 µl, and for the control it was 283 µl after 80 min. The O_2 uptake by mitochondria, preincubated with CoQ (0.1 mg/ml) for 15 min. before the addition of PF 2, PF 2 heated, or PF 30, was 30 µl and in the control it was 337 µl after 80 min. This result must be compared with those obtained using the polarograph, page 159.

THE EFFECT OF PSEUDOMONAS FRACTIONS ON TISSUE CULTURE CELLS; AND COMPARISON WITH THE EFFECT OF STAPHYLOCOCCAL PRODUCTS ON TISSUE CULTURE CELLS.

Two cell lines were used -

i) L "S" cells (a smooth suspension from subcutaneous mouse liver), and

ii) BHK cells - strain C 13 (from baby hamster kidney tissue and forming a monolayer).

a) The effect on oxygen uptake caused by varying the number of tissue culture cells.

This preliminary experiment was necessary to determine the optimal number of L "S" or BHK cells per Warburg flask i.e. the number of cells which gave a measurable respiration rate. L "S" cells or BHK cells were suspended in 2.8 ml complete Eagle's medium (ETC) to give 17 x 10^6 to 80 x 10^6 cells per Warburg flask. The experiment was set up as outlined in Table 26.

An optimal respiration rate was obtained when the number of cells per flask was greater than 47 x 10^6; flasks containing this number of cells, 62 x 10^6 or 80 x 10^6 cells showed comparable respiration in 70 min., i.e. increasing the number of cells per flask above 47 x 10^6 did not increase the respiration. The O_2 uptake in 70 min. was 300 μl. Figure 44.

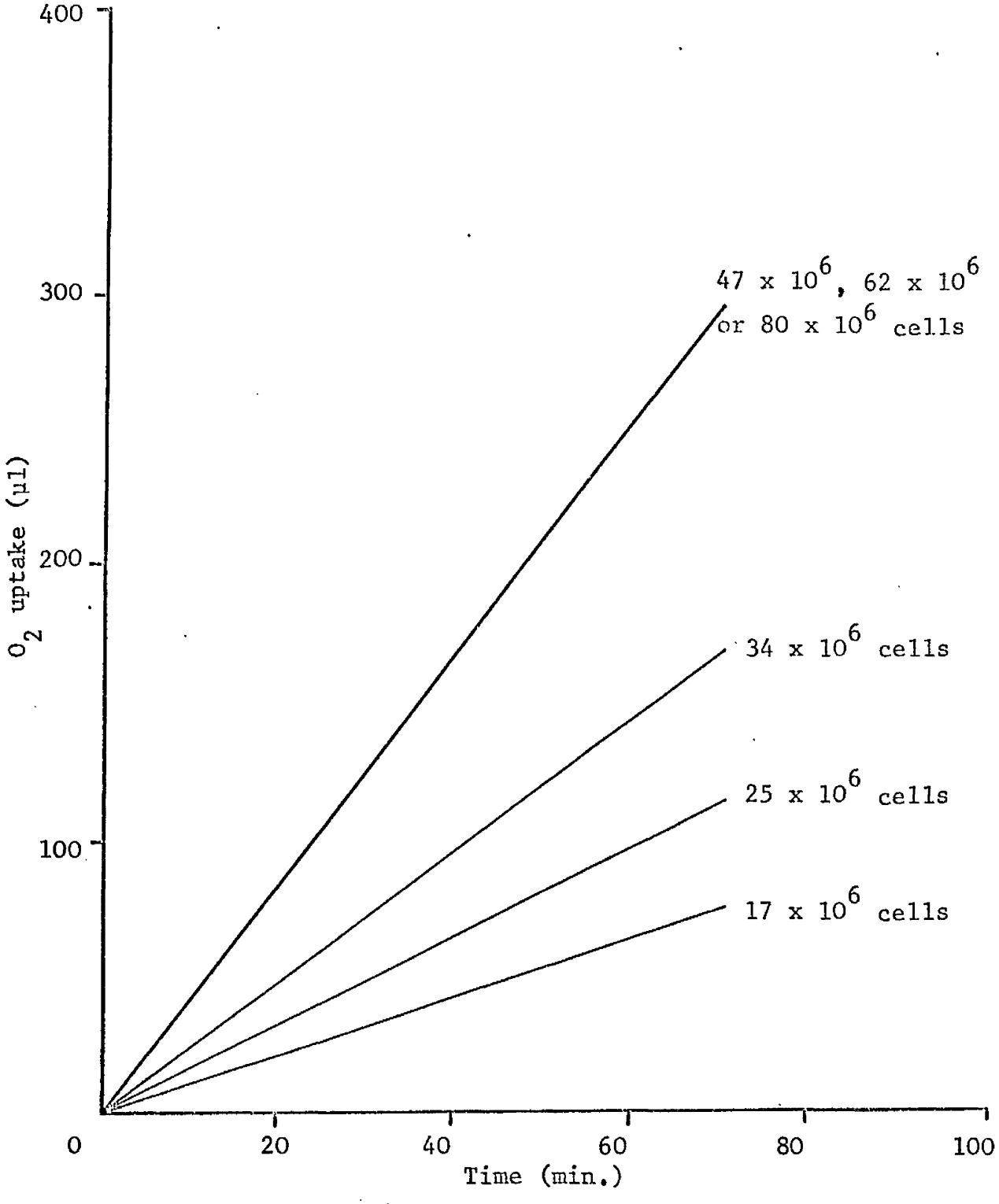


Figure 44 The variation in oxygen uptake caused by altering the number of L"S" or BHK tissue culture cells

Below 47 x 10^6 cells per flask, respiration fell off sharply:- 34 x 10^6 cells per flask took up 175 µl oxygen in 70 min.; 25 x 10^6 cells per flask took up 120 µl oxygen in 70 min.; 17 x 10^6 cells per flask took up 80 µl oxygen in 70 min.

b) <u>The effect of adding Pseudomonas fractions, Wood 46 (α toxin plus SOF) and SOF to tissue culture cells.</u>

The cells were suspended in Eagle's salts, amino-acids, vitamins and calf serum. Glucose or succinate was either contained in the main well (see Table 27) or was tipped from the side arm at zero time (see Table 28).

i) <u>The effect of Pseudomonas fractions, PF 2 or PF 30, on L "S" cells.</u>

The uptake of oxygen by L "S" cells respiring on succinate was inhibited by either PF 2 or PF 30; the O_2 uptake was 18 µl in 90 min., whereas in control flasks it was 240 µl in 90 min.

The uptake of oxygen by cells respiring on glucose was not inhibited by either PF 2 or PF 30; respiration was similar to that in the control flasks, containing PF 2C, Figure 45.

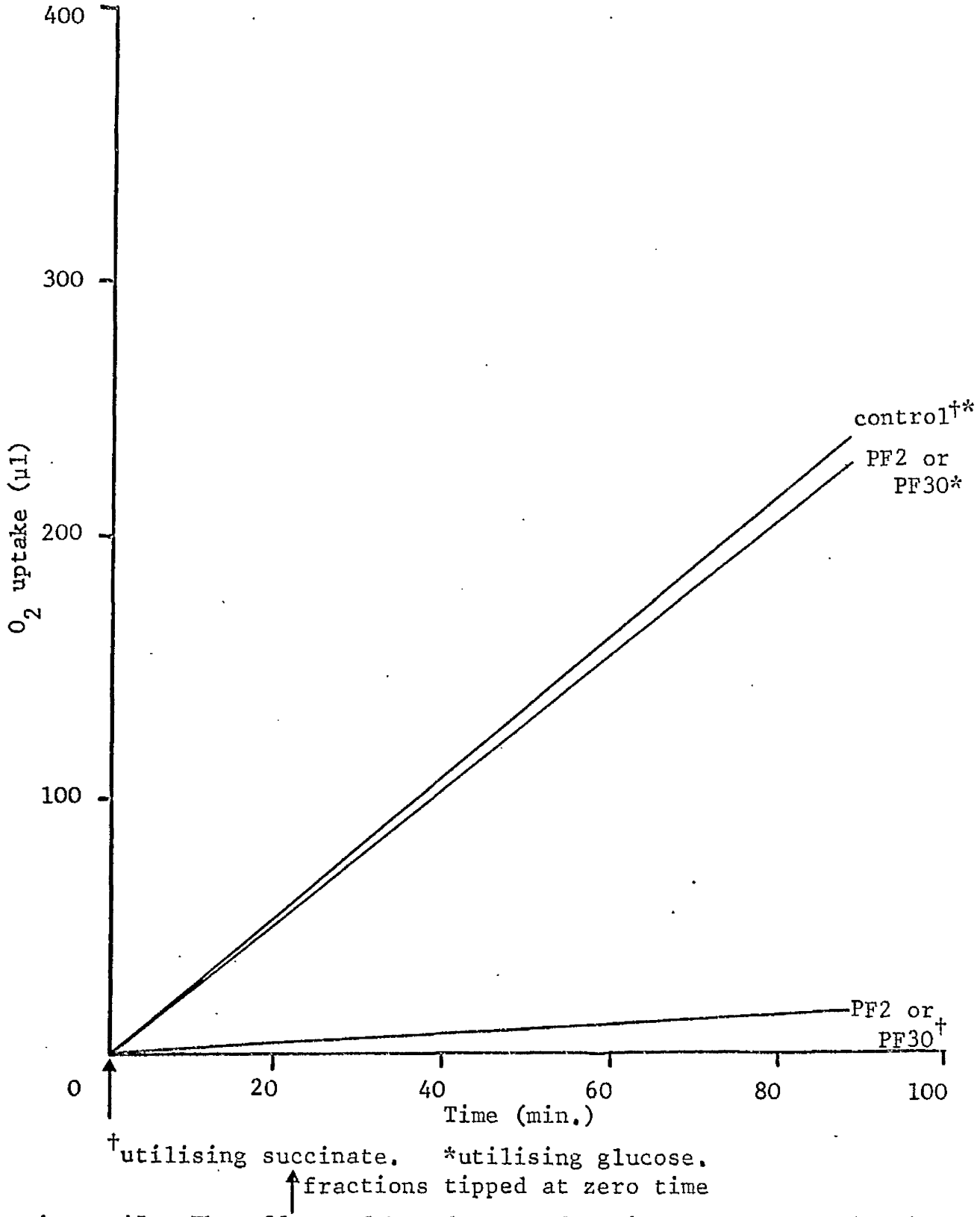


Figure 45 The effect of Pseudomonas fractions on the respiration of L"S" cells

ii) The effect of Pseudomonas fractions, PF 2 or PF 30, on BHK CELLS.

BHK cells utilised glucose but not succinate; therefore experiments with BHK cells were restricted to inhibition of glucose respiration by PF 2 or PF 30.

There was a lag period before the PF 2 or PF 30 inhibited the uptake of oxygen by BHK cells respiring on glucose. In 100 min., the O_2 uptake by cells treated with PF 2 or PF 30 was 160 µl, whereas in the control flasks it was 220 µl, Figure 46.

iii) The effect of Wood 46 on L "S" cells.

The inhibition of O_2 uptake by L "S" cells was greater when glucose was the substrate than found when succinate was substrate.

L "S" cells, treated with Wood 46, and respiring on succinate took up 140 µl oxygen in 80 min.; whereas untreated cells took up 180 µl oxygen in 80 min. On the other hand, L "S" cells, treated with Wood 46, and respiring on glucose took up 40 µl oxygen in 80 min.; whereas untreated cells took up 186 µl oxygen in 80 min. Figure 47.

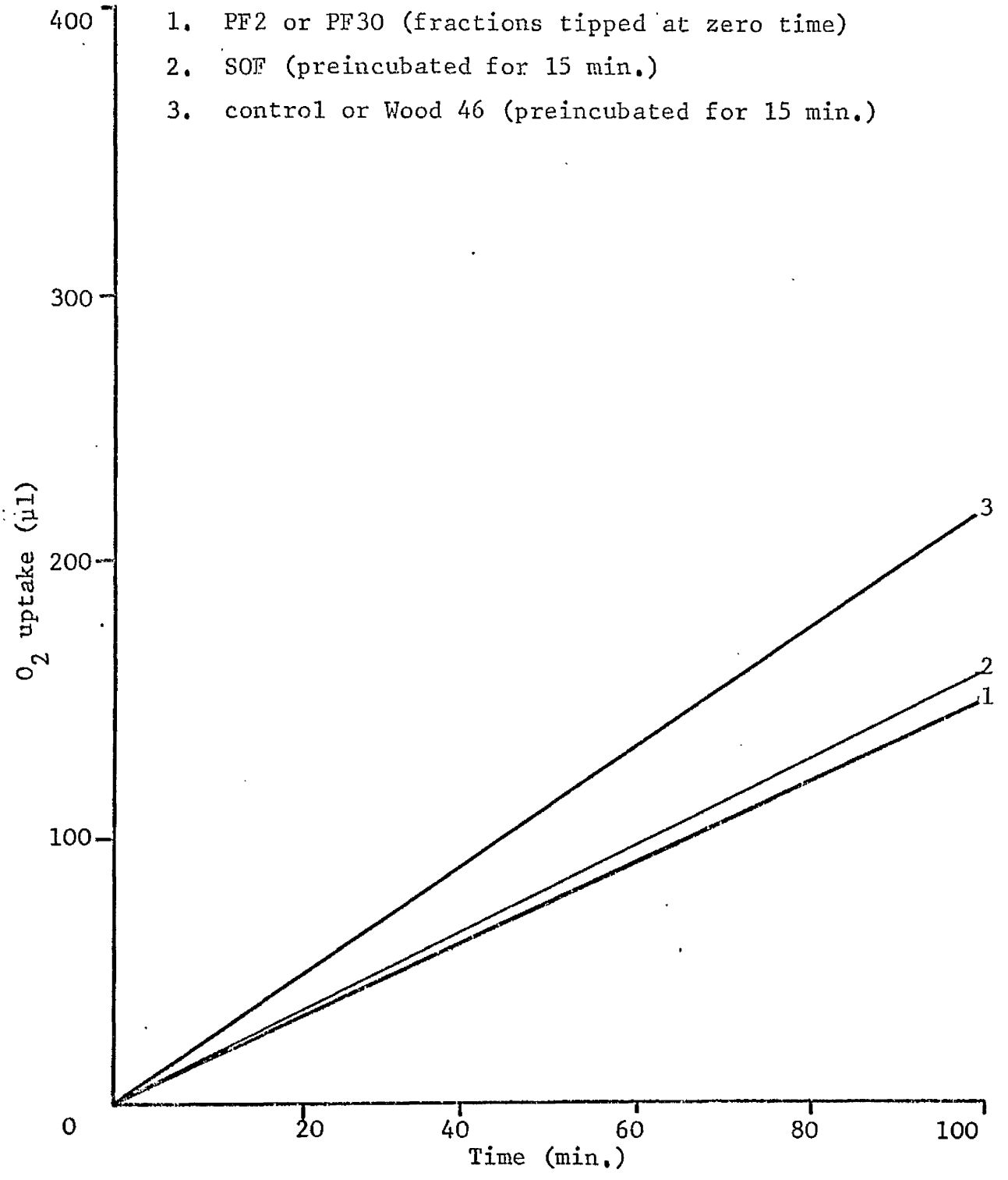
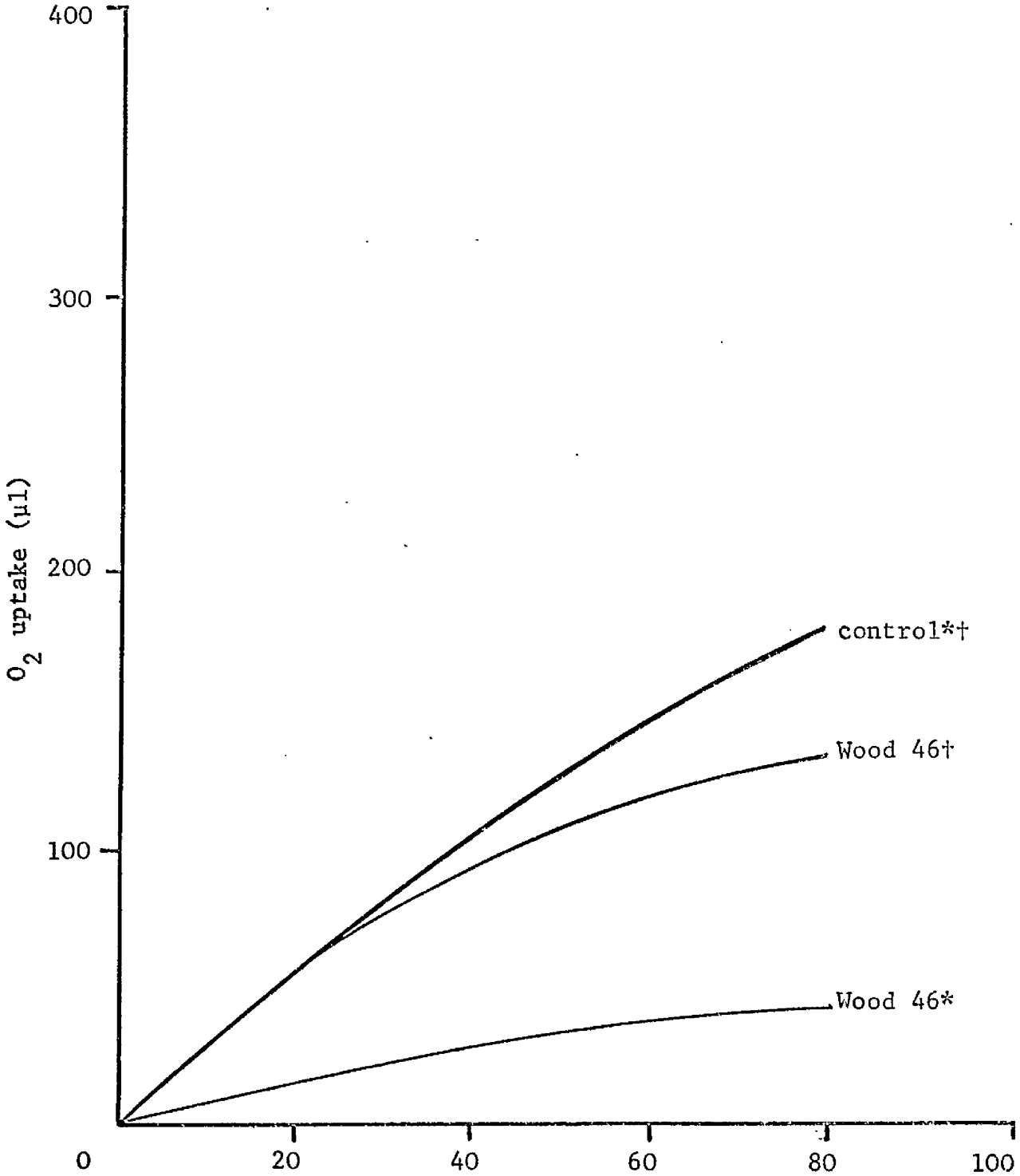


Figure 46 The effect of Pseudomonas fractions, Wood 46 or SOF on the respiration of BHK cells



*utilising glucose. †utilising succinate.
fractions preincubated for 15 min.

Figure 47 The effect of Wood 46 on the respiration of L"S" cells

(iv) The effect of Wood 46 on BHK Cells.

The uptake of oxygen by BHK cells respiring on glucose was not inhibited by Wood 46, Figure 46.

(v) The effect of SOF on L "S" cells.

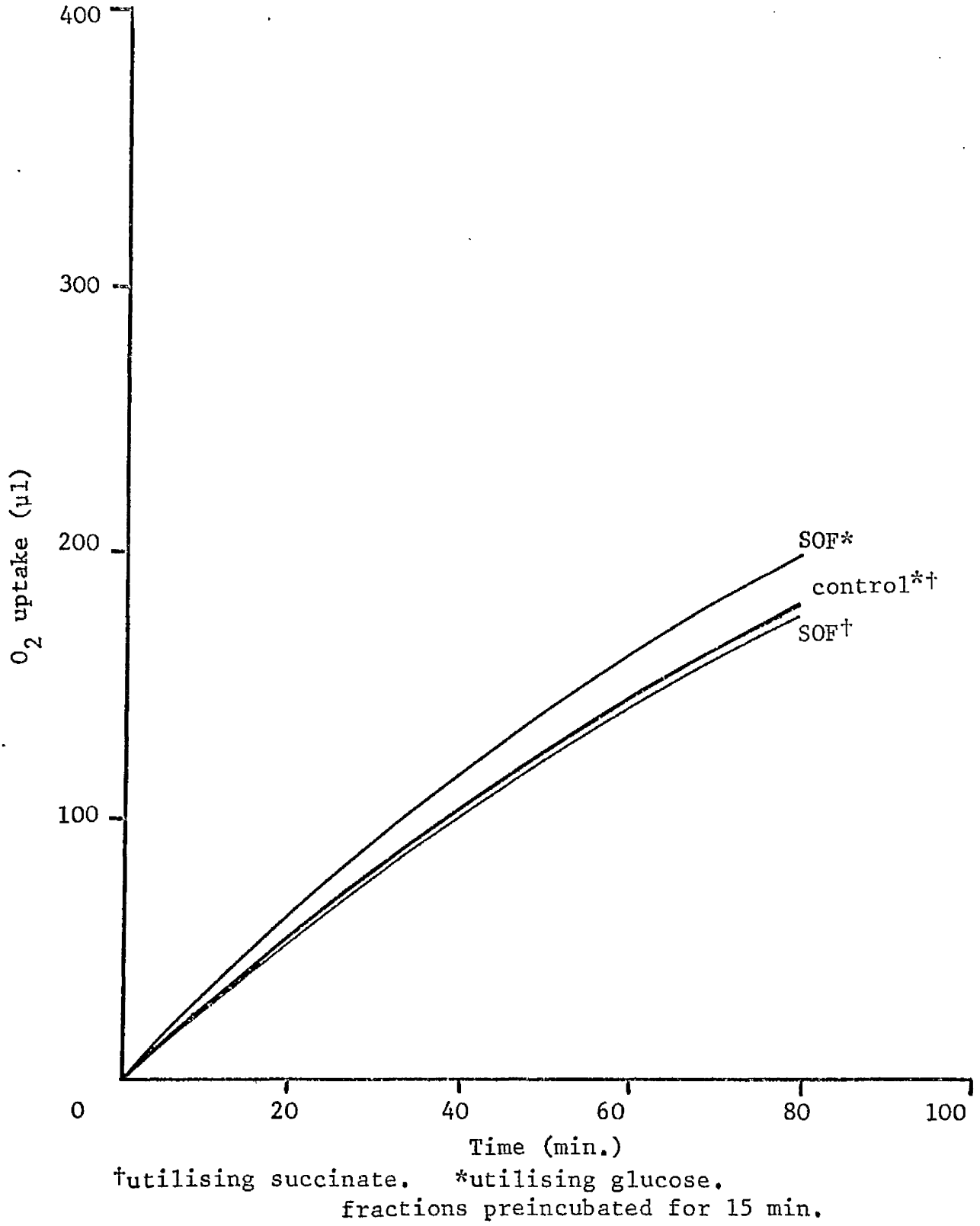
The uptake of oxygen by L "S" cells respiring on either glucose or succinate was not inhibited by the addition of SOF. In fact, the presence of SOF appeared to stimulate oxygen uptake by L "S" cells respiring on glucose, Figure 48.

(vi) The effect of SOF on BHK cells.

There was a lag period before SOF inhibited the oxygen uptake by BHK cells respiring on glucose. After 100 min., BHK cells treated with SOF took up 165 μ l oxygen whereas untreated cells took up 220 μ l oxygen, Figure 46.

(vii) The effect of Pseudomonas fraction PF 2 on the respiration of ascites cells.

In addition, the effect of Pseudomonas fraction PF 2 on the respiration of ascites cells was tested in collaboration with Miss Dorothy Symington. As shown in Figure 49, the uptake of oxygen by ascites cells utilising succinate was inhibited by Pseudomonas fraction PF 2.



†utilising succinate. *utilising glucose.
fractions preincubated for 15 min.

Figure 48 The effect of SOF on the respiration of L"S" cells

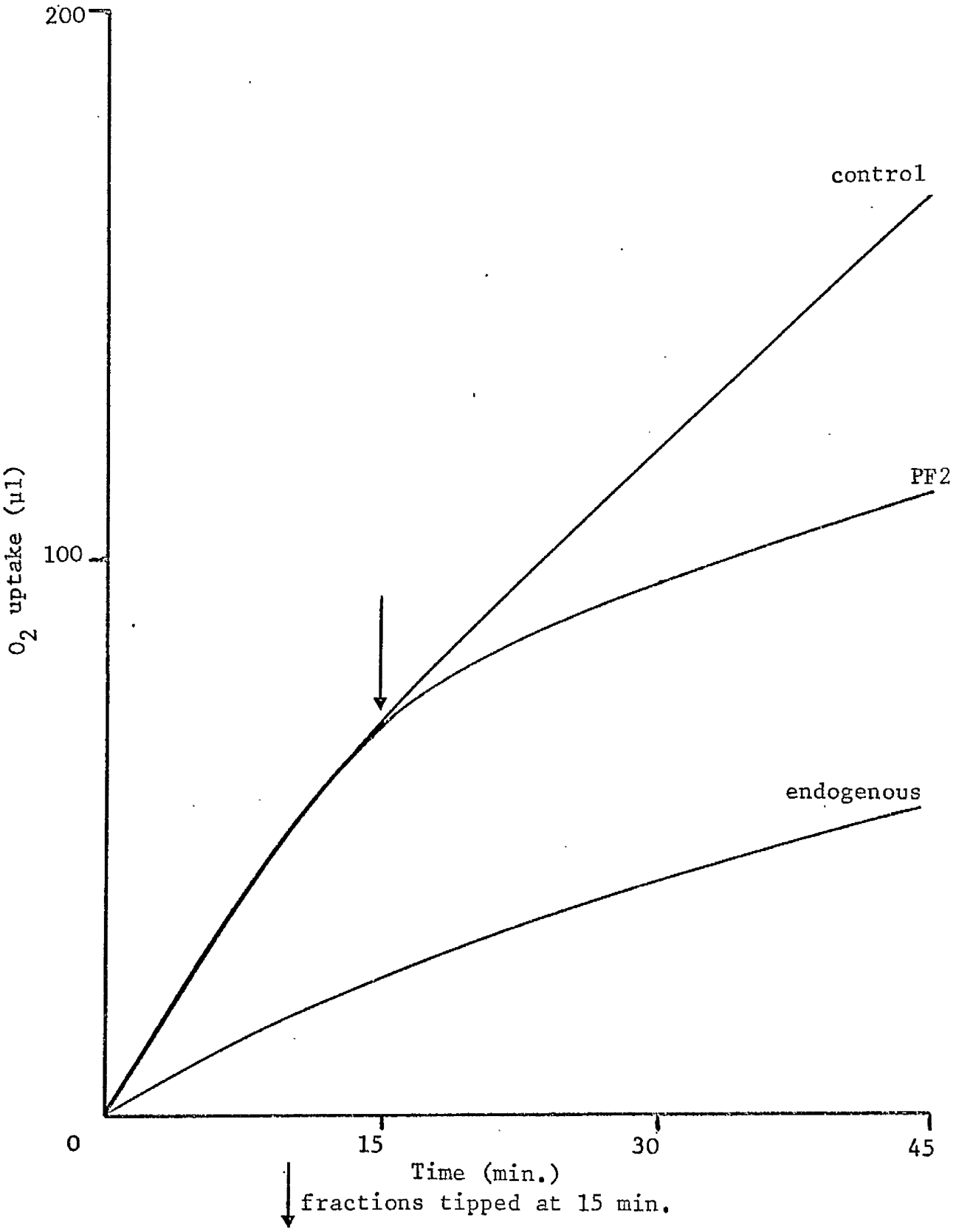


Figure 49 The effect of PF2 on succinate utilisation by Krebs 2 ascites tumour cells

THE EFFECT OF PSEUDOMONAS FRACTIONS, PYOCYANIN AND ITS DERIVATIVES ON MITOCHONDRIAL RESPIRATION, USING THE POLARAGRAPHIC TECHNIQUE

A series of polaragraphic experiments were carried out to examine the effect of various Pseudomonas fractions and chemically synthesised compounds on mitochondrial respiration. Oxygen uptake in biological systems can be monitored more accurately using the polarograph. It was decided to compare the results obtained by Warburg manometry with those obtained by this method. The O_2 uptake in each experiment was calculated per minute, Table 31.

A biological preparation of 1-hydroxyphenazine (PF 43) completely inhibited mitochondrial respiration at a concentration of 0.025 mg/ml or 0.033 mg/ml, Figure 50, whereas a biological preparation of pyocyanin (PF 42 or PF 44) showed no inhibition at a concentration of 0.016 mg/ml, little inhibition at a concentration of 0.033 mg/ml and a more marked inhibition at a concentration of 0.05 mg/ml, Figure 51, see Experiments 7, 8, 9, 22, 23 and 24.

A similar result was obtained using a chemical preparation of 1-hydroxyphenazine (PF 48),

Experiment	substance	amount added (mg)	µl O_2/min. consumed before addition	µl O_2/min. consumed after addition
1	PF 2	0.016	5.85	6.75
2		0.033	4.5	0.45
3		0.05	6.45	0.45
4	PF 2C	0.016	5.4	5.7
5		0.033	4.27	4.05
6		0.05	4.35	4.2
7	PF 43	0.016	5.17	2.4
8		0.025	5.5	0
9		0.033	6.5	0
10	PF 48	0.016	5.6	3.15
11		0.025	4.5	0
12		0.033	4.75	0
13	PF 47	0.016	5.1	4.65
14		0.033	5.6	4.95
15		0.05	5.4	4.35
16	PF 46	0.016	4.95	5.6
17		0.033	3.6	3.15
18		0.05	4.05	5.85
19	PF 49	0.016	4.05	4.35
20		0.033	4.5	4.05
21		0.05	4.7	5.7
22	PF 42 or PF 44	0.016	5.4 or 4.05	5.85 or 4.95
23		0.033	5.1 or 5.4	4.7 or 5.1
24		0.05	5.1 or 4.5	2.25 or 2.1
25	*1) PF 43	0.016	6.6	0
	2) CoQ	0.1	0	0.15
26	1) CoQ	0.1	†	5.4
	2) PF 43	0.033	5.4	0.45
27	1) PF 43 or PF 48	0.033	6.5 or 4.75	0 or 0
	2) CoQ	0.033	0	0.15
28	1) PF 43 or PF 48	0.025	5.5 or 4.5	0 or 0
	2) CoQ	0.033	0	0.3

† CoQ added before experiment commenced.
* The numbers 1) and 2) refer to the order of addition of the fractions or CoQ.

TABLE 31: The effect of Pseudomonas fractions, pyocyanin and its derivatives on mitochondrial respiration, using the polarographic technique

Legend for Figure 50 overleaf

1. control
2. 1-hydroxyphenazine (0.016 mg/ml)
3. 1-hydroxyphenazine (0.025 mg/ml or 0.033 mg/ml)

↓* addition of fractions

↓† addition of ubiquinone (~~0.33~~ 0.033 mg/ml)

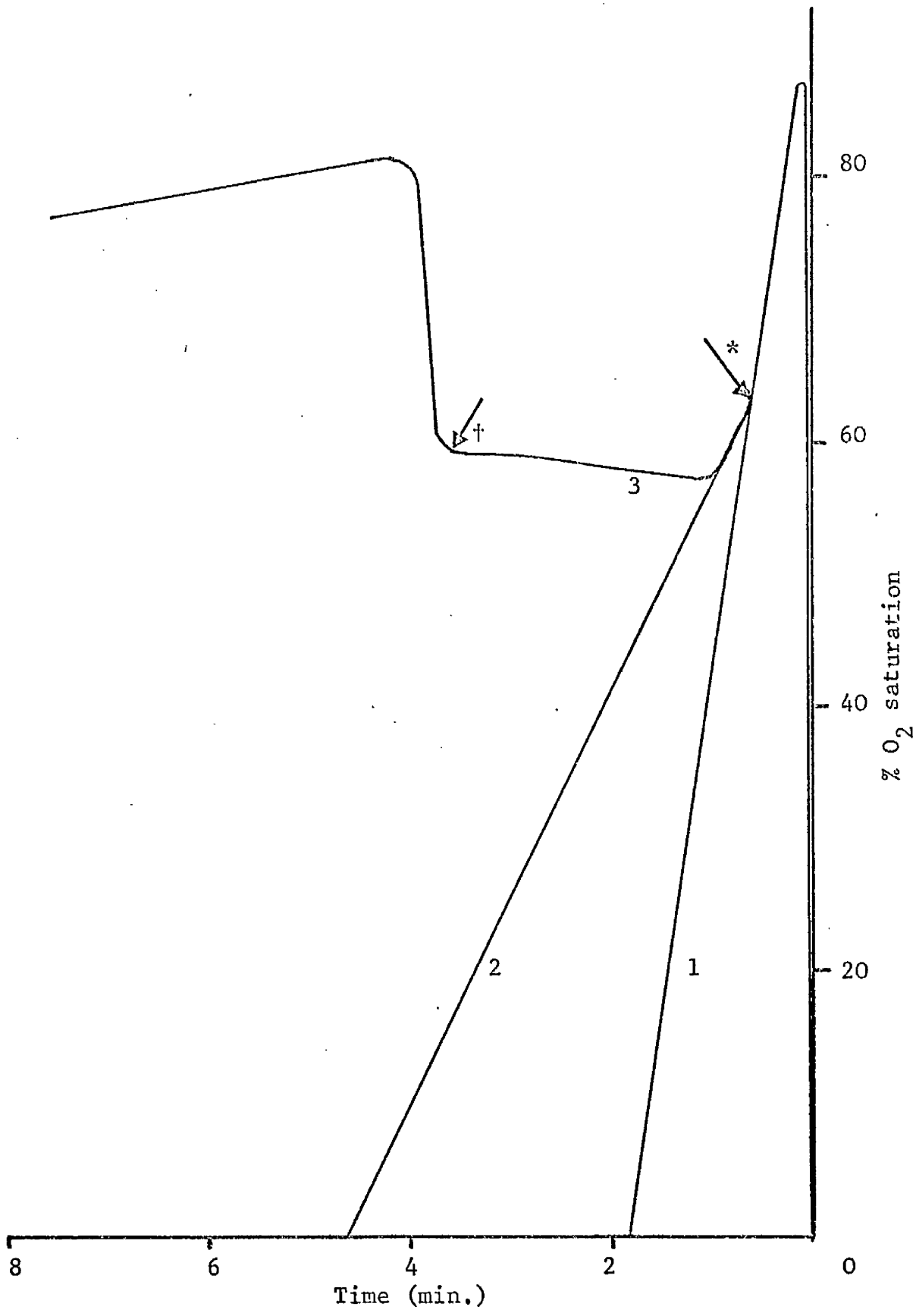


Figure 50 The effect of varying concentrations of biological or chemical preparations of 1-hydroxyphenazine (PF43 or PF48) at the site of ubiquinone in the electron transport chain, measured in the polarograph

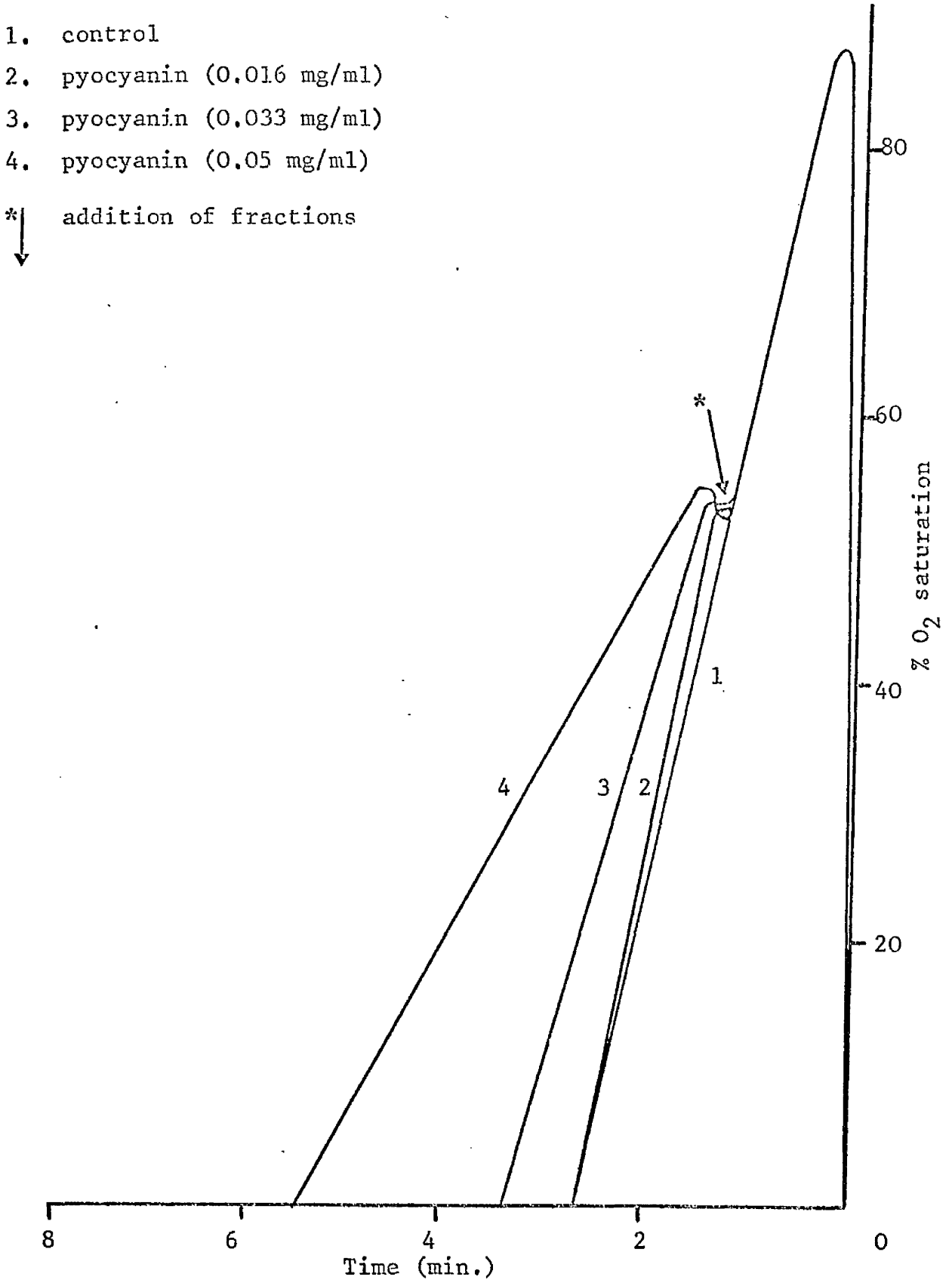


Figure 51 The effect of varying concentrations of a biological preparation of pyocyanin, PF42 or PF44, on the respiration of mouse liver mitochondria, measured in the polarograph

this completely inhibited mitochondrial respiration at a concentration of 0.025mg/ml or 0.033 mg/ml. An insignificant inhibition was obtained when chemical preparations of pyrogallol monomethylether (PF 46), 1-methoxyphenazine(PF 47) or pyocyanin (PF 49) in concentrations of 0.016 mg/ml, 0.033 mg/ml or 0.05 mg/ml were each added to mouse liver mitochondria, see Experiments 10 - 21.

In order to check the site of activity in the electron transport system, experiments were carried out in the presence of ubiquinone, see Experiments 25-28. From these experiments, it was seen that ubiquinone partially restored mitochondrial respiration, Figure 50. In addition, it was found that ubiquinone protected the mitochondria from the inhibitory effects of 1-hydroxyphenazine, see Experiment 26 and Figure 52.

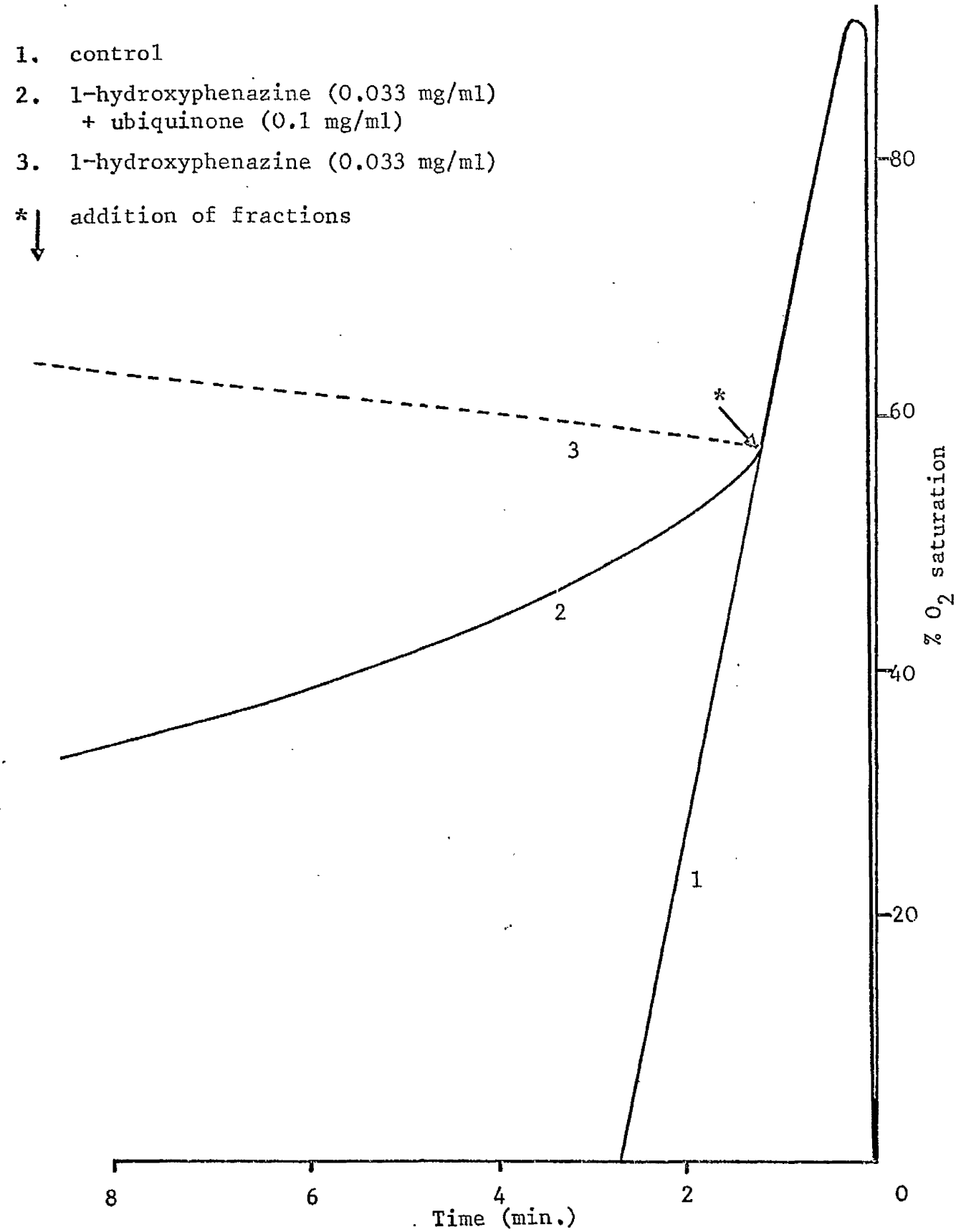


Figure 52 The effect on respiration of adding ubiquinone to mouse liver mitochondria before adding 1-hydroxyphenazine, measured in the polarograph.

IN VIVO EXPERIMENTS USING PSEUDOMONAS FRACTIONS

i) The pathogenic effect of various Pseudomonas spp. on mice.

Mice were injected intraperitoneally with 0.5 ml of a culture containing 40 x 10^8 organisms/ml. of the following organisms:-

a) Pseudomonas aeruginosa, NCTC 6750

b) Pseudomonas diminuta, NCTC 8545

c) Pseudomonas spp. (MSU 5306, Western Infirmary)

Mice injected with Pseudomonas aeruginosa, NCTC 6750 or Pseudomonas spp., MSU 5306 died within 12 hours. At post mortem, there were no apparent lesions in the internal organs. Swabs of the heart, liver and kidneys were taken but a bacteriological examination of these yielded negative results; no Pseudomonas organisms were isolated. Mice injected with Pseudomonas diminuta, NCTC 8545 were alive three weeks after injection and appeared to be healthy.

ii) The effect of a 24 hour Pseudomonas aeruginosa broth culture supernatant fluid on mice.

The supernatant fluid (0.5ml) was injected either i/v or i/p into groups of 6 mice. Immediately after i/v injection, there were signs of shock in test and control animals. These shock symptoms

subsided after 1 - 2 hours and the mice remained healthy during the three week test period.

After i/p injection, there were no signs of shock and the mice remained healthy.

iii) The effect of PF 2 and PF 30 on mice.

The mice were injected with the Pseudomonas fractions either intraperitoneally, intravenously or subcutaneously.

Mice injected i/p with PF 2 or PF 30 remained healthy during the three week test period.

Mice injected i/v with PF 2 or PF 30 and control mice showed some signs of shock immediately after injection, but these symptoms subsided after 1-2 hours, and the animals remained healthy.

Subcutaneous injection of the PF 2 or PF 30 did not cause necrosis, the skin remained clean, firm and pink in colour.

When the sterilised PF 2 and PF 30 used in this experiment were checked for their effect on O_2 uptake by mouse liver mitochondria, the results were similar to those shown in Figure 36, i.e. sterilised PF 2 and PF 30 inhibited the uptake of oxygen by mouse liver mitochondria.

iv) The effect of pyocyanin and 1 - hydroxyphenazine (biological preparation) on mice.

Mice were injected with 0.25 mg of each preparation either intraperitoneally, intravenously or subcutaneously.

Mice injected i/p with either pyocyanin or 1 - hydroxyphenazine showed no ill effects immediately after injection and remained healthy during the three week test period.

Mice injected i/v with either pyocyanin or 1 - hydroxyphenazine showed some shock effects immediately after injection, but these subsided in less than an hour, and the mice remained healthy.

v) The respiration of mitochondria prepared from mice injected with Pseudomonas aeruginosa.

Mice were injected i/p with 1×10^8 Pseudomonas aeruginosa organisms (0.5 ml) and mitochondria were prepared from the livers at 2, 4 and 17 hours after injection.

1×10^8 organisms was chosen after mice were injected with varying numbers of Pseudomonas aeruginosa organisms, varying from 0.04×10^8 to 28×10^8 organisms, and it was decided to use 1×10^8 organisms, since at this concentration, mice showed ill effects and an LD 50 was obtained.

Mice died 24 hours after receiving either 28×10^8 organisms or 5.6×10^8 organisms, whereas none died after receiving 0.22×10^8 and 0.04×10^8 organisms. It was found that mitochondria prepared from injected animals possessed a lower respiratory activity than those from uninjected animals. In addition, the Pseudomonas fraction, PF 2, inhibited the in vitro respiration of mitochondria from both sources, Figure 53.

vi) Testing of urine samples from mice injected with Pseudomonas aeruginosa.

Mice were injected i/p with 1.3×10^8 organisms; urine samples were tested at 2, 5 and 24 hours after inoculation using Hema-Combistix; urine samples from uninoculated mice were used as controls. No differences were observed in urine samples from uninoculated or inoculated animals.

vii) The effect of PF 2 and PF 30 on guinea pigs.

Guinea pigs were injected intraperitoneally with PF 2 or PF 30 and intramuscularly with PF 2 or PF 30, but no ill effects were observed during the four week test period.

Legend for Figure 53 overleaf

1. mitochondria from mice injected with *Pseudomonas aeruginosa* + PF2

2. mitochondria from control mice + PF2

3. mitochondria from mice injected with *Pseudomonas aeruginosa* + control fluid

4. mitochondria from control mice + control fluid

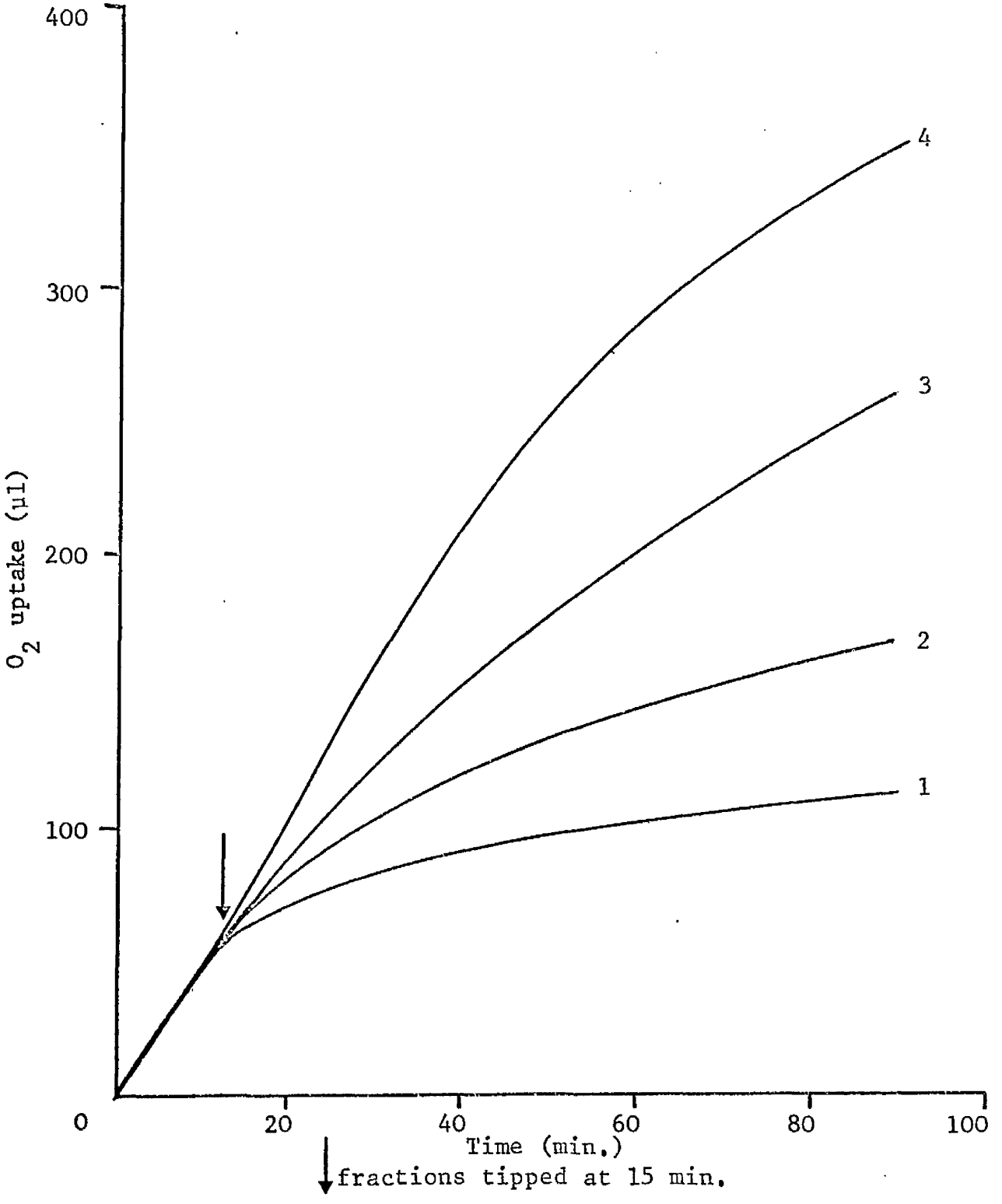


Figure 53 The respiration of mitochondria prepared from mice injected with Pseudomonas aeruginosa

PF 2 and PF 30 were sterilised for use in this experiment; however, the sterilised fractions inhibited the uptake of oxygen by mouse liver mitochondria; a similar result to that shown in Figure 36 was obtained.

(viii) The respiration of mitochondria prepared from mice injected with pyocyanin or 1-hydroxyphenazine (chemical preparations).

The mice injected i/v with 0.2 ml pyocyanin (containing 6.6mg) or 1-hydroxyphenazine (containing 2mg) died within two minutes of injection. The livers were removed and mitochondrial suspensions were prepared. An interesting point to note was that on centrifuging the disintegrated liver the supernatant fluid was pale green, in the case of pyocyanin injected animals, and a brownish-yellow, in the case of animals injected with 1-hydroxyphenazine.

The mice injected i/v with 0.1ml 1-hydroxy-phenazine (containing 1mg) or i/p with 0.2ml 1-hydroxyphenazine (containing 2 mg) showed no ill-effects immediately after injection. However, these mice were killed by cervical dislocation three hours after injection, their livers removed and mitochondrial suspensions were prepared.

The mice injected i/p with 0.2 ml pyocyanin (containing 6.6mg) died within ten minutes of injection, and again mitochondrial suspensions were prepared.

The mice injected i/p or i/v with 0.2 ml diluent showed no ill-effects after injection and were killed three hours later and mitochondrial suspensions were prepared.

The respiration of the various preparations of mitochondria were tested in the polarograph and the O_2 uptake per minute was calculated, Table 32. The majority of mitochondrial suspensions showed a similar oxygen uptake to that of the control. However, i/v injection of pyocyanin or 1-hydroxyphenazine caused a reduced O_2 uptake by mitochondria, particularly those treated with 0.1ml i/v 1-hydroxyphenazine where O_2 uptake was halved.

(ix) <u>The effect of pyocyanin or 1-hydroxyphenazine on the respiration of macrophages prepared from a guinea-pig injected with sterile paraffin-oil</u>.

The respiration of macrophages utilising 0.2 M sodium succinate was measured in the polarograph. As shown in Table 33, 0.15ml pyocyanin or 1-hydroxy-

Fractions	mode of injection	O_2 uptake per minute	death after injection
control (diluent	0.2 ml i/v or i/p	7.65 or 7.2	killed at 3 hours
1-hydroxyphenazine (2 mg)	0.2 ml i/v	4.95	died within 2 min.
1-hydroxyphenazine (1 mg)	0.1 ml i/v	3.15	killed at 3 hours
pyocyanin (6.6 mg)	0.2 ml i/v	4.5	died within 2 min.
1-hydroxyphenazine (2 mg)	0.2 ml i/p	7.05	killed at 3 hours
pyocyanin (6.6 mg)	0.2 ml i/p	6.3	died within 10 min.

TABLE 32: The respiration of mitochondria prepared from mice injected with pyocyanin or 1-hydroxyphenazine (chemical preparation)

Experiment	Fractions (0.025 mg/ml.)	O_2 uptake per hour	
		before addition	after addition
1	1-hydroxyphenazine (chem)	18	18
2	pyocyanin (chem)	18	18
		preincubation with sample	
3	1-hydroxyphenazine (biol)	9	
4	1-hydroxyphenazine (chem)	9	
5	pyocyanin (biol)	9	
6	pyocyanin (chem)	9	

TABLE 33: The effect of pyocyanin or 1-hydroxyphenazine on the respiration of macrophages prepared from a guinea-pig injected with sterile paraffin oil

phenazine had no effect when added to respiring macrophages. However, preincubation of the macrophages with pyocyanin or 1-hydroxyphenazine (biological or chemical preparations) for 1 hour at 37° resulted in the O_2 uptake per hour being halved.

DISCUSSION.

The original aim of this research project was to determine whether species of the genus Pseudomonas, in particular Ps.aeruginosa, produced a factor or factors which affected the electron transport system of host animals, and thus could be implicated in the pathogenic process.

FACTORS CONTROLLING THE PRODUCTION OF MITOCHONDRIAL RESPIRATORY INHIBITORS.

Initially, experiments were carried out using six species namely, Ps.aeruginosa NCTC 6750, Ps.aeruginosa MSU 14388, Stobhill Hospital, and Pseudomonas spp. MSU 5306,Western Infirmary. The other three species, which were not associated with pathogenic conditions, included Ps.diminuta NCTC 8545, Ps. hydrophila NCTC 7810 and Pseudomonas spp. NCTC 7452 (achromogenic). Four methods of production of fractions from Pseudomonas cultures, using three different media were used (see pages 70-74). Three of the six organisms, namely, Ps.aeruginosa NCTC 6750, Ps.aeruginosa MSU 14388 and Pseudomonas spp. MSU 5306 produced a diffusible pigment, Table 30. The culture supernatant fluid from these organisma caused a 90-100% inhibition of oxygen uptake by mouse liver mitochondria. The other three

species produced no pigment, even after 72 hours incubation, and at no time did the culture supernatant fluid from any of these organisms inhibit the uptake of oxygen by mouse liver mitochondria. From these results, it would appear that a factor, active in the inhibition of oxygen uptake by mouse liver mitochondria, was produced but only when a diffusible pigment was also produced. This observation was supported by the fact that a culture supernatant fluid from Ps. aeruginosa NCTC 6750 caused 90-100% inhibition of oxygen uptake by mouse liver mitochondria, when the organism was producing a blue-green diffusible pigment. However, Ps.aeruginosa NCTC 6750 can temporarily or permanently lose the power to produce this pigment, and the culture supernatant fluid from such strains caused no inhibition of oxygen uptake by mouse liver mitochondria.

The various methods of production of fractions,I to III,were carried out to determine the conditions necessary for maximum pigment production. In addition, the maximum inhibitory activity of culture supernatant fluids was determined. Both sloppy agar King,Ward and Raney medium A, for pyocyanin (page 70) and sloppy agar standard medium (page 70), method I, stimulated good pigment production

by Ps.aeruginosa NCTC 6750, in 24 hours, and the supernatant fluids of cultures grown in these media gave 90-100% inhibition of oxygen uptake by mouse liver mitochondria, Figure 28. An interesting point was that the particular peptone used in the King,Ward and Raney medium A, was crucial with regard to pigment production. Various peptones were separately incorporated into different batches of this medium. These included Oxoid peptone, Evans peptone, Eupeptone, Difco Neopeptone and B.D.H. peptone. The various batches of medium A were inoculated with Ps.aeruginosa NCTC 6750 and it was found that the colour of the pigment varied from a pale yellow-green with B.D.H. peptone to the typical deep blue-green with Oxoid peptone. Culture supernatnat fluids from the latter source caused 90-100% inhibition of oxygen uptake by mouse liver mitochondria. The inhibitory activity of supernatant fluids from cultures grown in the medium containing different peptones, was related to the degree of pigment formation. This observation also confirmed an association between pigment and the mitochondrial inhibitory factor.

Static liquid cultures of Ps.aeruginosa NCTC 6750, method II, resulted in neither pigment production nor production of a mitochondrial inhibitory factor.

Shaking liquid cultures of Ps.aeruginosa NCTC 6750, method III, required 48 hours incubation before pigment production was evident and the associated inhibition of oxygen uptake by mouse liver mitochondria was low, in the region of 50%, even after the 48 hours incubation.

Disintegrates of pseudomonas organisms, method IV, did not cause inhibition of mitochondrial respiration; it would appear that the factor responsible for the inhibition was extracellular and not an endotoxin moiety. This result agreed with the previous work carried out by Liu, Abe and Bates (1961) and by Elrod and Braun (1941) who showed that the dead cells of Ps.aeruginosa were non-toxic to mice. Liu,Abe and Bates (1961) believed that the toxicity was primarily due to the slime layer on the surface of Ps.aeruginosa cells.

Since maximum pigment production and inhibitory activity was obtained using method I, page 70, it was decided to prepare all biological

fractions by this procedure. In addition, further study of the mitochondrial inhibitory factor was carried out, using Ps.aeruginosa NCTC 6750, which constantly produced a deep blue-green pigment. Pseudomonas spp. MSU 5306, which produced a red-brown pigment, was a variant of Ps.aeruginosa, which instead of producing pyocyanin, directly produced a breakdown product of pyocyanin, 1-hydroxyphenazine. This was confirmed by acidification of the red-brown supernatant fluids, which led to the deposition of the yellow needles of 1-hydroxyphenazine. It will be shown later that, in itself, this result partially reveals the nature of the active factor, see page 183.

CHARACTERISATION OF A FACTOR PRODUCED BY PS.AERUGINOSA, INHIBITING THE MAMMALIAN ELECTRON TRANSPORT SYSTEM.

Methanol precipitation (page 75) of pseudomonas culture fluids, PF 1 and PF 36, yielded preparations which did not inhibit oxygen uptake by mitochondria. No mitochondrial inhibitory activity was found in the supernatant methanol fractions, probably due to dilution, since the

active principle was shown to be concentration dependent, Figure 29.

Extraction of supernatant fluids from pseudomonas cultures showed that the factor responsible for inhibition of oxygen uptake by mitochondria was chloroform-soluble; no activity was found in the chloroform-insoluble portion, Figure 36. Schoental (1941) showed that chloroform extraction of Ps.aeruginosa cultures or dried bacteria yielded three antibacterial substances, pyocyanin, α-oxyphenazine and a colourless bacteriolytic substance. It could therefore be tentatively suggested,at this stage, that the factor responsible for the inhibition of oxygen uptake by mouse liver mitochondria might also be one of these three substances.

Further study of chloroform extracts was attempted by fractionation of these extracts on an alumina column (page 78). Three fractions PF 33 (yellow), PF 34 (green) and PF 35(blue) were separated and eluted with chloroform. When these fractions were added to mouse liver mitochondria it was found that most of the activity was eluted in the yellow fraction (PF 33), Figure 37. However, there was still some inhibitory activity in PF 34

and PF 35. This was expected since, as shown in Figure 21, the solubilities of these fractions in various solvents, in particular chloroform, was very similar. Complete separation of PF 33, PF 34 or PF 35,using chloroform, was therefore impossible. However, this result is very interesting since Schoental (1941) produced α-oxyphenazine by chloroform extraction of an old culture of Ps.aeruginosa ; the extract was passed through a column of aluminium oxide on which a yellow substance was separated. Elution of this substance with alkali, acidification and purificationresulted in the yellow crystals of α-oxyphenazine being obtained. This was the first indication during the present investigation that an association existed between α-oxyphenazine, or one of its derivatives, and the factor responsible for the inhibition of oxygen uptake by mouse liver mitochondria.

At this stage, an attempt was made to determine the molecular weight of the mitochondrial inhibitory factor by Sephadex gel filtration of supernatant fluids from Ps.aeruginosa cultures. Three series of coloured fractions from G 10, G 15 and G 25 Sephadex columns were obtained - PF 36

(brown), PF 37 (colourless) and PF 38 (blue). The inhibitory activity was eluted in PF 36, Figure 38. Three sets of fractions were also obtained from a G 75 Sephadex column - PF 39 (colourless), PF 40 (brown) and PF 41 (green). Most of the inhibitory activity was eluted in PF 41, although some activity remained in PF 39 and PF 40. Results obtained by this method are invariably inconclusive, due to the difficulties of calibrating columns for molecular weight determinations. Substances of low molecular weight often deviate in behaviour from theoretical gel filtration, in fact, many aromatic and heterocyclic compounds will be retarded in a Sephadex column owing to their molecular structure. This retardation is largely unaffected by the elution medium, In addition, hydroxyl ions are also retarded because of complex formation. The results obtained in this investigation might indicate that a low molecular weight substance was responsible for the inhibition of mouse liver mitochondrial respiration. If so, this would strengthen the hypothesis that 1-hydroxyphenazine, or a derivative, is responsible for the inhibition of oxygen uptake by mouse liver mitochondria.

Fractions PF 2 (supernatant fluid from a culture grown in King,Ward and Raney sloppy agar medium A) or PF 30 (chloroform extract of PF 2) were resistant to 100° for 30 minutes and to 121° for 15 minutes. There was no difference in mitochondrial inhibition between heated and unheated fractions. Schoental (1941) showed that both pyocyanin and α-oxyphenazine were resistant to boiling (page 22). In 1924, Wrede and Strack showed that dry-heating or boiling of pyocyanin resulted in the production of 1-hydroxyphenazine. This heat resistance provided further evidence for the chemical nature of the factor responsible for mitochondrial inhibition, since it would be expected that if the factor were an enzyme, the activity would have been destroyed during the heat treatment. This observation was borne out by the results obtained after dialysis of PF 2. Neither dialysed PF2 nor dialysing solution inhibited the oxygen uptake by mouse liver mitochondria. These two fractions when combined had no effect on the respiration of mitochondria. Since the dialysed fraction was inactive,the active fraction was dialysable and

therefore not a protein. Paper chromatography of the pseudomonas fractions also indicated the non-protein nature of the active principle since no trace of amino acids was obtained with PF 30 (chloroform extract of PF 2) or with PF 30 trypsinised.

Treatment of PF 2 or PF 30 with trypsin or pepsin did not affect the inhibitory activity of these fractions on mouse liver mitochondrial respiration; inhibition was in the order of 90-100%. When PF 2 or PF 30 were treated with proteinase, it was found that the inhibitory activity was slightly reduced, Figure 31. This result is relevant if the active inhibitory factor is 1-hydroxyphenazine, since it is possible that proteinase binds to the hydroxyl group of 1-hydroxy-phenazine thus reducing the inhibitory activity. This phenomenon has been observed with substances like phenol which destroy the activity of certain enzymes by binding to the protein through the hydroxyl group. A similar result was obtained with PF 2 absorbed with mouse liver mitochondria and added to respiring mitochondria, Figure 35. It would appear that the active constituent was taken into the mitochondri during the period of absorption in an irreversible

manner. Indeed, there was no evidence of reversibility of inhibition, when mitochondria were exposed to the action of the inhibitory factor for periods of time up to two hours.

Acid hydrolysis for 18 hours at 105° of PF 2, PF 30 or PF 30 trypsinised removed most of the inhibitory activity of these fractions. It is probable that this result was due to the action of the heat alone; both the control fluid and the pseudomonas fractions were affected, Figure 33.

Pyocyanin (PF 42) and 1-hydroxyphenazine (PF 43) were prepared biologically using a modification of the method of Wrede and Strack (1924,1928), page 82. When these were tested on mouse liver mitochondria it was found that 1-hydroxy-phenazine completely inhibited the uptake of oxygen regardless of the concentration tested, Figure 40. Pyocyanin showed a lesser inhibitory effect on oyxgen uptake but the effect was concentration dependent. Similar results were obtained with biological preparations of pyocyanin (PF 44) and 1-hydroxy-phenazine (PF 45) obtained from Dr R Schoental. The ultraviolet spectra of these compounds were obtained using methanol as solvent. Pyocyanin (both PF 42

and PF 44) showed a peak at 320 nm and 1-hydroxyphenazine (PF 43) showed a peak at 297nm and 1-hydroxyphenazine (PF 45) showed a peak at 295nm, Figure 25. Corbett (1964) showed that the peak for pyocyanin in methanol was at 325nm and for 1-hydroxyphenazine it was at 265 nm. However, 1-hydroxyphenazine in 0.1 M NaOH in 50% aqueous methanol showed a peak at 295 nm; it is possible that the technique used to prepare 1-hydroxyphenazine influenced the wavelength of maximum absorption. Mass spectrometry of the samples PF 42 to PF 45 indicated that none of them were chemically pure. In fact, the samples of pyocyanin were found to contain 1-hydroxyphenazine, which would account for the slight inhibitory activity observed with biological pyocyanin. It would also account for the increase in the inhibitory activity when the concentration of biological pyocyanin was increased to 0.25 mg/ml, Figure 40. Since impurities were found in biological preparations it was decided to synthesise pure chemical preparations of pyocyanin and its derivatives in order to ensure a complete characterisation of the inhibitory factor. Four chemical compounds were synthesised, namely,

pyrogallol monomethylether, PF 46, 1-methoxy-phenazine, PF 47, 1-hydroxyphenazine, PF 48 and pyocyanin, PF 49, by the method of Surrey (1946) - see pages 83-89 and Figure 17. The effect of these preparations on the oxygen uptake by mouse liver mitochondria was tested by Warburg manometry, it was found that these fractions, at a concentration of 0.08 mg/ml, 0.16mg/ml or 0.25mg/ml, caused inhibition of respiration. Since oxygen uptake in biological systems can be monitored more accurately using the polarograph, it was decided to compare the results obtained by polarography with those obtained by Warburg manometry. Both the biological and chemical preparations of 1-hydroxyphenazine, PF 43 and PF 48 respectively, completely inhibited mitochondrial respiration at a concentration of 0.025 mg/ml or 0.033 mg/ml (Figure 50) whereas biological preparations of pyocyanin, PF 42 or PF 44, (Figure 51), caused no inhibition at a concentration of 0.016mg/ml, little inhibition at a concentration of 0.033mg/ml and marked inhibition at a concentration of 0.05 mg/ml. The inhibition was insignificant with the chemical preparations of pyrogallol monomethylether, PF 46, 1-methoxyphenazine,PF 47, or pyocyanin, PF 49, at

concentrations of 0.016mg/ml, 0.033mg/ml or 0.05mg/ml. The ultraviolet spectra of these chemical preparations was obtained using methanol as solvent. As shown in Figures 26 and 27, the peaks for pyocyanin, PF 49, 1-hydroxyphenazine, PF 48, 1-methoxyphenazine, PF 47 and pyrogallol monomethyl-ether, PF 46 was at 320nm, 263nm, 260nm and 268nm respectively. These figures compared favourably with the values published by Corbett (1964). Mass spectrophotometry of the chemical preparations, ie. PF 46 to PF 49, indicated that they were chemically pure. Accurate molecular weights were obtained, namely 140 for PF 46, 210 for PF 47, and 196 for PF 48. An anomally was found for pyocyanin, PF 49, since the expected molecular weight was 210 and the mass spectrometric analysis indicated a molecular weight of 224. However, pyocyanin can form an unstable dimer, which could break down in the mass spectrometer with no significant peaks in the higher regions. As the compound breaks down to give the monomeric form, molecular weight 210, stabilisation would occur and peaks would be visible. This could account for the difference in molecular weights even though the ultraviolet spectrum of the preparation agreed with published results. As a final check on the sample,

the ultraviolet spectrum was read in 0.1 M HCl in 50% aqueous methanol; a peak was obtained at 388nm which agreed with the published figure of 387nm (Corbett, 1964). It would therefore appear that the preparation of pyocyanin was also pure. Consequently, the results indicate that pyocyanin had no effect on the uptake of oxygen by mitochondria. A biological preparation of pyocyanin showed inhibitory activity at high concentration; this could be due to the presence of 1-hydroxyphenazine in the preparation as shown by mass spectrometry. It was also shown that addition of 0.225mg/ml $CaCO_3$ to mitochondria in a Warburg assay, along with crystalline pyocyanin,did not increase the activity of the pyocyanin on mitochondrial respiration; inhibition was insignificant. Since the addition of calcium ions to mitochondria would partially remove the permeability barrier to N-alkylphenazonium dyes shown by intact mitochondria, see page 55, the inactivity of pyocyanin was not due to its inability to enter mitochondria.

Experiments presented in this thesis have proved, for the first time, that the factor produced by Ps.aeruginosa responsible for the

inhibition of oxygen uptake by mouse liver mitochondria was 1-hydroxyphenazine (α-oxyphenazine, α-hydroxyphenazine or 1-phenazinol).

SITE OF ACTION OF 1-HYDROXYPHENAZINE IN THE ELECTRON TRANSPORT SYSTEM.

Although the experiments devised to characterise the active compound produced by Ps.aeruginosa showed that mitochondrial respiration could be inhibited by 1-hydroxyphenazine, they did not reveal the site of activity in the electron transport chain. A preliminary experiment was carried out to determine whether there was any difference in inhibitory activity if the pseudomonas fractions were preincubated with mitochondria, tipped at zero time or tipped on to respiring mitochondria, Figure 29; the inhibitory action was immediate in all instances. This suggested an immediate block in the electron transport chain, or an "electron shunt". Warburg manometry experiments revealed that the 1-hydroxyphenazine was acting at a site corresponding to ubiquinone (CoQ) or (CoQ.cyt b). No inhibition of succinic dehydrogenase or cytochrome

oxidase was observed. When CoQ was added to mitochondria inhibited by 1-hydroxyphenazine the uptake of oxygen was restored to a level comparable with that in the control flasks; this restoration lasted for 20 minutes. However, attempts to protect mitochondria by adding excess CoQ before the addition of 1-hydroxyphenazine were inconclusive. It was decided to repeat these experiments in the more sensitive polarograph; it was found that the addition of CoQ to mitochondria, inhibited by 1-hydroxyphenazine, restored respiration, Figure 50. To some extent, CoQ could also protect the mitochondria from the inhibitory effect of 1-hydroxyphenazine, Figure 52. This indicated that 1-hydroxyphenazine acted at a site corresponding to CoQ or CoQ.cyt b in the electron transport system. It also appeared that the relative concentrations of 1-hydroxyphenazine and CoQ were important; since it would seem that CoQ and 1-hydroxyphenazine compete for the same site in the electron transport chain. As stated previously, page 51, some phenazines because of their semi-reduced state can act as transfer agents or electron-carriers for oxygen in the metabolic processes of biological systems. It is therefore possible that

1-hydroxyphenazine might act as an"electron shunt" at the site of CoQ, ie. it would accept electrons from complexes I and II and transport them to a 'dead end' since it could not transfer them to cytochrome c and the rest of the chain, see page 46 and Figure 8. This hypothesis was tested by the fact that CoQ protected mitochondria, to some extent, from the inhibitory effects of 1-hydroxy-phenazine. In other words, the preliminary addition of an excess amount of CoQ to the mitochondria, would saturate the site in the electron transport chain and prevent all the electrons being"shunted off"by the 1-hydroxyphenazine. A relatively large amount of CoQ (0.1mg/ml) was required in comparison to 1-hydroxyphenazine (0.025mg/ml and 0.033mg/ml) which might suggest that the latter was more active than CoQ in competing for the site on the electron transport chain.

THE EFFECT OF PSEUDOMONAS FRACTIONS ON TISSUE CULTURE CELLS.

Preliminary experiments were carried out to test the effect of pseudomonas fractions on L"S" and BHK tissue culture cells, since these cells simulate, to some extent, the permeability harrier which often exists in the animal body. However, the limitations of the synthetic medium and the effects of the glass on cell monolayers must be borne in mind, as must the varying properties of cells from different tissue sources.

When L"S" cells were respiring on succinate the uptake of oxygen was inhibited by either PF 2 (supernatant fluid from a culture grown in King,Ward and Raney medium A) or PF 30 (chloroform extract of PF 2), whereas the respiration of these cells respiring on glucose was not inhibited by either PF 2 or PF 30. These results were compared with the effect of Wood 46 (α -toxin + SOF) and SOF on the uptake of oxygen by cells respiring on either glucose or succinate, because of their known activity in the electron transport chain. The uptake of oxygen by L"S" cells respiring on

either glucose or succinate was not inhibited by the addition of SOF alone, whereas treatment of L"S" cells with Wood 46 resulted in a greater inhibition of oxygen uptake when the substrate was glucose, than when the substrate was succinate. These results suggest that SOF alone could not penetrate the L"S" cells, but when α-toxin was also present SOF could enter the cells and inhibit electron transport. The inhibition of L"S" cell respiration by Wood 46, when glucose was the substrate, was probably due to cell damage as well as inhibition of electron transport, because Wood 46 is known to cause cytolysis. When BHK cells, respiring on glucose, were treated with pseudomonas fractions, Wood 46 or SOF it was found that they were resistant to Wood 46, and that a lag period was observed before pseudomonas fractions or SOF inhibited oxygen uptake because the glucose must be broken down before these compounds can act. These results indicate that the fractions prepared from Ps.aeruginosa can penetrate both L"S" cells and BHK cells and inhibit electron transport.

THE EFFECT OF PSEUDOMONAS FRACTIONS IN VIVO.

When mice were injected intraperitoneally with a culture of Ps.aeruginosa NCTC 6750, containing 40 x 10^8 organisms per ml, the mice died within 12 hours. At post mortem, there were no apparent lesions in the internal organs and no Pseudomonas organisms were isolated from swabs of the heart, liver and kidneys. This result agreed with the previous work of Elrod and Braun (1941) and Liu, Abe and Bates (1961) who showed that the cells of Ps.aeruginosa were non-toxic, ie. the pathogenicity of this organism was not due to an endotoxin moiety but is due to products of the organism other than this cell component. Liu, Abe and Bates (1961) showed that an extracellular slime (Table 7) was the most important fraction in the pathogenesis of Ps.aeruginosa, although both the haemolysin and extracellular enzymes (eg. lecithinase, protease and lipase) were contributing factors.

In order to determine whether there had been any mitochondrial inhibition during the period before death, mice were injected with

1×10^8 organisms of Ps.aeruginosa , and mitochondrial suspensions were prepared from sacrificed animals at two,four and seventeen hours after injection; these mitochondrial suspensions possessed a lower respiratory activity. However, PF 2 caused further inhibition in vitro, Figure 53; in other words the quantities of 1-hydroxyphenazine produced in vivo were too small to inhibit the electron transport system to any significant extent, so that the in vitro addition of 1-hydroxyphenazine potentiated the inhibitory effect.

Results obtained when mice or guinea-pigs were injected intraperitoneally, sub-cutaneously or intravenously with crude pseudomonas fractions, 1-hydroxyphenazine (0.25 mg biological preparation) or pyocyanin (0.25mg biological preparation) were inconclusive. The animals were not affected by any of these preparations. However, it is possible that the quantities of pyocyanin and 1-hydroxyphenazine injected were too small. Polarographic results revealed that the effect of 1-hydroxyphenazine was concentration dependent,

and it was considered to be essential to inject larger quantities of the preparations in order to determine whether the lethal effect of Ps.aeruginosa was to cause a generalised block in mitochondrial respiration, using chemical preparations of pyocyanin and 1-hydroxyphenazine. These were injected into mice either i/v or i/p. The mice died within two minutes of i/v injection of pyocyanin (6.6mg) or 1-hydroxyphenazine (2 mg), and when mitochondria were prepared from the livers of these mice, it was found that there was a reduction in oxygen uptake even after such a short period of exposure, Table 32. There was evidence that these chemical preparations had reached the livers, since, on centrifugation of the mitochondria the supernatant fluids were seen to be green (in the case of pyocyanin) and yellowish-brown (in the case of 1-hydroxyphenazine).

Mice injected i/v with 1-hydroxyphenazine (1mg) did not die after injection. These mice were killed three hours after injection and mitochondrial suspensions were prepared; it was found that the rate of oxygen uptake by the

mitochondria was halved. Intraperitoneal injection of pyocyanin (6.6mg) caused the death of mice within 10 minutes, but mitochondria prepared from the livers of these animals showed a similar level of oxygen uptake as the controls. In addition, i/p injection of 1-hydroxyphenazine (2 mg) neither caused death of the animals nor inhibition of oxygen uptake by mitochondrial suspensions prepared three hours after injection.

The results obtained after in vivo injection of living organisms indicated that insufficient 1-hydroxyphenazine was synthesised to cause a significant inhibition of mitochondrial respiration. At first sight, this would eliminate 1-hydroxyphenazine as an important factor in the pathogenicity of Ps.aeruginosa, since it would be unreasonable to propose that vast amounts could be synthesised during an infection. However, subsequent in vivo experiments showed that there was less inhibition of mitochondrial respiration after the injection of large quantities of 1-hydroxyphenazine, even though the animals died. On the other hand, the injection of smaller quantities

of 1-hydroxyphenazine caused a greater inhibition of mitochondrial respiration due to the prolonged exposure of the liver cells to the action of the chemical. Therefore, it is reasonable to propose that during an infection there would be a cumulative effect caused by the slow release of 1-hydroxy-phenazine.

THE EFFECT OF PYOCYANIN OR 1-HYDROXY-PHENAZINE ON MACROPHAGES OBTAINED FROM GUINEA-PIGS.

Macrophage cells are the first line of defence when bacterial cells invade the animal body. It was therefore decided to test whether pyocyanin or 1-hydroxyphenazine released by Ps.aeruginosa would inhibit the mitochondria of macrophage cells and thus interfere with the animal body defences.

When the macrophage cells, obtained by i/p injection of guinea-pigs with sterile paraffin oil, were preincubated with biological or chemical preparations of pyocyanin or 1-hydroxyphenazine (0.025mg) for 1 hour at 37°, the rate of oxygen uptake was halved, Table 33. Addition of either

preparation to respiring mitochondria had no effect. This result indicates that a time lag occurs to enable the absorption of the chemical by the macrophage after which there is inhibition of respiration. Symington (personal communication) has shown that the respiration of Krebs 2 ascites tumour cells is inhibited by preincubation with Streptolysin S; there was little effect on actively respiring cells. At this point, it is interesting to note that pseudomonas fractions will inhibit the respiration of Krebs 2 ascites tumour cells. It would seem that there is no permeability barrier to 1-hydroxyphenazine and that macrophages may well be destroyed in large numbers during a Ps.aeruginosa infection.

A POSSIBLE MECHANISM OF THE PATHOGENIC ACTION OF PSEUDOMONAS AERUGINOSA.

Many alternative theories for the mechanism of pathogenicity of Ps.aeruginosa have been put forward. The earlier workers (from Bouchard, 1888 to Young,1947) laid great emphasis

on the antibacterial properties of products from this organism (see pages 19-22). The importance of these properties was not realised with respect to the pathogenic action of Ps.aeruginosa. It is possible that in vivo, competing bacteria in an infection would be eliminated, thus allowing the unimpeded growth of Ps.aeruginosa. Schoental (1941) extended her work to show that the antibacterial products of Ps.aeruginosa exerted a strong bactericidal action towards both Vibrio cholerae and Staph.aureus, when tested manometrically. It was stated that chloroform extraction of Ps.aeruginosa cultures yielded three anti-bacterial substances, namely,pyocyanin, α-oxyphenazine and a colourless bacteriolytic substance. However, as shown in Plates 1 -6, Schoental's work was not confirmed in this thesis, since it was shown that the antibiotic effect was n associated with the chloroform extract of a culture supernatant fluid of Ps.aeruginosa, PF 30, but was associated with the crude pseudomonas fraction, PF 2, and the chloroform-insoluble fraction, PF 32. In addition, results shown in Plates 5 and 6 indicated that a pyocine, as

described by Osman (1964) was not associated with these chemical components, since there was no inhibition of growth of Pseudomonas species.

Recent investigations into the mechanism of pathogenicity of Ps.aeruginosa favour the pathogenic role of extracellular enzymes or extracellular toxins. Liu, Abe and Bates (1961) believed that an extracellular slime, haemolysin and extracellular enzymes were responsible for the overall pathogenic process of this organism. In 1963, Liu and Mercer stated that the relative resistance of man to infections was probably due to both passive immunity acquired via the placenta and active immunity acquired through exposure to latent infections, although this would seem to be a doubtful hypothesis. Gaines and Landy (1955) reported that antibody titres to the lipopolysaccharide of Ps.aeruginosa increased as an individual aged. Liu and Mercer (1963) believed that the virulence of Ps.aeruginosa depended both on its ability to grow in the serum of animals and its ability to produce various types of extracellular toxins,

eg. lecithinase and protease. No strain of Ps.aeruginosa lacking either of these characteristics was ever found to be virulent to animals. These authors also believed that the susceptibility of animals to infections with Ps.aeruginosa varied considerably depending on the ability of the sera of these animals to inhibit the growth of the organism. This also depended on the content of specific antibodies, to each serological type of surface antigen, in the serum.

Liu (1966a) separated the lecithinase and protease of Ps.aeruginosa and showed that the lecithinase was primarily responsible for oedema and liver necrosis, and that the protease was responsible for the haemorrhagic lesions of the intestines and lungs. Liu (1966b) also demonstrated the in vivo presence of a lethal toxin, produced by Ps.aeruginosa, which appeared to be protein in nature. It was suggested that the lethal effect of pseudomonas infections involving a large area of skin was probably due to absorption of the lethal toxin produced in the skin, and not due to the effects of the resultant bacteraemia.

Jones, Jackson and Lowbury (1966) believed that the failure of antibiotic therapy in the treatment of Ps.aeruginosa burn infections was due to the irreparable damage (cellulitis, invasion of the walls of small blood vessels and septicaemia), which had often occurred by the time the infection was diagnosed. This would also tend to suggest that extracellular products are of great significance in the pathogenicity of Ps.aeruginosa.

However, these theories do not take into account the precise biochemical action of certain products of Ps.aeruginosa in vivo. As already mentioned, 1-hydroxyphenazine an extracellular product of Ps.aeruginosa, inhibits the electron transport system at a site corresponding to CoQ or (CoQ - cyt b), probably due to competition for this site in the chain. The relative concentrations of 1-hydroxyphenazine and CoQ were found to be important in in vitro experiments. This was confirmed in the series of in vivo experiments carried out during this investigation. Phillips, Hoppner, Murray and

Campbell (1969) studied the ubiquinone levels in the liver of healthy and diseased individuals. They found that age influenced liver ubiquinone content. At birth, the ubiquinone level was low and increased during the first year of life. This interesting finding could account for the extremely high susceptibility of premature infants to fatal Ps.aeruginosa infection, see pages 3 to 7. Ubiquinone levels in liver from children (over one year) and adults did not show a difference between healthy and diseased individuals (causes of death studied included accidental, heart and coronary diseases, cancer and respiratory diseases). These results might indicate that the levels of ubiquinone in premature babies would influence their susceptibility to Ps.aeruginosa infections, but they would not account for lethal infections caused in children, over one year, and adults.

Nelson and Berk (1960) suggested that the development of lesions involved an alternation between toxic destruction of cells and bacterial multiplication. This hypothesis could explain

why Ps.aeruginosa lesions persist even after antibiotic treatment, since an unspecified toxic substance would be unaffected by the antibiotics and could cause cytolysis with consequent lesions. Thus the release of 1-hydroxyphenazine or of pyocyanin, which breaks down to 1-hydroxyphenazine, could cause cellular damage, even while antibiotic treatment was being carried out.

One of the most serious problems of Ps.aeruginosa infection is that of burn wound sepsis, often leading to fatal complications. In 1968, it was shown by Atik, Liu, Hanson, Amini and Rosenberg that a lethal exotoxin from Ps.aeruginosa caused two types of reaction in the dog, an immediate anaphylactoid reaction from which the dogs usually recovered, and a late hypotensive reaction which persisted and slowly progressed to death in 24 hours in untreated dogs. The circulatory and biochemical changes studied in association with this exotoxin shock were similar to those described for endotoxin shock. These authors postulated that the pseudomonas organisms grew in superficial

wounds, secreting into the blood stream the exotoxins which caused shock and death. Thus patients suffering from diabetic gangrene or burn wound sepsis, together with a pseudomonas infection may go into shock and die.

Studies on tissue culture cells, ascites cells and guinea-pig macrophage cells showed that pseudomonas fractions could enter intact cells and inhibit the uptake of oxygen. In vivo experiments indicated that an i/v injection of a sublethal dose of 1-hydroxyphenazine (1 mg) into mice caused a 50% impairment of oxygen uptake by mouse liver mitochondria, whereas a large lethal dose killed animals but did not affect the oxygen uptake by their liver mitochondria to the same extent. In addition, incubation of macrophage cells with 1-hydroxyphenazine or pyocyanin for 1 hour at 37^{0} resulted in a 50% impairment of oxygen uptake by these cells.

AN HYPOTHESIS TO EXPLAIN SOME ASPECTS OF PSEUDOMONAS AERUGINOSA PATHOGENICITY.

It is possible to postulate that in a superficial wound or burn infected with Ps.aeruginosa, first, 1-hydroxyphenazine would be produced which would cause an impairment of efficiency and possibly death of macrophage cells; thus removing the first line of defence of the body. Secondly, this would be followed by the further destruction of tissue cells and finally 1-hydroxyphenazine would be released into the blood-stream. The 1-hydroxyphenazine, produced by the organisms at the site of infection, would reach the liver, and accumulate in ever-increasing quantities in the liver cells. Eventually, the liver mitochondria would be totally inhibited with subsequent cytolysis and death of the animal. This hypothesis may not account for the total pathogenic activity of Ps.aeruginosa, since there are many extra-cellular substances produced which may play some part in such a complex mechanism. However, the sequence of events, which have been outlined,

would certainly explain why pseudomonas infections are so persistent.

Pseudomonas aeruginosa, a primary or secondary invader?.

As mentioned in the review of the literature, the definition of primary and secondary invader is rather tenuous. In a mixed infection, Ps.aeruginosa, because of its ability to synthesise antibacterial compounds, would gradually dominate other pathogenic bacteria, eg. Staph.aureus. Consequently, it would appear that the pseudomonas infection was secondary even though small numbers could possibly have existed with other organisms for a much longer time. Therefore, in the opinion of the author, the designation of Ps.aeruginosa as a secondary invader would seem to be invalid.

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