PLASMA ASPIRIN ESTERASE AND ASSOCIATED PLASMA

ESTERASES IN OLD AGE AND FRAILTY.

A thesis submitted in accordance with the conditions governing candidates for the degree of

PHILOSOPHIAE DOCTOR

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by

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For my Parents

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Abstract

The elderly form a physiological heterogeneous group. This thesis is concerned with the activity of plasma aspirin esterase and several other plasma esterases in the fit, community-dwelling and frail hospitalized elderly.

Several studies have produced evidence to suggest that drug metabolism is altered in the frail elderly and some of this work has centred around the plasma esterases. Kinetic analysis of plasma from young people, and fit and frail elderly people showed that the reduced plasma aspirin esterase in the latter group was most likely due to a reduced amount of cholinesterase enzyme (a reduced maximal activity) rather than that of a compromised affinity of the enzyme (increased Km).

Purification of whole plasma achieved the removal of 97% of the albumin component of plasma aspirin esterase. Subsequent kinetic analysis confirmed that there was no change in the Km value of plasma aspirin esterase in the three groups as a result of isolating the cholinesterase enzyme. Following this, it was postulated that the reduced activity of several plasma esterases in the frail elderly may be due to their often poor nutritional status.

A group of frail elderly people were randomised and half received a supplemented hospital diet. Plasma aspirin esterase, cholinesterase, paraoxonase, phenylacetate esterase, red blood cell intracellular esterase and red blood cell acetylcholinesterase in addition to anthropometric measurements were measured at 0, 4 and 8 weeks of the study period. The control group did not gain weight whereas the group who received a supplemented diet gained an average 1.3Kg (non-significant). The post study

weight and TSF measurements between the fed and control groups differed signifcantly at $p < 0.05$.

There were no significant changes in any of the esterases at 8 weeks , however plasma cholinesterase did show a significant increase in activity at 4 week ($p < 0.05$) and plasma paraoxonase showed a trend towards an improved activity.

Publications

Summerbell J, Yelland C, Woodhouse KW. The kinetics of plasma aspirin esterase in relation to old Age and frailty. Age Ageing. 1990. 19: 128-130

Summerbell J, Wiliams FM, Hankey CR, Wynne H. The effect of age and frailty upon paraoxonase and arylesterase activities and their response to a period on nutritional supplementation. Proceedings of the EURAGE meeting. Cardiff 1991. In press.

CERTIFICATE

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where reference has been made to published literature.

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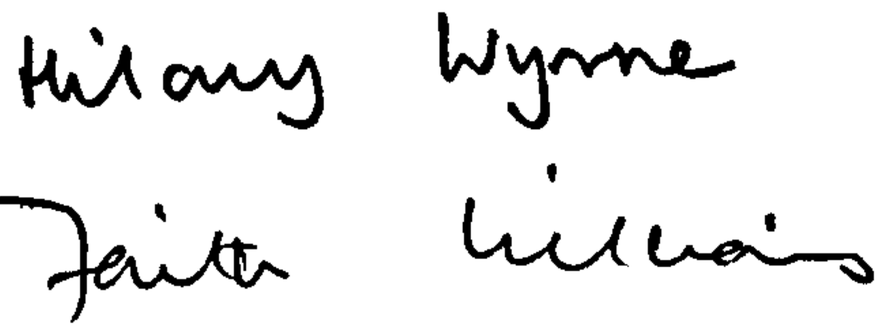

Directors of Studies

DECLARATION

I hereby declare that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in canditature for any other degree.

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CHAPTER 1

Clinical and Pharmacological Ageing

Chapter 1

Clinical and Pharmacological Ageing

The meaning of the term 'elderly' or 'aged' is open to discussion. Classification as 'old' is commonly based upon our expectations or knowledge of the average lifespan of the species under scrutiny.

Ageing can be regarded as the loss of the ability of an organism to adapt to its surroundings over a period of time (Evans, 1985). In human society this is complicated by the more severe challenges elderly people are expected to face, usually in the form of poverty (Fox et al, 1973).

Observation and breeding experiments have shown that every species of animal has a maximum life span. The vast improvements in public health, expecially during the last century have extended the *average* human life expectancy to 77 years (Walford, 1976) but there has been no change in the *maximum* lifespan recorded (Evans, 1985).

For most purposes people are defined as elderly when they reach 65 years of age. This is an imprecise definition since individuals from the middle years onwards show very different biological characteristics in response to variations in genetic, social and environmental factors (Woodhouse et al, 1988). With advancing years, the body is less able to maintain and repair body tissue with the result that cells die and organ function declines. This process occurs at different rates in different organs and individuals suffer varying degrees of organ failure as age progresses (Exton-Smith, 1985).

Rather than being a unifying concept therefore, the elderly are a very heterogenous population and generalities regarding chronological age associated concepts are simplistic and can be unsatisfactory. An appreciation of biological differences between the elderly is essential in pharmacological investigations of this age-group.

1.1 Definition of Frailty

Woodhouse et al (1988) proposed definitions of 'fit' and 'frailty' which have been used to define such populations throughout this course of study:

"The fit elderly are individuals, over 65 years of age, living independently at home or in sheltered accommodation. They are freely ambulant and without significant hepatic, renal, cardiac, respiratory or metabolic disorder on either clinical examination or laboratory investigation. They do not receive regular prescribed medication.

The frail elderly are individuals, over 65 years of age, dependent on others for activities of daily living, and often in institutional care. They are not independently mobile - whilst they do not have overt cardiac, respiratory, hepatic, renal or metabolic disease minor abnormalities may be revealed on laboratory investigation. They may require regular prescribed drug therapy. Conditions contributing to frailty commonly include Alzheimer's disease, multi-infarct cerebrovascular disease, Parkinsonism, osteoporosis, osteoarthritis, and healed fracture events".

Although the definition is based primarily upon social and functional criteria it has proved useful in illustrating two extremes of the elderly population. The frail elderly have probably become evident as a sub-population of those over 65 years in the developed world during this century. This is a result of much improved social and medical care which has vastly altered the average age to which we can expect to live as inhabitants of a developed country.

CHAPTER 2

Drug Use in the Frail Elderly Population

Chapter 2

Drug Use in the Elderly Population

2.1 Adverse Drug Reactions

Over recent years concern has grown over the increasing use of medicines by elderly people. The elderly, as a population, require more drug therapy than the young because of the age-related increase in pathology. Adverse reactions to these medications occur not only because of the multiple pathology of old age, but also because of the age-related changes in drug clearance and polypharmacy. The ability to clear the drug may fall due to compromised renal and/or hepatic function and with multiple drug therapy chances of a drug-interaction are increased.

The incidence of adverse drug reactions (ADRs) in the elderly has been studied most extensively in hospitalized elderly. A large muliticentre investigation arranged by the British Geriatrics Society in 1975 found that of 1998 elderly patients admitted to Geriatric Units, 81.3% were taking drugs. ADRs were noted in 15.3% of these patients and in 10.3% an ADR was the deemed to be the reason for their hospital admission (William and Chopin, 1980). In this study, ADRs were being particularly recorded by the clinicians. It is interesting to speculate upon how many of these, in more routine circumstances, would have gone unnoticed.

Fewer community based studies than hospital based have been reported. They are very important however, for example because of the less stringent controls

on multiple prescribing for the elderly in the community. In 1985, the proportion of elderly people in the UK population was 18% and they received 39% of all the prescription items dispensed (Cartwright et al 1988).

This is in direct contrast to a disproportionately low consultation rate by elderly people with their GP (General Household Survey, 1986), reflecting the number of repeat prescriptions issued to this group.

In many studies of ADRs drugs with a wide therapeutic index which are often implicated eg antibiotics, laxatives and analgesics (Brown and Castleden, 1990).

Castleden and Pickles (1988) correlated the frequency of ADR reporting with patient age. Of interest was the fact that in 75% of ADR reports involving a gastrointestinal tract (GIT) trauma, a non steroidal anti inflammatory drug (NSAID), was suspected and 91% of GIT bleeds and perforations in patients over 60 years were NSAID related. NSAIDs were also more frequently taken by older people admitted to hospital with GI bleeds than in age matched controls (Somerville, 1986).

CHAPTER 3

Aspirin

Chapter 3

Aspirin

3.1 Clinical and Pharmacological Aspects of Aspirin Use

Aspirin (acetylsalicylic acid) is almost entirely administered orally. This first pass metabolism by hepatic enzymes is an essential part of its pharmacokinetics. It has analgesic, antipyretic, anti-inflammatory and anti-platelet properties, all of which can be attributed to inhibition of prostaglandin synthesis (Ferreira and Vane, 1974). The analgesia is due to peripheral and central effects, and that of antipyrea to central action only.

Doses required for analgesia vary between 600 mg to 1000 mg given every three hours, to a maximum daily dose of 8 g (BNF 1991). Salicylates, in general, inhibit the synthesis of prostaglandins in inflamed tissues and prevent sensitisation of pain receptors to the action of substances such as bradykinin which mediates the pain response (Vane, 1974).

The antiplatelet properties of aspirin have received particular attention in recent years (UK TIA, 1988). Platelets and blood-vessel walls contain cyclo-oxygenase enzymes that convert arachidonic acid into thromboxanes and prostaglandins respectively through intermediary pathways. The end-product in platelets is Thromboxane A2 (TX A2) (Hamberg et al, 1975) and that in the vessels is prostacyclin (Moncada et al, 1976). TXA_2 is considered prothrombotic because it induces platelets to aggregate (Hamberg et al, 1975) and vessels to constrict (Ellis et al, 1976). Prostacyclin is thought to inhibit platelet aggregation since it

prevents the normal effects of platelets (Moncada et al , 1976) and is a vasodilator (Bergman et al, 1981).

Aspirin intially reacts with a supplemental binding site in cyclooxygenase (Cerletti et al, 1982) and then irreversibly acetylates an amino acid residue of the active site (Roth and Siok, 1978). This effect lasts for the lifespan of the platelet but in contrast the nucleated endothelial cells of the vessel walls synthesise new enzyme and the effect lasts only a few hours (Jaffe and Weksler, 1979) .

De Gaetano et al (1982) suggest that platelet TXA_2 synthesis may be more sensitive to the effects of aspirin than endothelial cell prostaglandin formation. Low doses of aspirin differentially inhibit TXA_2 synthesis and result in platelet disaggregation.

Aspirin has a very short half-life of only 12-20 minutes (Eadie, 1987) before it is converted to salicylate. The selected dose of aspirin should aim to maximally inhibit platelet formation of TX A_2 but minimally effect prostacyclin formation.

The most recent comprehensive cohort study was the Antiplatelet Trialist's Collaboration (1988) which carried out 31 randomised trials of anti-platelet treatment for patients with a history of transient ischaemic attacks (TIA), occlusive stroke, unstable angina or myocardial infarction. The aspirin dose used (300-325 mg or 900-1500 mg daily) was still probably far in excess of that required or desirable considering the risk of gastrotoxicity.

3.2 Adverse Effects of Aspirin

Many patients cannot tolerate aspirin at high doses. Frequent side effects of the main metabolite, salicylate, include tinnitis (Mongan et al, 1973), nausea, gastrointestinal irritation, haematemesis and melaena (BNF 1991). At such high doses however, small adjustments in aspirin dosage may be sufficient to eliminate toxic symptoms and yet maintain therapeutic plasma levels. The reason for this is discussed in 3.5.

Reye's Syndrome was first described in 1963 (Reye et al 1963). It is an acute encephalopathy associated with fatty degeneration of the viscera (especially the liver). It has been reported to occur after infectious illnesses, particularly chickenpox and influenza A or B, and usually in small children. Aspirin consumption has been implicated as a possible precurser of this complication, especially since it is often used in these disorders (Hurwitz et al 1985). Junior aspirin products have been removed from general sale to the public in the US and UK.

Some individuals are particularly sensitive to the effects of aspirin, and develop an aspirin sensitive urticaria or asthma. Asad et al (1983, 1984) showed that the plasma of these patients contained elevated levels of prostaglandins (PGF2) and histamine. Plasma aspirin esterase activity was reduced in this group compared with a control group of non-aspirin sensitive individuals ($p < 0.01$, aspirin sensitive asthma; aspirin sensitive urticaria, $p < 0.001$; Williams et al, 1987). The reason for this did not appear to be due to genetically determined atypical

enzyme or a lack of induction of plasma aspirin esterase as the result of aspirin abstention (Rainsford et al, 1980).

3.3 Use of Aspirin by Elderly People

Aspirin is widely used for its analgesic and anti-inflammatory properties. Because of its availability and cheapness as an "Over the Counter" (OTC) medication, it is perhaps more liable to misuse than many prescriptions only drugs (POM) and is commonly perceived by members of the public as a "safe" drug. In a General Practice survey by Law and Chalmers (1976), 39% of self-prescribed medication in a population of 151 people of 75 years and over were found to be analgesics. Aspirin and senna were the most regularly taken, self-prescribed drugs.

Regular prescribed use of aspirin is more likely in the elderly who suffer from chronic and painful osteo- and rheumatoid arthritis more frequently. Such disorders require a long term commitment to aspirin or similar NSAIDs at quite high doses (Karsh, 1990) of up to a maximum of 8g daily (BNF 1991). In recent years low dose aspirin has seen increasing use as a successful antiplatelet agent in secondary prevention of a variety of cardiovascular related disorders, (UK - TIA Study Group, 1988). Used as such it is given at sub-analgesic doses but regularly and for a long period of time (Hanley 1982).

3.4 Absorption of Aspirin

Labelling legislation requires aspirin supplied to the general public to bear the instructions 'with or after food'. Koch et al (1978) investigated the absorption of

a single 650 mg dose of aspirin after various meals each consisting predominantly of one of the three major food groups with large or small volumes of water.

The absorption rate of aspirin was greatest when taken with food and the high fat diet seemed to promote the greatest absorption. This may be the result of an increased bile flow and improved drug solubilization. Various enteric coated (EC) formulations of aspirin have been introduced to minimize the gastrointestinal problems associated with aspirin. With doses of 300 mg aspirin EC it is possible to eliminate mucosal injury completely and to substantially reduce it with higher doses (Hawthorne et al, 1991). These authors and Ross Lee (1982) found EC formulations resulted in much lower peak concentrations of aspirin. This is of great importance when it is used for analgesic and anti-inflammatory purposes but only very small plasma concentrations are required to inhibit platelet aggregation (De Gaetano et al, 1982).

Proost et al (1983) showed that a pH buffered formulation of aspirin produced higher levels of aspirin *in vivo* than a comparable dose of plain aspirin tablets (BP). This is contrary to the expected result as an alkali medium could be expected to provide more of the ionized species and hence slower absorption (Rowland et al, 1972). In addition buffered aspirin formulations cause less damage to the GI mucosa.

Rowland et al (1972) compared the availability of aspirin administered as a 650 mg tablet in 250 ml water with 650 mg aspirin given intravenously (IV). The amount of drug reaching the site of action after ingestion was only 68% of that obtained following IV administration. Incomplete absorption was excluded by

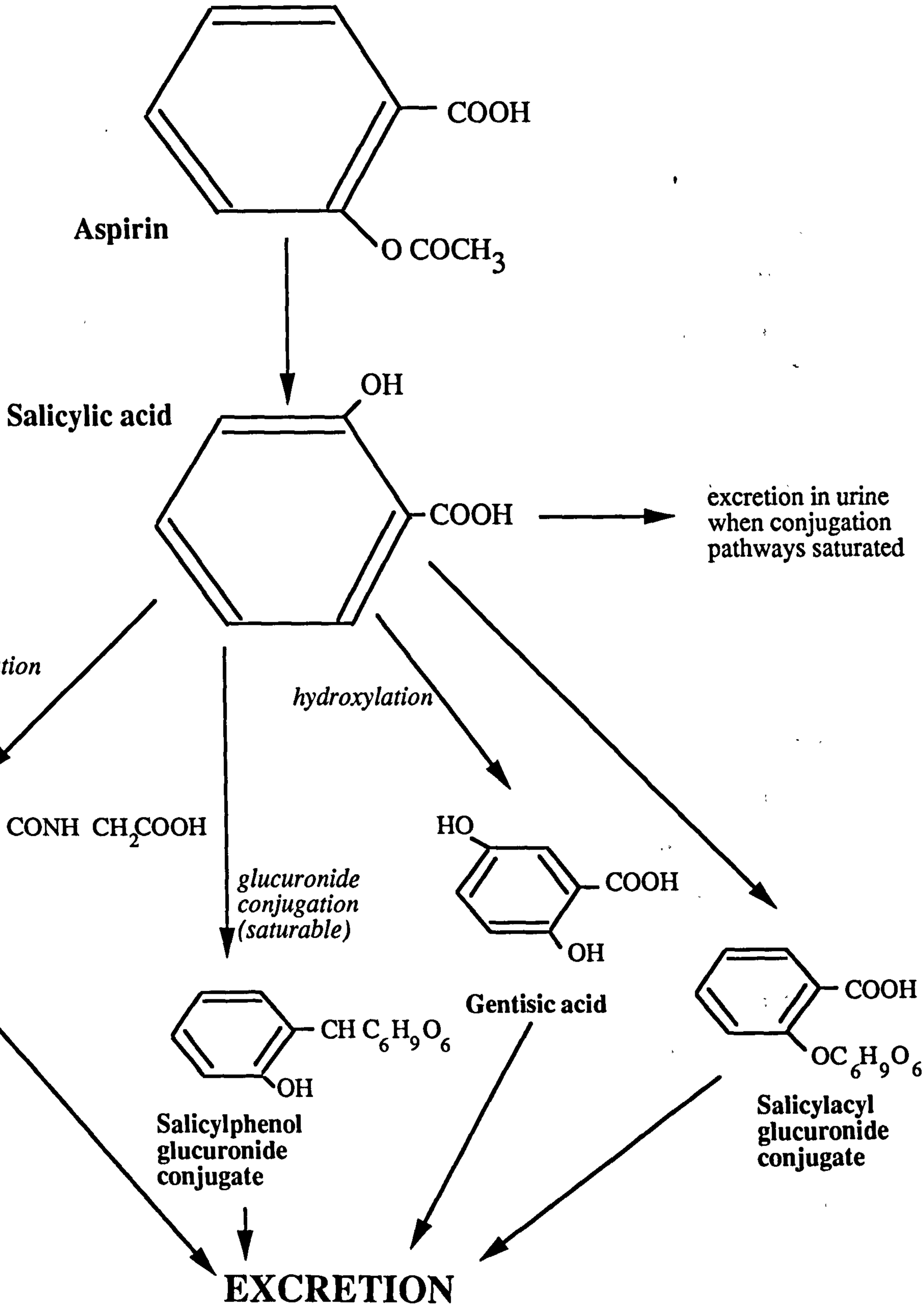
the investigators as the Area Under the Curve (AUC) was the same following either route. Esterase activity in the GI fluid was also rejected since the mean half lives of aspirin at 37oC in samples of gastric and duodenal juices were 16 and 17 hours respectively. Esterase activity in the gut wall seemed the most likely explanation. Builder et al (1977) investigated the possible role of gut mucosal aspirin esterase activity in the aetiology of chronic peptic ulcer. Tissue samples from different parts of the stomach and from both sexes varying in age, gastric disease type, and aspirin intake were studied. No difference in mucosal aspirin esterase activity was found in these groups. Aspirin esterase activity in the gut wall is thus unlikely to protect against gastric ulcers or to significantly alter the inter-individual variation in aspirin absorption. Rowland et al (1972) examined the whole GIT in rabbits and demonstrated aspirin esterase activity along the entire length. The results are given as the percentage of aspirin hydrolysed by each part of the GIT. Thus comparisons of absolute activity are not possible. In this case the duodenum shows the maximum activity but this is probably due to the large surface area.

In the same study wide variations were found in the absorption half-life of aspirin due to intra-individual differences in stomach emptying times, gastric blood flow, the amount of fluid in the stomach and degree of intestinal motility. These findings have been confirmed by Levy (1980) who examined data by Hollister (1972).

3.5 The Pharmacokinetics of Aspirin

The pharmacokinetics of salicylate are a major consideration in the elimination of aspirin from the body. The main metabolites are shown in Fig. 3.1.

Figure 3.1 Metabolism of Aspirin



The hydrolysis product salicylic acid is eliminated by the formation of salicyluric acid (glycine conjugate), salicylphenol glucuronide, salicylacyl glucuronide and gentisic acid (Levy, 1980), a small proportion is excreted unchanged (Levy, 1965).

The conjugates of salicylate are cleared more rapidly through the kidney and are thus a more effective pathway for excretion (Schachter and Manis, 1958). Renal excretion of salicylate is dependent on urine pH, being higher in alkaline than acid urine. Concurrent administration of antacids for dypepsia significantly reduces plasma levels due to the more rapid renal excretion of the drug (Levy et al, 1975).

Antiplatelet therapy requires small doses of aspirin and subsequent elimination of salicylate proceeds by first order kinetics. The major metabolite in this instance is salicyluric acid (Levy 1965).

When the dose is increased to 1 g or more per day the formation of salicyluric acid becomes saturated and consequently its contribution (as a zero-order process) becomes negligible to the elimination of salicylate. In such circumstances elimination continues by the first order processes of excretion of unchanged salicylate and salicyl glucuronide formation. (Levy, 1965).

Salicyluric acid only becomes a significant elimination product when the administered dose is reduced in size or during the terminal phase of salicylate elimination following a large dose. At small doses the half life of salicylate elimination is around 2.9 hour when all processes proceed by first order kinetics. At high doses the elimination half-life increases to 22 hours due to the now

negligible contribution of salicyluric acid to the elimination process (Levy, 1965).

The steady-state level of salicylate does not increase in proportion when dosing frequency is increased due to the saturation of the enzymatic processes involved in the formation of the principle metabolite (Levy 1975).

The complexity of salicylate pharmacokinetics helps to explain the substantial interindividual differences in steady-state plasma or serum salicylate concentrations in healthy, normal subjects receiving the same daily dose of aspirin (Levy, 1979; Gibson et al, 1975).

The survival of aspirin *in vivo* is also dependent upon the presence of blood esterases. These have been found in red blood cells (arylesterases) (Costello and Green, 1982) and plasma (cholinesterases) (Rainsford et al, 1980; Morikawa et al, 1979). Costello and Green (1982) suggested that it is the activity of the erythrocyte arylesterase which controls the overall rate of aspirin hydrolysis in the blood.

The role of esterases in aspirin metabolism will be discussed in more detail in Chapter 4.

3.6 The Effect of Old Age on Salicylate Pharmacokinetics

Salicylate is 80-90% bound to plasma proteins especially albumin. Hence, any condition which results in hypoalbuminaemia, including old age may result in high levels of free salicylate with resulting toxicity (Karsh, 1990).

Roberts et al (1983) found little difference between the pharmacokinetics parameters of aspirin/salicylate in elderly people. The higher peak salicylate concentration in young people was attributed to the higher apparent volume of distribution in the elderly.

An unpublished study by Baillie et al (personal communication) compared aspirin pharmacokinetics in fit elderly with those of the frail elderly and found a reduced AUC for aspirin and salicylate in the latter. One explanation of the results could be impaired aspirin absorption which, if confirmed may be an important observation in view of the widespread therapeutic use of aspirin in this group.

Age *per se* does not appear to be an important factor in aspirin absorption. Salem and Stevenson (1977) found the AUC of salicylate elimination in a group of elderly was approximately twice that for young subjects. Netter et al (1985) and Castleden et al (1977) found a tendency for an increased AUC. This implies that salicylate clearance is slower in the elderly. Netter et al (1985) found no statistically significant difference between salicylate clearance in young and elderly subjects. However there did appear to be a greater variability in the kinetics, with a greater number of elderly individuals showing a reduced salicylate clearance.

Ho et al (1985) found higher concentrations of salicyluric acid plasma concentrations in the elderly. This was substantiated with correlations between the renal clearance of salicyluric acid, free salicylate and renal clearance.

3.7 Summary

The changes in pharmacokinetics which occur with ageing cannot fully explain the increased predisposition of the elderly to toxicities such as confusion or GI bleeding (Somerville, 1986). Altered end organ sensitivity may account for the correlation between some toxic side effects, ingested dose and serum salicylate concentrations (Owen et al 1989).

The fall in serum albumin with age is probably not of significant magnitude to affect salicylate concentrations (Wallace et al, 1976), although its effect cannot be discounted completely (Netter,1985).

Renal function declines with age (Davies and Shock, 1950) and thus renally excreted salicylate and the metabolites may exhibit a slower renal elimination rate. Certain drugs such as uricosuric agents compete for tubular secretion and can hence increase salicylate concentrations (Karsh, 1990).

The elderly kidney is more sensitive to the inhibition of renal prostaglandin by aspirin. This can result in a reduced renal blood flow and glomerular filtration which, in turn reduces salicylate excretion, hence a cycle due to age and aspirin use can quickly raise salicylate concentrations to toxic levels (Baily and Jones, 1989).

Aspirin is a very valuable drug but like any other ingested foreign compound is not without risk. Before being prescribed, whether for chronic low dose antiplatelet therapy or in higher doses for analgesic/anti-inflammatory effects, an assessment of risk versus benefit must be made. Concurrent medication,

previous history of GI disturbances and the patient's age must all be considered. When used for self-limited conditions (for which most over-the-counter purchases are made) duration of therapy should be minimal. When used for long-term treatment, the patient should be reviewed at regular intervals.

CHAPTER 4

Esterases

Chapter 4

Esterases

4.1 Nomenclature

A simple, clear classification of esterases is extremely difficult to set out since many have overlapping substrate specificity and will hydrolyse more than one substrate, albeit to varying degrees.

Esters, amides, hydrazides and carbamates can be hydrolysed by various esterase enzymes present in body tissues and fluids. The reaction catalyzed by esters is as follows:

$$RC00R^1 + H_2O \text{ -----> } RCOOH + R^1OH$$

$RCOOR^1$ is the hydrolysable substrate, RCOOH and R^1OH are the products.

Their classification by the International Enzyme Commission (EC) is based on the substrate type for which the esterase shows the greatest affinity.

Of those listed in Table 4.1, arylesterase, cholinesterase (formerly pseudocholinesterase) and acetylcholinesterase (formerly cholinesterase) are found in the blood. Acetylcholinesterase is found membrane bound to red blood cells, arylesterase in the plasma and red blood cell lysate and cholinesterase in the plasma alone.

Table 4.1

Classification of Esterases

EC number	Systematic name	Trivial name	Typical name
3.1.1.1	carboxylic ester hydrolase	carboxylesterase	aliphatic esters
3.1.1.2	aryl ester hydrolase	arylesterase	aromatic esters
3.1.1.3	glycerol- ester hydrolase	lipase	triglycerides
3.1.1.6	acetic ester	acetylesterase	acetic esters
3.1.1.7	acetylcholine hydrolase	acetylcholin-esterase	acetylcholine
3.1.1.8	acylcholine acyl- hydrolase	cholinesterase	acylcholine

The E.C number is the International Enzyme Commission Classification

An additional classification introduced by Aldridge in 1953 is used mainly in toxicological literature and parallels the EC system. It is based on the observation that paraoxon (an irreversible inhibitor of serine esterases) may, in fact, act as a substrate for others. Thus paraoxon is a substrate for A-esterases (arylesterases) an inhibitor of B-esterases (carboxylesterases and cholinesterase) and without influence on C-esterases (acetyl esterases).

Many substrates have been used in an attempt to further classify esters which has resulted in a very confusing picture. This has particularly been the case with carboxylesterases which predominate in mammals both qualitatively and quantitatively, possessing a high degree of multiplicity and a large number of isozymes coded for by separate genetic loci (Hedrich and von Diemling, 1987).

Heymann (1989) reasoned that carboxylesterases must be present to hydrolyse endogenous compounds thus attempting to simplify the nomenclature by identifying such substrates. He proposed that these substrates alone should form the basis of a new classification system, hence eliminating the present state whereby homologous enzymes have different names between species.

4.2 Human Liver Esterases

The liver of humans and other mammals contains a number of carboxylesterases (EC 3.1.1.1) (Williams, 1985). These are non-specific serine hydrolases with differing substrate and inhibitor specificities. In common with most plasma esterases their biological role remains unknown although a role in lipid metabolism has been tentatively suggested. This is due largely to their ability to hydrolyse medium and long chain fatty acid esters (Ketterman et al, 1989,

Tsujita and Okuda, 1983). They also play an important part in the hydrolysis of many xenobiotic esters and amides (Heymann, 1982). Most of the work to date has involved small mammal studies with very limited work on human livers.

Junge et al (1974) and Tsujita and Okuda (1983) both report only one form of the esterase in human livers which Tsujita and Okuda (1983) propose to be present in the microsomal fraction. Junge et al (1974) observed some heterogeneity in the purified enzyme preparation when isoelectric focussing was carried out but further investigations were not undertaken.

A trimeric structure was proposed by both workers with each monomer having a molecular weight in the region of 60,000 Daltons.

In contrast, Ketterman et al (1989), Inoue et al (1980) and Ali and Kaur (1983) all provide evidence of at least two human liver carboxylesterases. Most studies have concentrated on the microsomal fraction. Kinetic studies by Williams et al (1989) on microsomal and cytosolic fractions using aspirin as a substrate suggest carboxylesterase is present in both. Although only two livers were studied, the cytosolic enzyme showed a significantly higher affinity and higher predicted maximal activity for aspirin than did the microsomal enzyme. In a further eight livers, the esterase of the cytosolic fraction showed five times the activity of the microsomal fraction at 1mM aspirin concentration.

White and Hope (1984a) also investigated different carboxylesterases in the cytosolic and microsomal fractions of guinea pig liver cells. Activity in the microsomes was found to be due to a carboxylesterase which is a serine hydrolase requiring a free thiol group for activity (White and Hope, 1984b).

That in the cytosol, however, has a lower molecular weight (35,000 Daltons compared with the usual value of 60,000 Daltons for liver carboxylesterases) and a different degree of sensitivity to the specific carboxylesterase inhibitor bis (4-nitrophenyl) phosphate (White and Hope, 1984a).

Interestingly Ketterman (1989) claims microsomal and cytosolic esterases are identical and the two esterases found in purification procedures are present in both fractions.

4.3 Human Blood Esterases

Esterases constitute a significant proportion of the many protein and enzymatic components in blood. Esterases present in both plasma and red blood cells (RBC) and one or both may be involved in the elimination of an endogenous or foreign compound from the blood.

A role for esterases of considerable clinical significance has been confirmed and this is central to the following studies. The biological role of many esterases is, as yet, undefined, however.

4.3.1 Aspirin Esterase

When aspirin is administered orally it is presystemically metabolised in the liver and gut wall so that only 65% of the original dose reaches the systemic circulation (Rowland et al, 1972). Subsequent hydrolysis by plasma, blood esterases and the liver on recirculation is rapid and the biological half-life of aspirin in man is only about 15-20 minutes (Levy, 1965).

The enzyme activity referred to as 'aspirin esterase' ie the enzymes resulting in hydrolysis of aspirin to salicylate is contributed to by both cholinesterase and albumin in the plasma (Morikawa et al, 1979; Rainsford et al, 1980).

Cholinesterase exhibits the greater activity at an estimated 80% and albumin contributes the other 20% (Rainsford et al, 1980). Hawkins et al (1969) suggested that aspirin acetylated the lysine residue of albumin thereby releasing salicylate and acetate.

RBC aspirin esterase is an intracellular enzyme (Rylance and Wallace, 1981). It appears to be unrelated to membrane bound acetylcholinesterase (Costello and Green, 1983) as suggested by Harris and Riegelman (1967) and may, in fact be a modified arylesterase. Inhibition studies with mercury chloride and 5,5'-dithiobis - (2-nitrobenzoic acid) or DTNB (a sulphahydryl reagent) suggested that, unlike other arylesterases, RBC aspirin esterase requires functionally intact sulphahydryl groups. A greater degree of specificity is also suggested by the lack of inhibition by sodium fluoride which, again, inhibits many arylesterases (Costello and Green, 1983).

Further work by Costello et al (1984) in which the haemocrit of dogs was reduced by controlled bleeding and/or removal of the spleen demonstrated a negative correlation between aspirin survival in the blood and haemocrit. It is possible that these results could be extrapolated to humans. A similar reduction in erythrocyte lysate could lead to increases in aspirin survival *in vivo* and subsequently to a greater pharmacological effect. There is no evidence for this, however.

Plasma aspirin esterase has been observed to be a useful predictive tool of the latter's ability to hydrolyse aspirin (Williams et al, 1989). Despite two different enzymes being responsible in each case, Williams et al (1989) observed a parallel variation in plasma cholinesterases and liver carboxylesterases.

A number of workers have demonstrated correlations between plasma aspirin esterase activity and the pharmacological effects of aspirin. Seymour et al (1984a) found a significant negative correlation between plasma aspirin esterase activity and the area under the pain-score-time curve in a group of 15 patients who receive a single dose (1.2g) of aspirin for post operative dental pain. Similarly Seymour et al (1984b) showed a significant negative correlation between plasma aspirin esterase activity and prolongation of bleeding time at 24 hours and inhibition of platelet aggregation with collagen following a single 1g dose of aspirin. In a study of patients with rheumatoid arthritis, plasma aspirin esterase activity was also negatively correlated with pain relief achieved by females but not by males (Rainsford, 1980).

Therefore, despite the liver having the greater esterase capacity to hydrolysed aspirin, variations in the plasma enzyme appear to significantly influence the removal of aspirin from the blood and hence pharmacological effect. Aspirin esterase activity is also present in the liver (4.2) and gut wall (3.4).

4.3.2 Cholinesterases

The cholinesterases can be classified broadly into acetylcholinesterase (E. C. 3.1.1.7 AChE) and cholinesterase (E.C. 3.1.1.8.ChE), formerly pseudocholinesterase.

ChE is found in almost all major systems of the mammalian body although there is considerable variation in the level of ChE activity between species (Silver, 1974). Despite its widespread presence, no biological function has been established to date. Several studies investigating its role have been concerned with the control of neurological enzyme systems (Kutty, 1980) and lipid and lipoprotein metabolism (Chu et al, 1978).

ChE can be distinguished from AChE by substrate specificity and selective inhibitors. ChE will hydrolyse acetylcholine *in vivo* but benzoylcholine and butyrylcholine are the preferred substrates. It is inhibited specifically by 10-(1-diethylaminopropionyl) phenothiazine.

The specificity of ChE is due to the active centre which consists of an anionic site. Once the substrate is reversibly bound, the aryl group binds to the serine residues of the esteratic site, and the choline is released. Next, the acetylated intermediate reacts with water to yield the acid and regenerate the active enzyme (Williams, 1985).

Early work by Surgenor and Ellis (1954) suggested that human serum cholinesterase activity was due to a single protein. However, with the introduction of more sophisticated electrophoretic and chromatographic techniques, this now seems unlikely. Multiple molecular forms of cholinesterase have been identified using cholinesterase preparations of varying degrees of purity as the starting material. Various combinations of starch and polyacrylamide gel electrophoresis (PAGE) together with different staining techniques have been used to observe the different isozymes (Brown et al, 1981). So many different methods of study have led to conflicting conclusions.

However, at least five bands of different electrophoretic mobility have been isolated with minor additional bands sometimes found in human plasma (La Motta, 1968). Juul (1968) detected twelve bands of cholinesterase activity using a stain containing butyrylthiocholine and copper.

The least mobile bands have the greater proportion of enzyme activity (Gaffney, 1970). Inconsistent minor bands are most likely to be cleavage products of the main bands due to prolonged storage, especially when subject to intermittent freeze-thaw treatments (Gaffney, 1970). Such apparent quantitative changes in isozymes make any comparisons between individual plasmas fraught with difficulty.

Cholinesterase is made up of 4 identical subunits (tetramers) and has an approximate molecular weight of 342,136. It comprises of 574 amino acids with a molecular weight of 65,092 and 9 carbohydrate chains which vary in weight (Lockridge, 1987). Molecular weights have been calculated using methods such as ultracentrifugation (La Motta, 1970) and Sephadex gel filtration (La Du and Snady, 1971) but all agree quite closely with these results.

Protease contamination can cause cleavage of the residual, dimer subunits. If this mixture is then subjected to SDS gel electrophoresis two bands are evident: one due to the monomer and the other to the unreduced dimer (Lockridge, 1979). Proteolysis by trypsin results in disulphide-depleted tetramer, trimer, dimer and monomer. This disulphide bond does not appear to be essential for maintaining the activity (Lockridge et al, 1979) as the digestion products all exhibit activity (Lockridge and La Du 1982). The interchain disulphide bond is located only four amino acid residues from the end of the subunit

(Lockridge, 1987). Thus removal of the 4 amino acids does not significantly alter the weight, probably less than 5000 Daltons (Lockridge and La Du, 1982), and is imperceptable in sodium dodecyl sulphate (SDS) gels due to the high carbohydrate content (23.9%) which results in broad bands (Lockridge, 1990).

4.3.3 Acetylcholinesterase

The possible existence of this enzyme was predicted by Dale in 1914, following work in 1899 in which he showed acetylcholine (ACh) to be naturally present in animal tissue (Dale, 1914). It wasn't however until 1937 that Marney and Nachmansohn showed that nearly all the acetylcholinesterase present in frog muscle was concentrated at the nerve endings (Marney and Nachmansohn, 1937) and that this was present in sufficient concentration to hydrolyse ACh to the extent predicted in theory.

Later, the picture became more confused as acetylcholine hydrolysing enzymes were isolated from more tissue and organs. It appeared that they exhibited optimal activity at different substrate concentrations which led Alles and Hawes in 1940 to demonstrate the existence of two choline-esters.

Acetylcholinesterase (AChE) is present in nerve endings, striated muscle and red blood cells. Sorensen (1986) detected AChE in human serum using polyclonal and monoclonal antibodies. Its role in limiting ACh at cholinergic synapses and neuro muscular junctions is well established however that in red blood cells is not.

Red blood cell acetylcholinesterase (RBC AChE) is hydrophobic and detergent soluble (Dudai, 1972). Partially purified membrane enzyme preparations have yielded multiple molecular forms with molecular weights anywhere between 66000 and 562000 (Wright, 1973). Using immunological means to raise antibodies against intact human erythrocyte membrane it has been shown that dimeric acetylcholinesterase predominates in the native membrane (Ott, 1983).

Purified enzyme from red blood cell membranes (RBC) is particularly useful model system *in vitro* because of its accessibility and similarities with a number of neural enzymes. Fritze and Beckman (1987) made use of this in a study investigating possible genetic differences in the RBC AChE of psychiatric patients. However, no differences were found which again gave rise to the question of validity in using RBC activity as a marker. The effect of concomitant drug therapy was not investigated.

The AChE content of the cell membrane shows a very complex profile dependent upon the age of the cell (Lawson and Barr, 1987). Changes in AChE occur in parallel with those of lipid content of the RBC (Kamber et al, 1984). There is a decrease in the cholesterol:phosphate ratio as the cell ages and this is followed by a decrease in the activities of membrane bound enzymes including AChE.

4.3.4 Arylesterases

Arylesterases (formerly also known as 'A' esterases) are present in the plasma, erythrocytes and a wide variety of mammalian organs (Aldridge, 1953). Phenylacetate is most often used as a substrate although other substrates used

include alpha and beta-naphthylacetate.

Arylesterases are dependent upon the presence of Ca^{2+} ions and thus are inhibited by calcium chelating agents eg EDTA (Erdos et al, 1959). Wilde and Kekwick (1964) found that 10 mM Ca^{2+} in the presence of enzyme seemed to provide optimal activity *in vitro*.

Arylesterases do not appear to have a physiological role but they do have a major role in the metabolism of some drugs and organophosphate insecticides. It is the latter with which the literature on these esterases is particularly occupied. High levels of this esterase are an important safeguard against the toxic effects of such compounds.

In 1988 the classification of 'A' esterases and arylesterases was reviewed and it was recommended that 'A' esterases were removed from classification EC 3.1.1.2. (arylesterase) and together with the esterase DFPase form a new group - Organophosphorus Compound Hydrolase (Mackness, 1989).

This was the result of a number of studies which provided overwhelming evidence to suggest that 'A' esterase and arylesterases were in fact different enzymes.

4.3.5 Paraoxonase (A-esterase)

Extensive studies have since shown a differential distribution of arylesterase and paraoxonase activities in a wide range of human population groups, other mammalian species and birds.

Mackness and Walker (1983) carried out polyacrylamide gel electrophoresis of sheep serum and found that the fractions exhibiting paraoxonase and arylesterase activity were not coincidental.

Several other studies have also shown that paraoxonase and arylesterase have different peaks of activity in Caucasian populations. Human serum paraoxonase activity was found to be bimodally distributed (Eckerson et al, 1983b) and arylesterase activity to exhibit a unimodal distribution (Simpson, 1971; Lorentz et al, 1989).

In 1987 Mackness et al looked at aryl- and A-esterase activity in the sera of 14 species of bird. The serum arylesterase of at least ten of these birds possessed no measurable paraoxonase activity.

Gel filtration of human serum in the same study produced two major peaks with paraoxonase activity and three with arylesterase activity, none of which overlapped. Erdos and Boggs (1961) showed that hydrolysis of paraoxon in human serum is partly inhibited by ethylene diamine tetraacetic acid (EDTA). The reason for this was proposed by Ortizoga-Ferado et al (1984) who resolved human serum paraoxonase activity into two fractions using gel-filtration chromatography. The first peak of activity (peak I) was found to be a high molecular weight compound which co-eluted with arylesterase. The second fraction was closely associated with albumin (peak II).

The esterase activity of albumin is well known. However in purification it cannot be ruled out that a minor protein which cofractionates with albumin is responsible for the hydrolysis of paraoxon and not the albumin itself. The

likelihood of this was ruled out by investigating paraoxonase activity in an individual who was analbuminaemic. Ortizoga-Ferado et al (1984) found that, in this case, there was only one peak of paraoxonase activity which was completely inhibited by EDTA.

Earlier experiments by the same (Mueller et al, 1983) and other workers (Eiberg et al, 1981) demonstrated differences in the pH profiles of the two purified fractions. These pH profiles were very similar to the pH profiles of individuals with high and low phenotypes. In Caucasians the EDTA-sensitive enzyme (peak I) shows a genetically determined polymorphism governed by two alleles (Geldmacher-von-Mallinckrodt and Diepgen, 1988). Other ethnic populations show different distributions of the two phenotypes (see 5.3). However, the EDTA-stable paraoxonase (peak II-albumin) shows unimodal distribution. The determination of phenotype is discussed in 5.31. These different pH profiles are perhaps best explained by considering the peak of activity which predominates in each of these phenotypes. In an individual with serum homozygous for the high activity. Peak I accounted for up to 75% of the paraoxonase activity. Conversely Peak II is responsible for 70% of the paraoxonase activity in sera homozygous for the low activity allele.

Thus, it follows that the pH profile of paraoxonase activity in the serum of an individual most closely resembles the pH profile of the predominating peak. In a serum homozygous for the high activity this is Peak I and for the low activity allele Peak II. (Geldmacher-von-Mallinckrodt and Diepgen, 1988).

4.3.6 Conclusion

Individual variations in the level of esterase activity may determine the pharmacological effect of many prodrugs dependent on them for hydrolysis. Conversely, the therapeutic effect of an active drug may be terminated by the same or different esterases.

The inter-individual variation in some esterase activity (notably paraoxonase) has been well documented, with less information available to date on others.

Intra-individual variation due to seasonal, nutritional and pathological influences is largely circumstantial, except perhaps with regard to seasonal studies which are easier to control.

All these influence blood esterase activity and the ability of an individual to hydrolyse ester compounds. The following chapters consider these factors individually.

CHAPTER 5

Factors Affecting Blood Esterase Activity

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Factors Affecting Blood Esterase Activity

There have been several studies to investigate the intra-individual variation in cholinesterase, particularly in plasma, over different time periods. Sidell and Kaminskis (1975a) measured cholinesterase activity twice weekly in 22 subjects over 1 year. In a matter of weeks they claimed the activity changed by as much as 25-50% in a normal healthy population. Brock and Brock (1990) used a much larger population of 131 individuals and found the intra-individual variation was anything between 3% and 42% over an 8 month period. Mason et al (1989), however, using a much smaller group of 9 people (7 men, 2 women demonstrated only a 4% variation over a 13 month period. In the latter study, it may have been that the proportionally smaller number of women reduced the well documented hormone-related changes in cholinesterase activity (Lepage et al, 1985).

Brock and Brock (1990) and Mason et al (1989) both found a much smaller biological variation in erythrocyte AChE activity over their respective study periods. This may be due to the longer life span of red blood cells (120 days) compared with the 7 day life span of plasma ChE.

Paraoxonase intra-individual variability measured by Mutch et al, 1991 (in press) was found to be more variable than plasma cholinesterase and RBC AchE in 5 individuals measured 4 times over the span of one year. The maximum increase measured was obtained one week after Christmas and could be associated with

dietary excess, since paraoxonase activity has been linked with High Density Lipoprotein (HDL) (5.4). Over a period of only 35 days, Zech and Zucher (1974) found that paraoxonase activity was remarkably constant.

Seasonal variations may also occur. Ratner et al (1989) measured ChE activities in the whole blood of an Israeli population over one year (n = 228, summer; n = 117, winter). He found a reduced plasma cholinesterase activity in the summer compared with the winter months and attributed this to an increased exposure of the population to organophosphate insecticides on fruit crops during this period.

Plasma aspirin esterase activity is largely due to cholinesterase activity (Rainsford et al, 1980; Morikawa et al, 1979) and will thus follow a similar pattern of intra-individual variation with time.

5.1 Age

A number of plasma esterases have been shown to be affected by age but is not clear whether this occurs across the age spectrum. A reduced plasma aspirin esterase activity has been identified in frail elderly individuals but not in the fit elderly (Williams et al, 1989), when compared with fit young subjects. In the same study the authors identified a significant reduction in plasma arylesterase activity (using phenylacetate as a substrate) in the frail elderly.

In these observations, age *per se* was not an accountable factor in the reduced activity. Frailty, however, was, and this may be in important consideration at any age.

Mueller et al (1983) measured paraoxonase activity in 60 people aged between 3 months and 78 years. He found no significant alteration of paraoxonase activity over this age range. Analysis of 31 cord blood samples in the same study showed that paraoxonase activity is not fully developed at birth and is still reduced in infants less than 1 year old (Ecobiochen and Stephens, 1973).

Playfer (1977) found no change in paraoxonase activity with age in a cross section of an elderly population comprising healthy elderly, hospitalized elderly and nursing home residents aged 70-96 years.

Zech and Zurcher (1974) however found that the average paraoxonase activity was lower in older people but since they gave no details of the population type these individuals were drawn from, whether this was an age-related effect is unclear.

McCance et al (1949) and Lehmann et al (1957) observed serum cholinesterase activity to be slightly lower in the newborn than in the adult human. After approximately two months the activity reached adult levels and then continued to increase so that during childhood values were considerably higher than in adults.

In the study by Williams et al (1989) referred to earlier, plasma cholinesterase activity was also measured in fit and frail elderly and compared with that in young volunteers. The measured activity was not significantly lower in either of the elderly groups although there was a trend towards a reduced activity in the frail hospitalized group.

McWilliams et al (1990) recently confirmed no significant difference between 'normal' red blood cell AChE and that from demented or depressed elderly people. However, there did appear to be a reduced red blood cell AchE in normal women over 80 years.

Sidell and Kaminskis (1975b) found that RBC AChE actually increases with age in both sexes. Those individuals aged less than 30 years appear to have the lowest RBC AChE as a group, and although the authors did not find statistically significant differences between each successive age group, there was a demonstrable significant increase between the younger and older age groups. Lawson and Barr (1987) conclude a deficiency of RBC AChE has no pathogenic significance (see 6.3).

5.2 Sex

Some groups have reported a higher plasma aspirin esterase activity in males than females (Gupta and Gupta, 1977; Menguy et al, 1972a; Morikawa et al, 1979) but other reports show no apparent sex difference (Rainsford et al, 1980, Williams et al, 1989).

It has been well established over the years that plasma ChE activity is greater in men than women (Moses et al, 1986, Lepage et al, 1985; Sidell and Kaminskis, 1975b).

This has been explained by hormonal differences between women and men. Prior to puberty, the cholinesterase activity in males and females are similar. The activity decreases by about 10% after the onset of puberty and increases

again by about 16% in post - menopausal women reaching the same value for men (Lepage et al, 1985). Sidell and Kaminskis (1975b) reported similar ChE activities in post-menopausal women and men over 60 years old.

In a study looking at the possible effect of the menstrual cycle upon the inter-individual variation seen in females, Fairbrother et al (1989) concluded that the relationship between ChE activity and hormone concentration was a complex one. They suggested that there may be an interaction of progesterone and oestrogen with each other and with other hormones such as thyroxine. Certainly, a lower ChE activity has been demonstrated in young women (Sidell and Kaminskis, 1975b; Lepage et al, 1985) but this is further reduced by oral contraceptives (Sidell and Kaminskis, 1975b; Fairbrother et al, 1989).

Fairbrother et al (1989) also found weekly variations in ChE activity from as little as 4% to as much as 32% in some women. There was no apparent pattern of variation and activities did not alter with hormonal treatments. In contrast, RBC AChE has been found to be higher in women than men and higher still in women taking oral contraceptives (Sidell and Kaminskis, 1975b).

Apart from the possible hormonal effects *per se*, the fluctuating hydration status of females throughout their menstrual cycle and the dilution effect this will have on plasma proteins does not appear to be considered in the literature.

Following initial studies on the paraoxonase activity in different ethnic groups, Geldmacher-von-Mallinckrodt et al(1983) looked for sex differences in activity but found none. Agarwal et al (1982) found similar results in rats. Mueller et al (1983b) found a slightly higher mean value in females compared with males but

this was not statistically significant. Mutch et al (in press) suggest that the absence of a high activity group in their study population may be partly due to the absence of females but with little direct evidence.

5.3 Race, Genetics and Population Studies

There have been extensive studies into plasma esterase activities exhibited by various ethnic groups. In 1982 Kalow observed that ethnic groups afforded some degree of predictability with regard to intra-individual variation in the metabolism of some compounds.

Different races are also exposed to different environmental pressures (eg nutrition and xenobiotics) which will be discussed in greater depth later.

Williams et al (1986) measured plasma aspirin esterase activities in Caucasian and Ghanaian subjects and found these to be generally lower in the Ghanaian subjects. Interestingly, the cholinesterase activity was preserved in this group. The authors hence suggested other factors were more important in determining the rate of aspirin hydrolysis.

Similarly lower plasma aspirin esterase activities were also found within a Nigerian population (Isah et al, 1988) which was not accompanied by a reduced plasma cholinesterase activity.

The worldwide distribution of serum paraoxonase activity has been investigated (Geldmacher-von-Mallinckrodt and Diepgen, 1988). Different ethnic groups have been shown to exhibit different patterns of serum paraoxonase activity. Of

the populations studied Playfer et al (1976) found the Indians and Caucasians both exhibited a bimodal distribution. This, however, was not the case in African, Malaysian and Chinese populations where distribution was unimodal. Negroid and Mongoloid samples were also bimodally distributed but, compared with the Europeans, there was a lower percentage of the population in the low activity group (Geldmacher-von- Mallinkrodt and Diepgen, 1988).

In some of the ethnic groups studied no low activity group was detected (Hommel, 1978). This was particularly the case in some African, Aborigine, South Pacific and South American tribes. In fact the frequency of the low activity phenotype seems to reduce as the distance from Europe increases (Diepgen and Geldmacher-von-Mallinckrodt, 1986).

Arylesterase (phenylacetate esterase) has been studied to a much lesser degree. Williams et al (1986) found a significantly lower activity of plasma arylesterase in the same group of Ghanaian individuals mentioned above.

5.3.1 Phenotyping A-esterase Status

Eckerson et al (1982, 1983a) postulated that human serum paraoxonase is a polymorphic enzyme determined by two allelic genes at one autosomal locus. The two isozymes are known as 'A' and 'B' and the three phenotypes as A, AB and B.

Three qualitative differences in paraoxonase to arylesterase isoenzymes A and B are used to classify the phenotypes (arylesterase has a unimodal distribution).

1) 1M sodium chloride (NaCl): Isoenzyme B is stimulated 2-3 times whilst isoenzyme A is minimally affected (Eckerson et al, 1983a).

2) Ratio of paraoxonase to arylesterase activity: Isoenzyme ratio of isoenzyme B is approximately 7 times that of isoenzyme A. (Eckerson et al, 1983b).

3) 0.1mM chlorpromazine: The residual arylesterase activity of isoenzyme A is less than that of isoenzyme B.

5.4 Nutrition

As previously discussed the biological function of plasma cholinesterase has been the subject of much speculation. It is well known that a significantly reduced serum cholinesterase activity may be indicative of liver damage (Brown, 1981).

In 1950 Waterlow suggested that such damage could be the direct result of malnutrition when he observed an increase in the serum ChE activity of two malnourished infants fed on a high milk diet. This observation led to the suggestion that there was a relationship between ChE and food assimilation. A number of animal experiments have since followed.

One early such investigation involved fasting mice for 48 hours and then refeeding them at time intervals with bread and water. Liver ChE was monitored by histochemical methods and reached its peak after four hours followed by a decline (Gerebtzoff, 1959).

Later work in rodents has added to, and in some ways confused the ever mounting data on ChE and energy storage. For example, Kutty et al (1981) demonstrated an increased liver and serum ChE activity in normal, obese and diabetic mice fed on a high calorie (supplied as carbohydrate) diet. In contrast the ChE activity in the adipose tissue of these groups was reduced. Hyperglycaemia was a common feature in all these animals. Kutty suggested it may be a cause of ChE repression in this tissue and that adipose ChE may actually be related to the hormone sensitive lipase which is reduced in obesity. Osada et al (1989) carried out similar work on male Wistar rats fed on a high fat diet. They found a corresponding reduced serum ChE activity in these animals which is in agreement with those of Kean et al, 1986.

Most of the recent work has concentrated on circumstantial evidence involving the measurement of serum ChE activity in sub-populations of people with various genetically acquired hyperlipoproteinaemic conditions (Cucuianu et al, 1968; Jain et al, 1983; Schouten et al, 1988).

Kean et al (1986) attempted to elucidate exactly the optimum composition of food required to raise liver ChE in mice. A high protein diet enhanced the enzyme activity the greatest. Those mice fed high protein diets had twice the amount of absolute fat as those fed on a low protein diet. Obese animals show increased lipogenesis and hence increased by-products as the result of fatty acid metabolism - the significance of which is discussed below.

In addition to these, an observation by Burch et al, 1957 confirms the link of serum cholinesterase with dietary protein. In patients with Kwashiorkor, serum

ChE activity increased two fold when treated with a high protein diet compared with pretreatment levels.

A nutrition survey of elderly people in Great Britain (DHSS 1979a) found that, in men, serum pseudocholinesterase activity correlated significantly with arm circumference, skin fold thickness, protein intake and serum albumin concentration. This has been corroberated by Lepage et al (1985).

These factors are closely related to protein synthesis and hence ChE, as one of many enzymes, reflects them.

Chu et al (1978) correlated plasma ChE activity in whole plasma and low density lipoprotein fraction (LDL) with triglycerides and cholesterol levels in patients suffering various types of hyperlipoproteinaemias. The results of this and other studies led to suggestions that cholinesterase may be involved in lipid and lipoprotein metabolism.

A significantly increased ChE activity is most often the consequence of an individual exhibiting metabolic disorders related to the storage and mobilization of fat. Such disorders include obesity, hyperlipidaemia (Kutty et al, 1981), diabetes (Antopol et al, 1937), nephrotic syndrome - especially in association with hyperlipoproteinaemia (Way et al, 1975) and hyperthyroidism (Antopol et al, 1937). These individuals usually have increased serum levels of very low density lipoprotein (VLDL) or pre-betalipoprotein, which is the result of excess fatty acids derived from carbohydrate metabolism (Kutty et al, 1981).

In 1963 Clitherow postulated that butyryl Co A produced during fatty acid metabolism may form butyrylcholine in the presence of choline. The excess of these metabolites produced in susceptible individuals would therefore require detoxification to prevent possible nicotinic side effects. These would be eliminated by means of a compensatory increase in cholinesterase levels.

Serum ChE appears to form a close association with LDL by reacting with the phosphoryl choline site of lecithin (Kutty et al, 1973). Hence there may be two pools of ChE activity in serum - one is free and the other forms an integral part of LDL structure (Kutty et al, 1977). This may be formed via an unstable VLDL - ChE intermediate.

The ultimate product however, is very stable and only ultrasonification or treatment with phospholipase D will release free ChE.

Cucuianu et al (1968) proposed a link between raised serum ChE activity and high levels of lipoproteins. Schouten (1988) confirmed Cucuianu's proposal of this relationship and more particularly agreed that this relationship was strongest with VLDL.

Jain in 1983 measured serum ChE and lipoproteins in a patient who accidently ingested parathion - a powerful organophosphate anticholinesterase. ChE activity was reduced as was VLDL and LDL but there was an increase in high density lipoprotein (HDL). During the next 72 hours following ingestion ChE enzyme activity increased in parallel with VLDL and LDL, and a decrease in HDL.

Schouten and Cucuianu both agree "... there is probably no cause and effect relationship between these two parameters... the relationship... if there is any at all, is certainly not a simple one" (Schouten, 1988; Cucuianu, 1988). Kutty et al (1976) suggested a close relationship between AChE and lipoproteins in the cell membranes of red blood cells. Increased fragility of red cells has been demonstrated when treated with physostigmine. (Milstock et al, 1972). In addition AChE and lipoprotein are extracted together when the erythrocyte ghosts are treated with hypertonic saline (Kutty et al, 1976).

Paraoxonase, similarly, has been shown to be closely associated with lipids. Mackness et al (1983, 1989) undertook studies to investigate this association. In sheep they found most of the serum paraoxonase activity was closely linked with HDL. Ultracentrifugation of human serum caused paraoxonase activity to partition in to the lipoprotein fraction at a similar rate and manner to HDL.

Further links between HDL and paraoxonase activity have been based on observations in subgroups of patients with possible or real disturbances in lipid levels. Reduced serum paraoxonase levels were found in malnourished children compared with healthy children (Mackness and Clerc, unpublished).

Fish-eye disease is a familiar condition which involves, among other symptoms, abnormal plasma lipoproteins. The HDL concentration was found to be reduced by 90% and the paraoxonase activity by 89% in these individuals compared to controls (Mackness et al, 1987b).

Tangier disease is a condition in which levels of apolipoprotein AI and AII are 100 and 10 times respectively lower than controls. In a patient suffering from

this disease no paraoxonase activity could be detected (Dumon et al, 1986).

However, as La Du (1988) suggests, paraoxonase may not be stable without the presence of HDL with which to form a complex. The reduced paraoxonase activity may therefore be a secondary effect of a clinically low HDL level. A primary deficiency in this enzyme would establish the clinical features associated with, or requiring paraoxonase.

Although very difficult to substantiate, it is possible that the interethnic differences which exist in serum esterases are due to environmental factors such as nutrition (Kalow, 1982). Isah et al (1988) found Nigerians living in the UK had similar plasma aspirin esterase activity and albumin concentrations to British subjects, but a low cholinesterase activity. Nigerians resident in Nigeria showed significantly lower plasma aspirin esterase and cholinesterase activity and albumin concentration than British subjects.

5.5 The Effect of Xenobiotic Exposure on Blood Esterases

Xenobiotics are defined as any substance which is foreign to the biologic system. This includes drugs and substances such as insecticides. Some substances as apparently innocuous as ethyl alcohol (ethanol) can thus be regarded as a xenobiotic since it is of minimum nutritional content.

A reduction in serum cholinesterase activity was observed by Ward et al (1976) in patients suffering chronic liver disease, including alcohol related damage, and also by Liu and Yuan (1985). The reduction would appear to be proportional to

the degree of liver damage (Foldes, 1956).

In 1980 Rainsford showed that serum aspirin esterase was severely depressed in patients with alcohol induced liver cirrhosis. Since aspirin esterase activity is due to cholinesterase and albumin, both indicators of liver function, this is hardly surprising.

Builder et al (1977) also demonstrated an inhibitory effect of alcohol on aspirin esterase activity in the gastric mucosa. This may partly explain the reason for the observed increase in blood loss induced by aspirin, since a local concentration of unionized aspirin in the mucosal cell initiates the chain of events leading to breakdown of the mucosal barrier. Red blood cell acetylcholinesterase was suggested to have possible use as a biochemical marker of alcoholism when it was found to be lower in a group of alcoholics (Haboubi et al, 1986). Abernethy et al (1986) disputed this and it is more likely that inappropriate storage is to blame.

The most extensive *in vitro* work on possible drug effects on serum aspirin esterase was carried out by Gupta et al (1979). The authors looked at a number of drugs chosen on the basis of most likely concurrent administration with aspirin. Only calcium and magnesium ions were found to be activators of this enzyme, the majority were inhibitory or had no effect.

Rainsford et al (1980) found that sodium aurothiomalate and D-penicillamine produced higher levels of plasma aspirin esterase activity in six patients.

Because these esterases originate in the liver it seems likely that any substance

capable of inducing liver enzymes may also induce esterase production. Puche et al (1989a) found the antiepileptic drugs sodium valproate, carbamazepine and phenytoin administered together raised serum aspirin esterase activity in epileptics when compared with normal controls. A subsequent study looked more particularly at serum cholinesterase in patients receiving only one of these drugs (Puche et al, 1989b). As expected activity increases were found with aspirin esterase and cholinesterase. No significant difference was seen between each of the individual drugs.

Habitual aspirin intake, often without an accepted clinical indication, is especially prevalent amongst women. Gupta et al (1977) investigated aspirin esterase activities in a group of pregnant women, against a control group of pregnant women who were occasional users only. Unfortunately the physiological variability contributed to by the 'hormonal' state of pregnancy and the fact that there were only 12 women in the control group compared with 59 in the experimental group make firm conclusions difficult. Nevertheless plasma aspirin esterase levels were reported to be significantly lower ($p < 0.05$) in the experimental group.

Rainsford et al (1980) found similar results in rheumatoid arthritic patients taking regular aspirin.

Exogenous hormones taken in the form of oral contraceptives can have a profound effect on some esterases. (See 5.2).

5.5.1 Organophosphate Insecticides

Organophosphate insecticides are very potent inhibitors of acetylcholinesterase and cholinesterase. The pharmacological effects of these substances are largely due to inhibition of acetylcholinesterase in the nervous system. Serum cholinesterase is the enzyme most readily inhibited (Namba, 1971) and as such is a very useful biochemical marker for possible exposure to such inhibitors (Health and Safety Executive, 1987).

Grob et al (1947) administered 2 mg diisopropyl fluorophosphate (DFP) intramuscularly or intraarterially and observed a drop of cholinesterase to 5% of its original activity. Red blood cell AChE in contrast only dropped by 65%. Within one hour the serum cholinesterase had dropped to near zero but it took 24 hours for the RBC AChE to show maximal depression. It is still not known how AChE differs structurally from ChE to make it more reactive than AChE to organophosphate esters (Lockridge, 1987). It may, in fact, be due to the inaccessibility of AChE to the substrate because it is membrane bound to RBC.

Serum paraoxonase has an important role to play in protecting cholinesterase against the organophosphate paraoxon by hydrolysis to paranitrophenol (Eckerson and La Du, 1984).

Not all xenobiotic effects are detrimental to the individual. A number of cholinesterase inhibitors are used clinically. Although they inhibit both cholinesterase and acetylcholinesterase, it is the effect on the latter which makes them clinically useful. Inhibitors such as physostigmine bear a close structural similarity to acetylcholine and they are used in conditions such as myasthenia

gravis and in eye drop formulations to treat glaucoma (Williams, 1985). Unlike the organophosphate insecticides where the effect is irreversible, the inhibition is terminated when the ester linkage is hydrolysed within a matter of hours.

Certain anti-cancer drugs eg cyclophosphamide irreversibly inhibit cholinesterase as a side effect. In such cases it can take 2 weeks to regain 70% of normal activity and 6-8 weeks to regain full activity, until the liver synthesises new cholinesterase.

CHAPTER 6

The Effects of Altered Blood Esterase Activity

Chapter 6

The Effects of Altered Blood Esterase Activity

6.1 The Effect of Impaired Blood Esterase Activity on Drug Handling

Any compromise in serum cholinesterase activity or other esterase activity will affect the pharmacological effects of xenobiotic compounds dependent on them for metabolism. If hydrolysis by esterases removes the active drug then impaired esterase function will result in reduced clearance and possible dose-dependent side effects. As an example, alcohol cirrhotics may experience gastrointestinal symptoms due to therapeutic doses of aspirin (Menguy 1972b). Alternatively if the esterase hydrolyses a prodrug, impaired esterase activity may mean that insufficient levels of the drug are released for pharmacological effect before the prodrug is cleared. Examples are shown in Tables 6.1 and 6.2.

Menguy et al (1972b) demonstrated a significantly reduced serum aspirin esterase in 22 individuals with portal cirrhosis which would imply that this group of individuals is especially vulnerable to complications from aspirin ingestion. This is worsened by hypoalbuminaemia and subsequent increased fraction of unbound salicylate (Roberts et al, 1983). Rainsford et al (1980) confirmed this finding.

Suxamethonium (succinylcholine) is a depolarizing muscle relaxant. Recovery of muscle control following administration is due to the rapid hydrolysis by plasma cholinesterase to succinylmonocholine and the slower conversion to succinic acid

Table 6.1 :

Hydrolysis of drugs to less active drugs.

Drug	Pharmacological action	Inactive /Less active type
Acetylsalicylic acid	analgesic	salicylic acid
Succinyl choline	muscle relaxant	choline
Procaine	local anaesthetic	p-aminobenzoic acid

choline (Tsuiji et al, 1955). Recovery from the effects of suxamethonium is usually complete within 3 to 20 minutes (Foldes et al, 1956). Shortly after its introduction in the early 1950s, several cases of prolonged apnoea were reported (Gould 1952,Harper, 1952). These patients were found to have low serum cholinesterase (Evans et al, 1952). Although the atypical gene variant of low plasma cholinesterase activity is a very important factor, it cannot be used as a sole indication of increased susceptibility to prolonged drug effects (Bauld et al, 1974). Other pathological conditions affecting cholinesterase activity have been discussed earlier and include liver diseases; malnutrition, malignancies etc (Brown, 1981).

Considerable evidence does exist to support the premise that it is *serum* cholinesterase which determines the likelihood of prolonged apnoea. Goedde et al (1968) normalized the duration of apnoea by injecting a highly purified pseudocholinesterase fraction. Cholase from Behringwerke AG is only 5.5% pure but is a preparation which has been used successfully by the Danish Cholinesterase Research Unit to reverse succinylcholine apnoea (Viby-Mogensen, 1981).

6.2 Atypical Serum Cholinesterase

Because cholinesterase has been identified using a very specific substrate, studies regarding its activity, mode of behaviour, inheritance pattern etc are far more extensive.

Shortly after the introduction of the muscle relaxant suxamethonium for use in anaesthesia in the early 1950s, there were reports of patients experiencing

prolonged apnoea as a result of its use (Gould, 1952; Harper, 1952). Numerous reasons were suggested for this phenomenon (Foldes, 1956) but since then an atypical cholinesterase variant has been found to be responsible.

There is only one gene for human cholinesterase so all cholinesterase genotypes must arise from this locus (Lockridge, 1990). The silent allele, atypical cholinesterase was first proposed by Liddell et al (1962) and since then there have been a number of quantitative variants identified. When in combination with the 'usual' gene, although exhibiting a reduced activity, they are indistinguishable from usual cholinesterase. It is not until they are in combination with silent cholinesterase occurring as a heterozygote phenotype that they are detectable (Lockridge, 1990).

Each variant appears with different frequency in sub-populations and imparts varying degrees of reduced activity. In addition, not all the variants have been positively identified as yet.

Population studies to determine the worldwide distribution of the atypical allele were initiated in the late 1950s. However a limited selection of small groups led to similar gene frequencies being proposed in the ethnic populations studied (Kattamis et al, 1962; Horsfall et al, 1963).

Whittaker (1986) presented a comprehensive summary of ChE allele frequencies in various populations throughout the world. The Jews of Iraq and Iran have amongst the highest occurrance of the atypical allele which contrasts with other Jewish populations who have the much lower gene frequency of the Northern European populations. The Alaskan Eskimos show the highest incidence of

the silent allele where 1603 people were tested , 28 were found homozygous silents and 301 were heterozgotes (Lockridge, 1990).

The C5 + variant is associated with up to a 30% increase in ChE activity (Robson and Harris, 1966) and is probably due to an association between ChE and a second unidentified protein (Scott and Powers, 1974). From the 40,000 people tested for this variant, 9% of Europeans, 3% of Asians, 5% of Africans and 7% of Americans have been identified as carriers (Lockridge, 1990).

At the molecular level, Clark et al (1968) showed that positively charged compounds affect the catalytic properties of normal and atypical ChE differently. Valentino et al (1981) predicted sensitivity to a number of drugs including aspirin, in individuals with usual and atypical cholinesterase. They found that atypical and typical cholinesterase have the same turnover number and Km (affinity of the enzyme) for positively charged substances and aspirin. This suggests that the esteratic sites are the same but the charged sites are altered in some way in atypical cholinesterase. Muensch et al (1978) suggested that atypical cholinesterase carried a positively charged residue instead of a negative one. In contrast, Das (1976) concluded that a mutation of a single amino acid residue may lead to a change in the positioning of the active centres with relation to one another and a modification of binding sites.

6.3 Clinical Significance of Blood Esterases and their Pathology

Serum cholinesterase is a direct and historically useful indicator of liver function since it is manufactured in the liver and immediately enters the general circulation (Brown, 1981). Any disease which causes reduced protein synthesis

therefore results in reduced serum cholinesterase activity. The usefulness of a single determination is limited by the occurrence of the silent gene (which is very rare) and variability related to sex, age, body fat etc. Its greatest value is as a prognostic tool when a series of measurements are made.

A number of authors have commented on the sharp reduction in serum cholinesterase activity (Hunt and Lehmann, 1961; LaMotta et al, 1957) and phenylacetate esterase activity (Takahashi et al, 1967) shortly before an individual dies. This is most likely to be due to a breakdown of protein synthesis. In a patient who dies from acute liver failure however, serum ChE activity may not fall below normal baseline values. This is probably due to the half-life of ChE of 12-15 days. Death may therefore occur before the activity has fallen enough to be evident (Ward et al, 1976).

Malignant disease can result in a reduced rate of protein synthesis and plasma cholinesterase levels always appear to fall (Brown, 1981). Whetstone et al (1960) observed a specific reduction in cholinesterase compared with other plasma esterases, albumin etc. McComb et al (1964), suggested that this may be due to a selective depression by the cancer cells on cholinesterase formation or activity.

Burn injuries have also been show to cause a fall in plasma cholinesterase activity (Price et al, 1970). The initial drop in activity is probably due to loss of the enzyme through capillaries and intensive fluid therapy. The persisting low activity may be due to a reduced cholinesterase synthesis since it is known that skin burns cause a disturbance in liver function (Brown, 1981).

Deficiency of erythrocyte AChE does not appear to have pathological significance and there are only a few conditions in which enzymes are abnormal. As one might expect, these are largely manifest in cell membrane abnormalities (see 4.3.3) and include paroxysmal nocturnal haemoglobinuria and hereditary spherocytosis (Lawson and Barr, 1987).

Attempts have been made to use plasma cholinesterase and RBC AChE as peripheral markers for the early diagnosis of patients with Alzheimer's disease. Results have been confused and often contradictory. Smith et al (1981) reported an apparent increase of plasma cholinesterase by as much as 101% in patients with Alzheimer's disease but no significant decrease in RBC AChE in these patients compared with "normal" age matched controls. Atak et al (1985) additionally looked at activities in individuals with neurotic and endogenous depression. They found no difference in RBC AChE activity in any of these groups which was in agreement with Smith et al (1981) and also no difference in plasma cholinesterase which was in direct conflict with Smith's findings.

Serum paraoxonase has been reported to be reduced after a recent myocardial infarction (McElveen et al, 1986) although this was not confirmed by Secchiero et al (1989). Premature coronary disease has been associated with low concentrations of HDL (Miller and Miller, 1975) which suggests that serum paraoxonase and HDL could be used to predict the susceptibility of an individual to developing atherosclerosis (Secchiero et al, 1989).

As discussed previously, in patients suffering from Fish-Eye disease, Mackness et al (1987b) found that HDL was reduced by 90% and that paraoxonase

apparently reduced in parallel by 89%. In Tangier disease (analalphaproteinaemia) no paraoxonase activity was detected (Dumon et al, 1986).

In 1985, Eiberg produced evidence to suggest that there was a genetic link between cystic fibrosis and a low paraoxonase activity. Unfortunately further genetic studies have resulted in no such confirmation (Tsui et al, 1985) and the current view is that there is no biological or physiological link between the two conditions.

Plasma esterases have thus been linked on numerous occasions with a variety of clinical conditions. Some of these associations are somewhat tenuous. Those which aren't tend to be when the esterase concerned directly reflects liver function and hence protein synthesis.

6.4 Pharmacological Significance of Plasma Esterases

The pharmacological significance of plasma esterases is not in dispute. Ester prodrugs have extensive use in medicine and by definition are hydrolysed by esterases. This occurs in the intestinal mucosa, liver, serum and locally at the site of action following subcutaneous administration. They have been formulated to overcome the factors which limit the concentration of active drug available for pharmacological action in the body and also to maintain a more controlled release of active drug. Examples of the latter include oil based depot injections of hormones and antipsychotic drugs. The lipophilic prodrug is dissolved in the oil and is slowly released. The rate of release is generally dependent in the structure of the ester group. Hydrolysis is due initially to tissue

carboxylesterases but some of the prodrug slowly perfuses into the blood where the majority is acted on by serum esterases.

Orally administered prodrugs have been formulated to overcome problems due to incomplete absorption. About 40% of the antibiotic ampicillin is absorbed from the gut due to its amphoteric nature. Esters of ampicillin such as pivampicillin have increased its lipophilicity and improved absorption across the gut wall (Williams, 1985).

Chloramphenicol is prepared as chloramphenicol palmitate which makes the bitter taste of the parent drug more palatable. Steroid creams are formulated as the more lipophilic ester to aid transdermal absorption. Release of the active steroid relies on tissue esterase activity.

Examples of such prodrugs and the corresponding active moiety are shown in Table 6.2.

6.5 Summary

As discussed above plasma esterases are of considerable clinical value in diagnostic, prognostic and pharmacological fields of medical research. As a direct product of the liver they are a valuable source of biochemical information. However there are still many aspects of their existence which have not yet been elucidated, not least of which is their physiological function.

Table 6.2

Hydrolysis of less Active Prodrugs to Active Drugs

Prodrug	Pharmacological type	Active Moiety
Heroin	analgesic	6 - monoacetylmorphine
Benorylate	analgesic	paracetamol / salicylate
Clofibrate	hyperlipidaemic	clofibric acid
Carbimazole	hyperthyroid	methimazole
Testosterone cyprionate	steroid	testosterone
Methylprednisolone acetate	steroid	methyl prednisolone
Erythromycin estolate	antimicrobial	erythromycin
Pivampicillin	antimicrobial	pivampicillin
Chloroamphenicol succinate	antimicrobial	chloramphenicol
Prochlorperazine valerate	neuroleptic	prochlorperazine
enalapril	angiotensin converting enzyme inhibitor	enalaprilat

CHAPTER 7

Aims and Outline of the Studies Undertaken

Chapter 7

Aims and Outline of the Studies Undertaken

The studies described in this thesis were conducted in order achieve the following aims:

1. To elucidate the nature of plasma aspirin esterase and determine the difference in activity between fit and frail elderly and the reason they exist.

2. To investigate the effects of frailty on blood esterases in general in the fit and frail elderly.

3. To investigate the effect of nutritional supplementation on blood esterase activity in the malnourished frail elderly individual.

The study in Chapter 8 was carried out in order to elucidate plasma aspirin esterase kinetics in the frail elderly individual and compare these with the results in fit elderly and young people. The work in Chapter 9 extended this investigation by isolating the enzymatic components and in Chapter 10 the kinetic analyses were repeated using the purified cholinesterase.

In Chapter 11 the activities of several blood esterases in frail individuals, fit elderly and young people were determined and compared. The study discussed in Chapter 12 aimed to increase the activity of several plasma esterases in hospitalized frail elderly people (who showed a reduced activity) by means of improving their nutrition. In addition anthropometric measurements were obtained which included weight, triceps skinfold thickness and mid arm circumference.

CHAPTER 8

Determination of Plasma Aspirin Esterase Kinetics in Young, Fit and Frail Elderly

Chapter 8

Determination of Plasma Aspirin Esterase Kinetics in Young, Fit and Frail Elderly - Identification of the enzymes involved.

8.1 Introduction

Plasma aspirin esterase activity is thought to be dependent on two components, albumin contributing a possible 20% of the activity and cholinesterase contributing 80% of the remaining activity (Rainsford et al, 1980). Plasma aspirin esterase activity in frail elderly individuals, as defined by the criteria of Woodhouse et al (1988), was found to be reduced by Williams et al (1989). Albumin concentrations were also low in these individuals. Cholinesterase activity, however, was not significantly reduced at the 5% level of significance but did show a trend towards a reduced activity.

It is possible therefore, that the reduced plasma aspirin esterase activity is due to a change in the nature of albumin or the cholinesterase enzyme (eg substrate affinity) or a reduction in the quantity of one or both these components.

The aim of the following study was to identify the enzyme(s) hydrolysing aspirin in human plasma; to separate them using kinetic and/or physical means; and to analyse the kinetic parameters Km and Vmax of one/both components in the whole plasma of young, fit elderlyand frail elderly individuals.

8.2 Methods

8.2.1 Plasma Aspirin Esterase Activity: Identification of the Enzyme Components.

a) In Whole Plasma

Physostigmine is a specific inhibitor of cholinesterase. Adding this to an incubation mixture of plasma and aspirin inhibits the cholinesterase so that remaining aspirin esterase activity is due to albumin.

Physostigmine at concentrations between 0.05-0.4 mM was added to incubations of plasma and aspirin solutions between 0.4 and 3mM. The formation of salicylate was monitored at 300 nm on a direct reading spectrophotometer (see 8.2.2b)

b) Human Albumin Fraction V

A fresh albumin solution at 40 g/l concentration was added to an incubation mixture containing 3mM aspirin concentration. Salicylate formation was monitored as before.

8.2.2. Kinetic Analysis for Plasma Aspirin Esterase.

a) Volunteers

These comprised 10 (5 female) young adults with a mean age 24 years (range

20-31 years), 7 (6 female) fit elderly adults with a mean age of 73 years (range 70-79 years) and 10 (7 female) frail elderly patients with a mean age of 79 years (range 72-86 years). The individual details are shown in Table 8.1.

Patient Blood Samples

All studies were approved by Newcastle Ethics Committee. All blood samples were taken with the individual's informed consent or the consent of a relative where appropriate.

Frail elderly volunteers were recruited from long-stay geriatric units in Newcastle District Health Authority. Patients were selected by Dr H Wynne (consultant geriatrician) following the criteria of Woodhouse et al (1988). Fit elderly were recruited from community day centres in and around Newcastle. Young healthy adults were recruited from hospital staff, students and colleagues.

All venous blood samples were collected by venepuncture by a clinician. The blood was dispensed into lithium heparin tubes, unless otherwise stated. Blood was centrifuged at 3,000g for 10 minutes and the plasma separated and stored at -80oC until required when it was thawed at room temperature.

The red blood cells were also required for some assays. The buffy coat (which forms the interface between the plasma and red blood cells, containing white cells and platelets) was carefully removed. An equal volume of normal saline was added, mixed gently and centrifuged as before. The supernatant was discarded and the red cells resuspended in saline and the wash procedure repeated.

Table 8.1

Volunteer Details

Volunteer (sex)	Age (years)	Albumin [1] (g/L)	Drugs (if any)
Frail Elderly			
1(F)	84	37	
2(F)	74	34	
3(M)	86	41	glibenclamide
4(M)	80	30	thioridazine
5(F)	75	35	terfenadine
6(F)	79	36	digoxin, thyroxine
7(F)	80	39	lactulose
8(F)	81	41	
9(F)	72	34	
10(M)	80	38	
Mean ±SEM	79±0.02*	37 ±1.09#	
Fit Elderly			
11(F)	74	42	
12(F)	74	41	temazepam
13(F)	71	43	
14(F)	77	41	
15(F)	73	48	
16(M)	70	45	co-codamol
17(F)	79	41	co-codamol
Mean ±SEM	73±0.94	43±1.00	

Table 8.1 continued

Volunteer (sex)	Age (years)	Albumin [1] (g/L)	Drugs (if any)
Young			
18(F)	23	50	
19(M)	24	46	
20(M)	24	47	
21(M)	20	48	
22(F)	20	43	
23(F)	22	49	
24(F)	30	45	
25(F)	31	46	
26(M)	24	47	
27(M)	26	49	
Mean $\pm$SEM	24$\pm$1.18	47$\pm$0.67	

[1] measured by Clinical Biochemistry ,Royal Victoria Infirmary, Newcastle Upon Tyne.

*Significant at $p<0.05$; $t=2.6$

#Significant at $p<0.001$; $t=4.2$

No subject was taking aspirin or other drugs known to affect plasma esterase activity. Sufficient blood was also withdrawn for haematological and biochemical profiles where these were deemed necessary and were not already available from recent investigations.

b) Plasma Aspirin Esterase Kinetics

Plasma aspirin esterase activity was determined by a direct reading spectrophotometric assay (Williams et al, 1989).

The aspirin concentration was varied between 0.4mM and 3mM using fresh aspirin solutions in Tris-HCl containing 200 mM calcium chloride at pH 7.4.

Each aspirin solution was incubated in duplicate at 37^{o}C with 300 ul plasma in a total volume of 3ml Tris-HCl buffer pH 7.4. The salicylate liberated was measured by monitoring at 300 nm at minute intervals over a period of five minutes. Spontaneously liberated salicylate was corrected for by the parallel incubation of aspirin solutions at each concentration in the absence of plasma.

The results were expressed as nmol salicylate produced/ml plasma/min.
The lower limit of detection was 4.1 nmol salicylate produced/ml plasma/min.
The interassay coefficient of variation (CV) was 4.7% (at a plasma aspirin esterase activity of 182 nmol salicylate/ml plasma/min at 3mM aspirin concentration; S.D. = 8.6; n = 5).

c) Plasma Cholinesterase Activity

This was measured by the method of Kalow and Lindsay (1955).

1.5ml 0.133 M phosphate buffer pH 7.4, 0.75 ml water, 15 µl plasma were incubated at 37oC until equilibration. The reaction was started by the additiion of 0.75ml of 0.2 mM benzoylcholine chloride (final concentration 0.05 mM). The final incubation volume was 3ml and each measurement was carried out in duplicate. The rate of benzoylcholine disappearance was monitored at 240 nm for a period of 2.5 min during which time the reaction was linear.

The results were expressed as nmol benzoylcholine chloride hydrolysed/ml plasma/min. The interassay variability CV was 2.7% (at a plasma cholinesterase activity of 1277 nmol benzoylcholine chloride hydrolysed/ml plasma/min; S.D. = 35.6; n=3).

8.2.3 Linearity of the Enzyme Activity with Time

Most of the enzyme measurements made in this thesis, unless otherwise stated involved continual monitoring of product formation at intervals over a certain time period. Conditions were selected to give linearity with time.

a) Linearity with Protein

Plasma Aspirin Esterase Activity

This was confirmed by incubating 100 µl, 200µl and 300µl of plasma with the lowest (0.4 mM) and highest aspirin concentration (3mM) used in the kinetic study. (Fig 8.1 and 8.2).

Plasma Cholinesterase Activity

2.5 µl, 5 µl, 10 µl, 15 µl, and 20 µl of plasma was incubated with a final concentration of 0.05 mM benzoylcholine chloride as described in 8.2.2 . Confirmation of linearity was thus established (Fig 8.3).

b) Substrate Curves

Single measurements of enzyme activity are usually carried out at a substrate concentration in excess of that producing maximal enzyme activity. Thus one is able to assume this remains constant because the enzyme - substrate complex present at any one time is so small by comparison to substrate. A discussion of Michaelis-Menten enzyme kinetics is included in Appendix II.

Aspirin Esterase Measurements

Williams et al (1989) used a 1mM aspirin concentration in order to compare plasma aspirin esterase activities. This was below Vmax chosen because at higher aspirin concentrations show proportionately greater spontaneous

hydrolysis than enzyme mediated hydrolysis was observed. It is desirable to minimise this in order to avoid erroneous results. I used concentrations of aspirin up to 3mM. Spontaneous hydrolysis reached a maximum of 17% of total enzyme hydrolysis in the plasma of lowest activity.

Cholinesterase Measurements

Benzoylcholine chloride does not degrade spontaneously and single enzyme measurements can be made at saturating substrate concentration (0.05 mM benzoylcholine chloride). Measurements below 0.03 mM substrate concentration were not possible due to non-linearity over a 2.5 minute time period . Figure 8.4 shows the effect of varying substrate concentration. There is a maximal activity of 1300nmol benzoylcholine chloride hydrolysed /mlplasma /min at 0.05mM substrate.

The methods used for enzyme kinetic and statistical analyses are described in Appendices II and III.

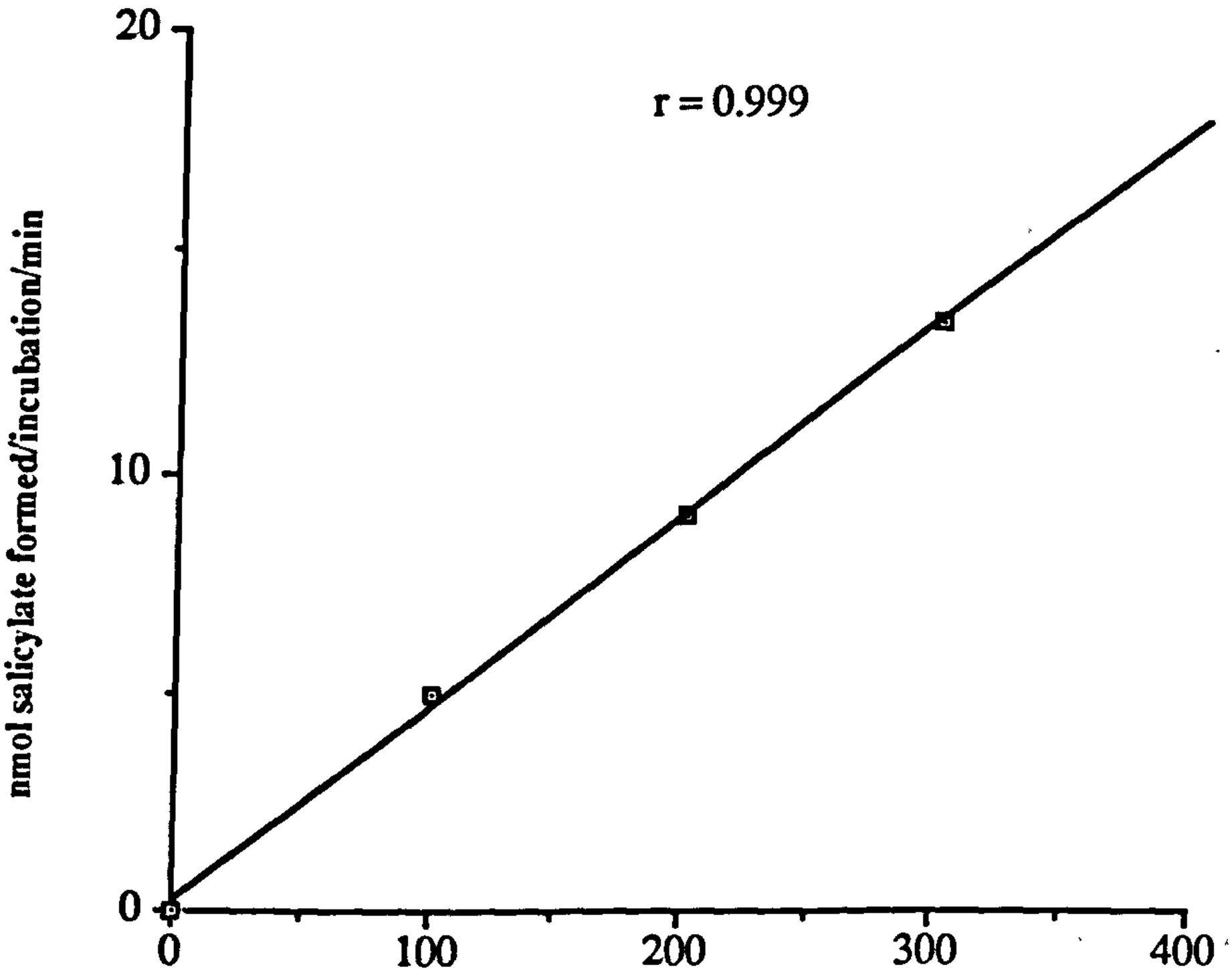


Figure 8.1

Title: The formation of salicylate: the effect of increasing plasma concentration in final incubation at an aspirin concentration of 0.4mM.

Ordinate: the amount of salicylate formed over a 1 min. time period at an aspirin concentration of 0.4mM and at 37°C.

Abscissa: volume of plasma in incubation

The values are the mean of two determinations. The solid line is the least square regression line

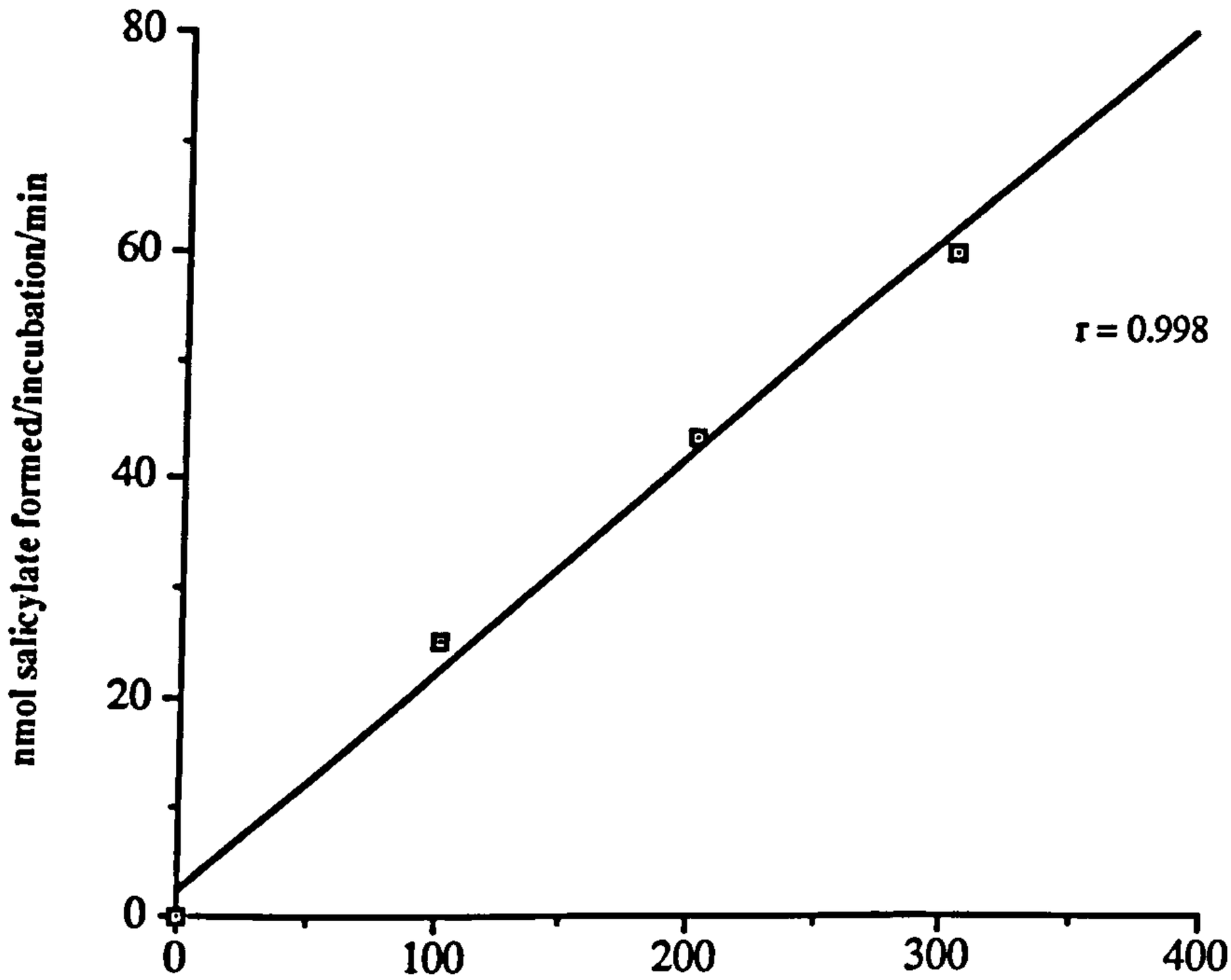


Figure 8.2

Title: The formation of salicylate; the effect of increasing plasma concentration in final incubation at an aspirin concentration of 3mM.

Ordinate: the amount of salicylate formed over a 1 min. time period at an aspirin concentration of 3mM and at 37°C.

Abscissa: volume of plasma in incubation.

The values are the mean of two determinations. The solid line is the least square regression line

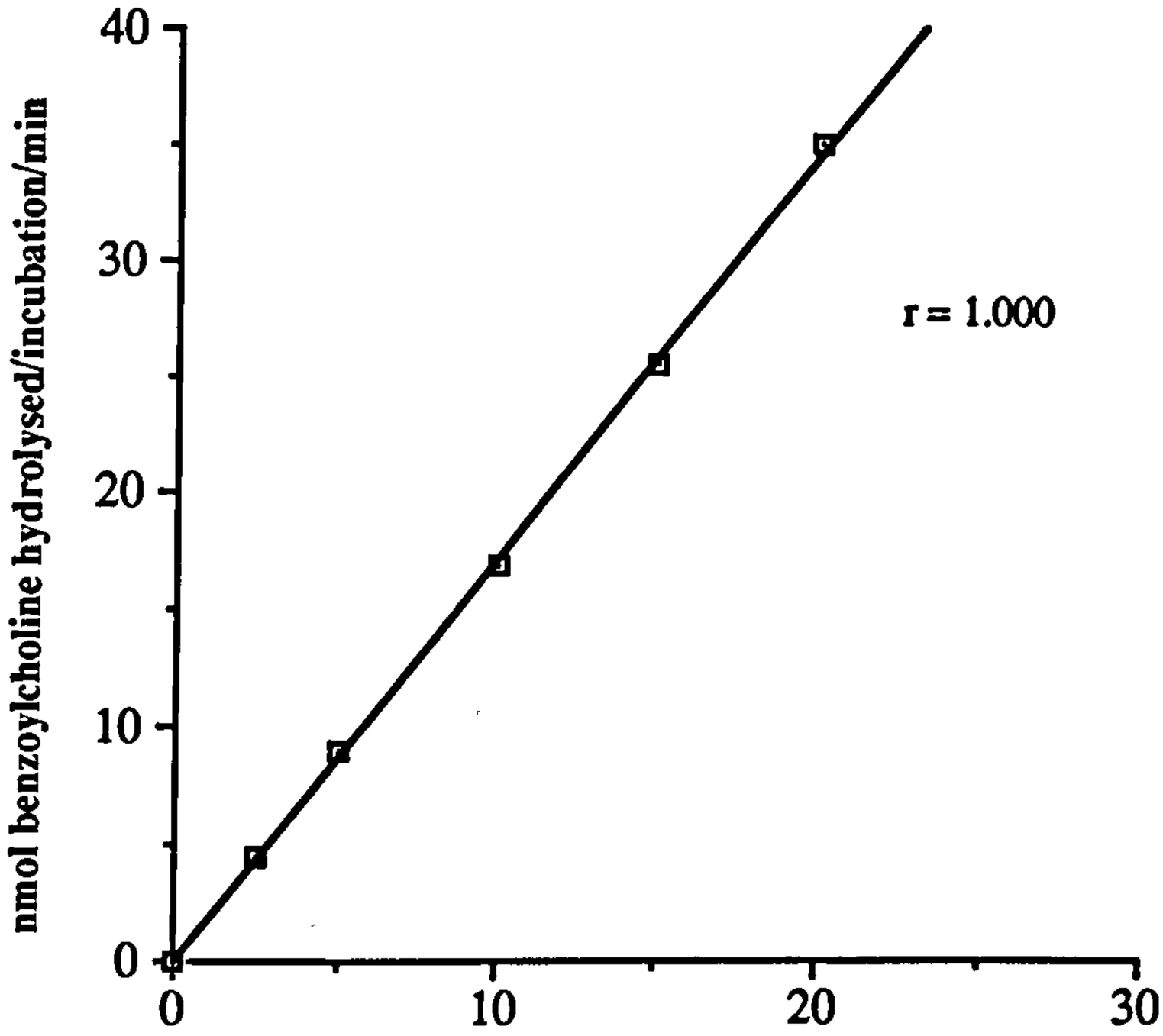


Figure 8.3

Title: The rate of disappearance of benzoylcholine chloride: the effect of increasing plasma concentration in final incubation at a substrate concentration of 0.05mM.

Ordinate: nmol benzoylcholine chloride hydrolysed over a one minute time period at a substrate concentration of 0.05mM and 30°C.

Abscissa: amount of plasma in incubation.

The values are the mean of two determinations. The solid line is the least square regression line.

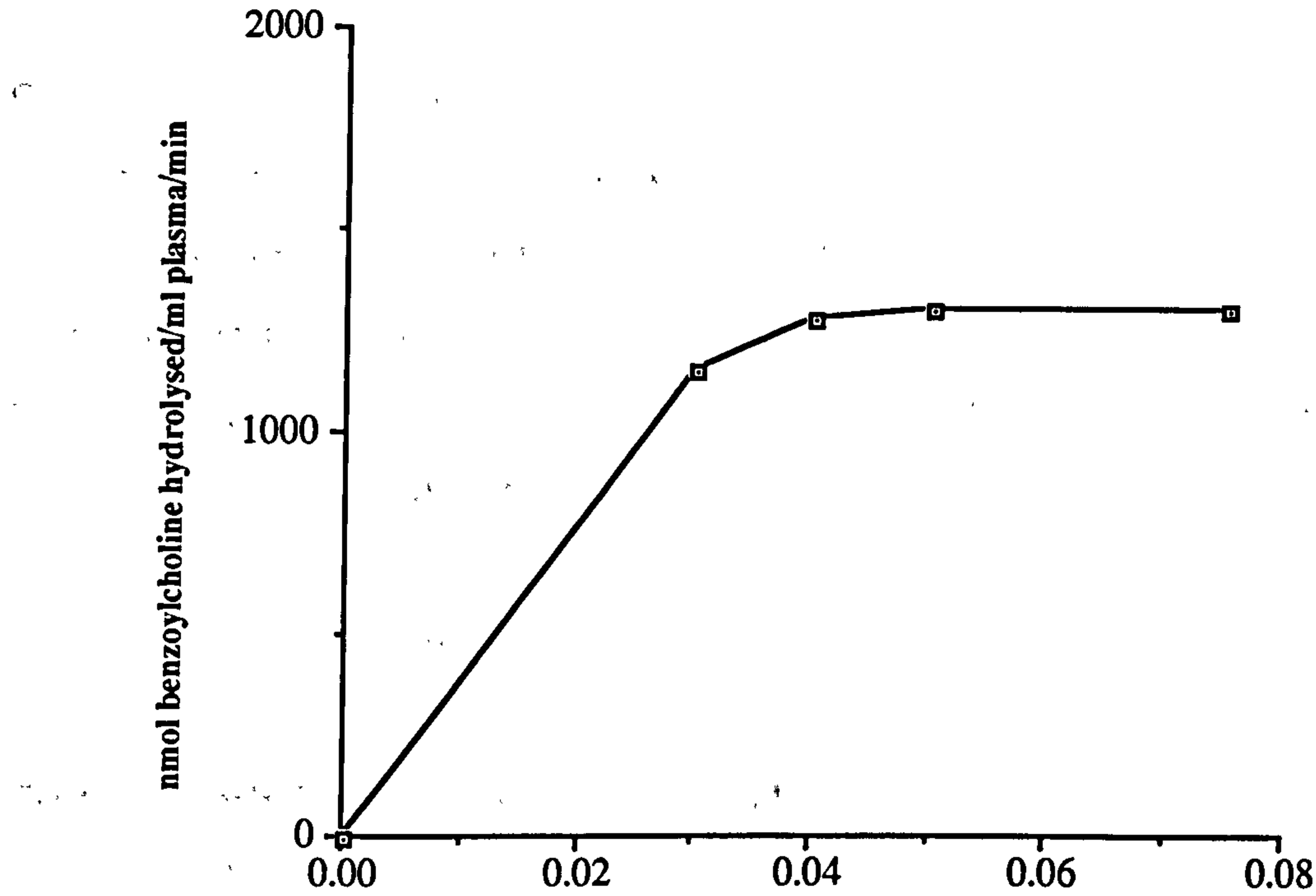


Figure 8.4

Title: The rate of disappearance of benzoylcholine chloride when incubated with 15µl of plasma: the effect of varying substrate concentration.

Ordinate: nmol benzoylcholine hydrolysed over a 1 min. time period by 15µl plasma at 30°C.

Abscissa: concentration of benzoylcholine chloride.

The values are the mean of two determinations.

8.3 Results

8.3.1 Aspirin Esterase Activity due to Albumin

From Table 8.2, it can be seen that aspirin esterase activity in plasma was completely inhibited by physostigmine (0.05-0.4mM). When albumin fraction V, at a concentration similar to that found in plasma was incubated with aspirin, there was a small proportion of enzyme hydrolysis of 20.8 nmol salicylate/ml albumin/min, in addition to spontaneous hydrolysis (Table 8.3). However, this was completely inhibited by the addition of 0.2 mM physostigmine.

8.3.2. Kinetic Analysis

The frail elderly individuals were significantly older (79 $\pm$ 0.4 years; $p < 0.05$). The frail elderly had a significantly lower albumin concentration (37 $\pm$ 1.09 g/l) than the fit elderly (43 $\pm$ 1.00 g/l) (Table 8.1).

Aspirin esterase activity increased with substrate concentration (after adjustment for spontaneous hydrolysis) to a maximum. Eadie-Hofstee analysis of the data indicated only one significant enzyme component. The Vmax and Km values were calculated from the Y-intercept and slope of the line respectively as shown in Fig 8.5. Vmax was reduced in the frail elderly (448 $\pm$ 40.2) compared to the fit elderly (694 $\pm$ 65.2; $p < 0.01$; $F = 5.71$). There were no significant differences between the fit elderly and fit young (598 $\pm$ 50.6), Table 8.4 and Fig. 8.6.

Table 8.2 :

Plasma Aspirin Esterase Activity in the Presence of Varying Concentrations of Physostigmine (inhibitor) and Aspirin (substrate).

Aspirin concentration (mM)	Physostigmine concentration (mM)	nmol salicylate / ml plasma /min
0.5	0	41.6
	0.05	<4
	0.2	<4
	0.4	<4
1.0	0	93.7
	0.05	<4
	0.2	<4
	0.4	<4
3.0	0	179
	0.05	<4
	0.2	<4
	0.4	<4

Table 8.3:

Asprin Esterase Activity due to Albumin Fraction V (40g/l) When Incubated with Aspirin (3mM)

Incubation contents	nmol salicylate/ml albumin / min	
	Physostigmine (0.2mM)	no Physostigmine
Albumin (fraction V) 40g / l	25.0	45.8
Blank (no protein)	25.0	25.0

Table 8.4

Kinetic Analysis of Plasma Aspirin Esterase

Frail Elderly	*Vmax* Plasma cholinesterase (nmol benzoyl-choline hydrolysed'min)	*Vmax* Plasma aspirin esterase (nmol salicylate/ml plasma /min)	*Km* Aspirin (mM)
1	-	550	4.1
2	-	395	4.6
3	1570	648	5.5
4	1015	240	2.6
5	-	455	3.1
6	1090	608	5.1
7	480	433	6.3
8	1500	424	3.5
9	1490	298	2.6
10	1135	431	3.5
Mean ±SEM	°1183± 145	*448 ± 40 .2	4.1 ± 0.4
Fit Elderly			
11	1680	541	4.6
12	1840	966	5.7
13	1410	541	4.6
14	1345	621	5.0
15	1515	559	4.7
16	1920	773	6.1
17	2410	861	3.9
Mean ±SEM	1731± 139	694± 65.4	4.9± 0.3

Table 8.4 continued

Young	*Vmax* Plasma cholinesterase (nmol benzoyl-choline hydrolysed/min)	*Vmax* Plasma aspirin esterase (nmol salicylate/ml plasma /min)	*Km* Aspirin (mM)
18	1600	576	4.6
19	1800	670	4.5
20	-	509	4.2
21	2065	503	2.8
22	1495	438	3.1
23	990	533	4.5
24	1375	419	3.6
25	1795	682	5.5
26	2435	961	6.3
27	1440	690	5.5
Mean ±SEM	1666± 140	598± 50.6	4.5 ± 0.3

° $p < 0.01$; $F = 5.7$, compared with fit elderly volunteers (ANOVA followed by the Scheffe test)

* $p < 0.05$; $F = 4.17$, compared with young and fit elderly volunteers (ANOVA followed by the Scheffe test)

Correlation coefficient between *V max* for plasma aspirin esterase and cholinesterase is $r = 0.77$. This is significant at $p < 0.001$

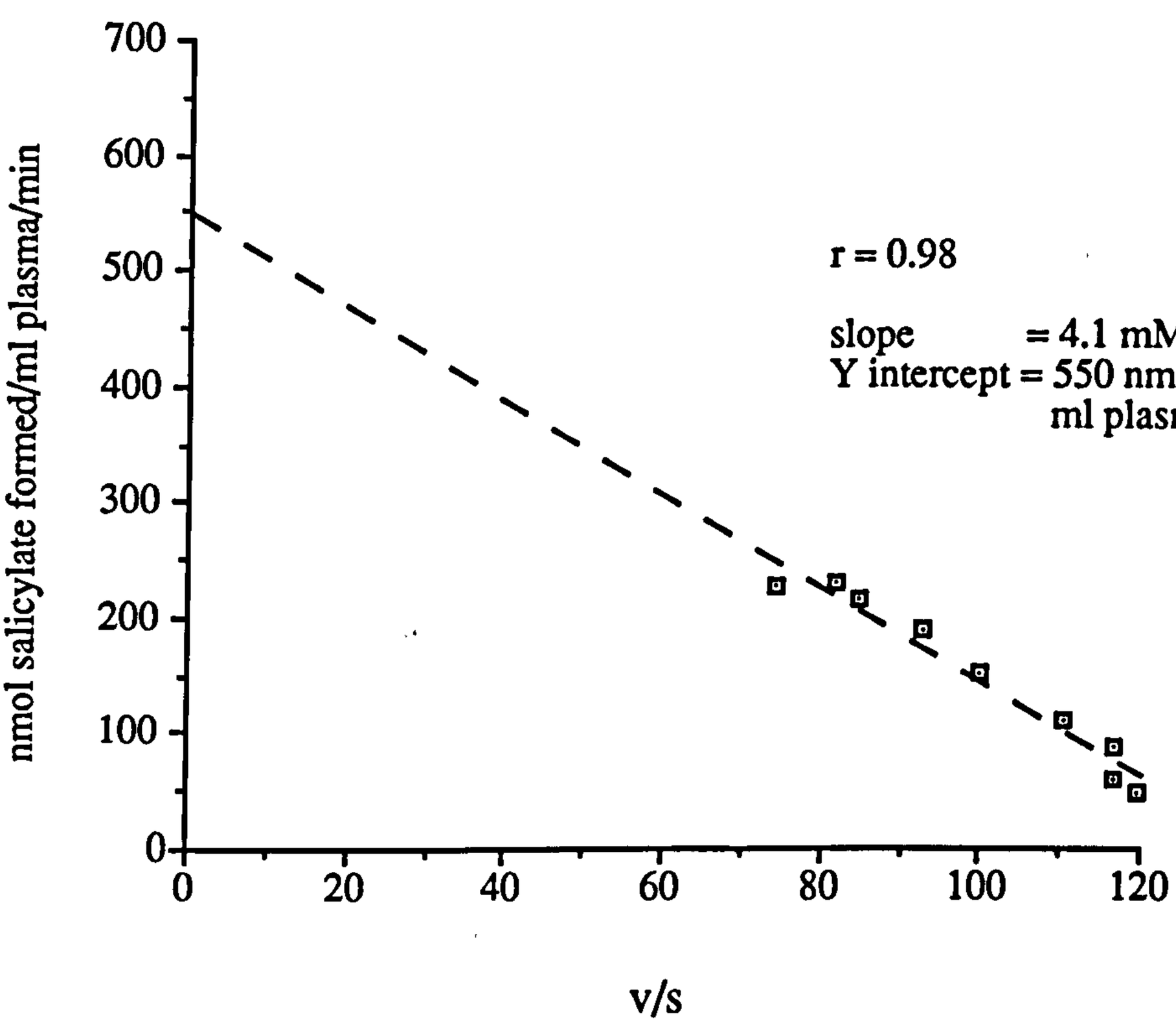


Figure 8.5

Title : An Eadie - Hofstee plot of the results obtained from a frail elderly volunteer (no 1)

Ordinate : V is the reaction velocity as nmol salicylate produced per ml plasma in one minute at 37°C

Abscissa : The reaction velocity V divided by the aspirin concentration (mM)

Figure 8.6

Title : Plasma aspirin esterase activity in young ; fit and frail elderly

Ordinate : Plasma aspirin esterase activity, nmol salicylate produced per ml plasma in a 1min. time period at 37°C

Abscissa : Values in young; fit elderly and frail elderly

The Km values did not differ significantly in any of the three groups (frail:4.1± 0.4mM; fit: 4.9± 0.3mM; young: 4.5± 0.3mM) as shown in Table 8.4 and Fig. 8.7 (F=1.26).

The Vmax for plasma cholinesterase was also lower in the frail elderly individuals (1183 ± 145; $p < 0.05$; $F = 4.17$) as shown in Table 8.4. and Fig.8.8.

Regression analysis of the Vmax values for plasma aspirin esterase and cholinesterase showed that the two are significantly correlated ($r = 0.77$; $n = 23$; $p < 0.001$) and this is shown in Fig 8.9 .

8.4 Discussion

The frail elderly volunteers were significantly older that the fit elderly at $p < 0.05$. As people age they become increasingly frail, thus finding suitable fit age matched controls was difficult. Plasma albumin concentration was significantly reduced in the frail group which is one of the characteristics of this population. (Woodhouse et al, 1988).

No plasma aspirin esterase activity was attributable to albumin. This was confirmed by incubating plasma with the specific cholinesterase inhibitor physostigmine and aspirin, and also incubating the purified preparation albumin fraction V with aspirin.

Kinetic analysis of plasma aspirin esterase by Eadie- Hostee revealed only one

Figure 8.7

Title : *Km* values for plasma aspirin esterase in young; fit and frail elderly patients

Ordinate : *Km* value (mM)

Abscissa : Values in young ; fit elderly and frail elderly

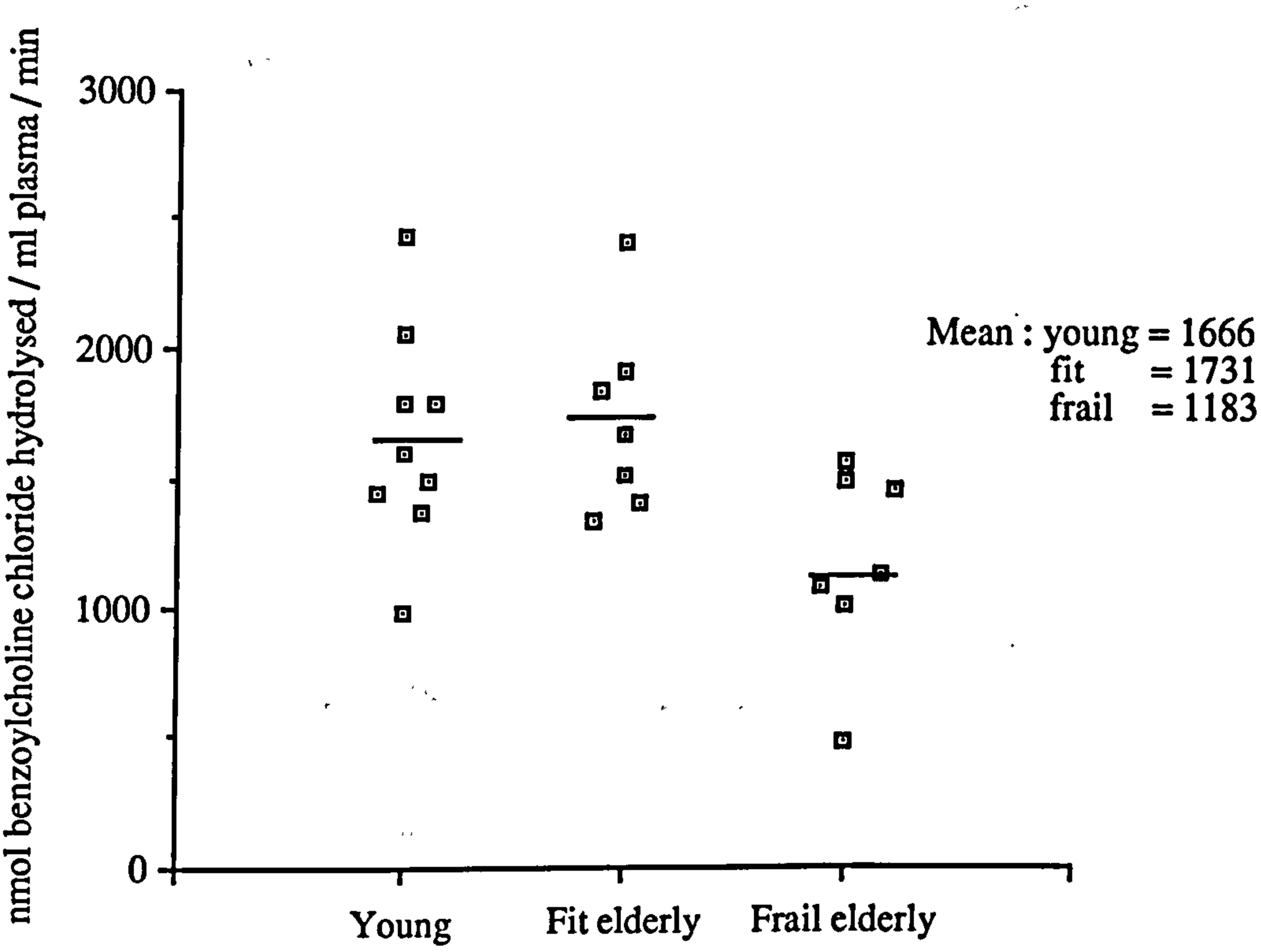


Figure 8.8

Title : Plasma cholinesterase activity in young : fit and frail elderly.

Ordinate : Plasma cholinesterase activity, nmol benzoylcholine hydrolysed per ml plasma in a 1 min. time period at 37^{o}C

Abscissa : Values in young : fit and frail elderly

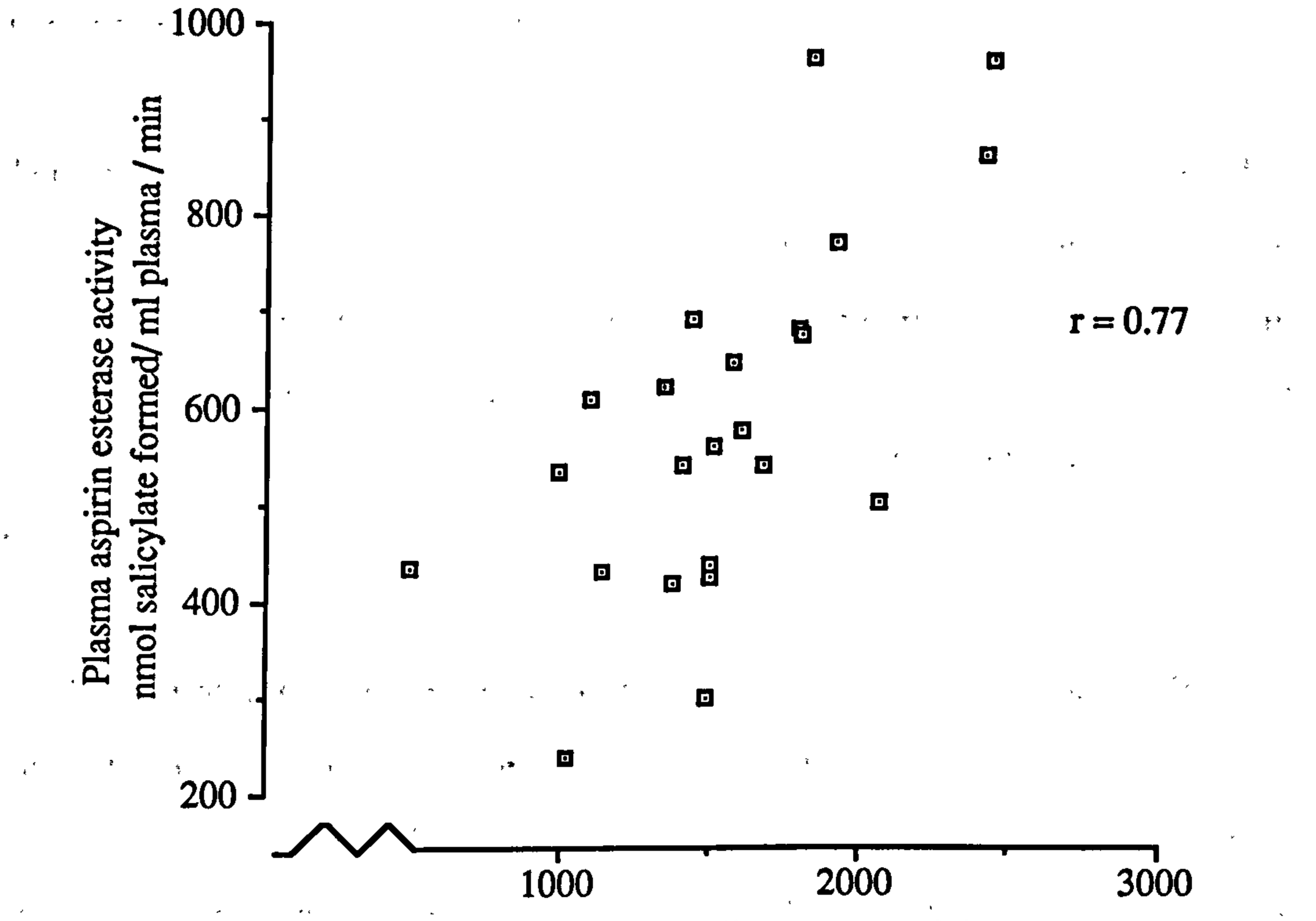


Figure 8.9

Title : The correlation between calculated *Vmax* for plasma aspirin esterase and plasma cholinesterase activity in young; fit and frail elderly

Ordinate : Calculated Vmax for plasma aspirin esterase at 37°C

Abscissa : Plasma cholinesterase activity at 37°C

Correlation significantly different fron zero at $p<0.001$

major enzyme component in all the plasma samples analyzed. These findings suggested that cholinesterase alone contributed to plasma aspirin esterase measurements using this spectrophotometric technique.

The reduced Vmax for plasma aspirin esterase and cholinesterase observed in the frail elderly group is in agreement with the results of Williams et al (1989) who measured plasma aspirin esterase at a 1mM aspirin concentration. They found a significantly reduced plasma aspirin esterase activity ($p < 0.01$) and although cholinesterase was not statistically significant at the 5% level there was a trend towards a reduced activity.

Production of abnormal proteins has been postulated as one of the fundamental mechanisms of ageing (Goldstein, 1977). However the Km values for plasma aspirin esterase measured here shows no significant change with age or frailty and were in good agreement with that obtained by Valentino et al (1981) who gave the value as 4.2 mM.

The reduced Vmax for aspirin esterase in the frail elderly group however compared with the fit elderly group strongly suggests that impaired aspirin esterase activity is due to a decrease in the total plasma content of cholinesterase rather than a detrimental change in enzyme protein contributing to a change in affinity of the active site.

CHAPTER 9

Semi-purification of Aspirin Esterase (Cholinesterase) from Whole Plasma Using Ion-Exchange Gel Chromatography

Chapter 9

Semi-purification of Aspirin Esterase (cholinesterase) from Whole Plasma using Ion-Exchange Gel Chromatography.

9.1 Introduction

Although there did not appear to be a significant contribution by albumin to plasma aspirin esterase activity (Chapter 8), it cannot be assumed to be absent.

The calculated Km value may be a composite of two very similar values, one for albumin and one for cholinesterase or another protein. Only separation of albumin and other proteins by a semi-purification technique would allow the calculation of a Km value for the "pure" cholinesterase contribution to plasma aspirin esterase.

The following chapter details ion exchange gel chromatography which was used to separate the enzyme components and SDS polyacrylamide gel chromatography which was used to visualize proteins and esterase activity. The preliminary studies sought to confirm the later methods.

These methods were used in order to achieve the aim of the following work which was to remove the albumin component and repeat kinetic studies on the plasma aspirin esterase in young, fit and frail elderly.

9.2 Purification of Cholinesterase (ChE).

Purification of ChE has been carried out over many years, using various combinations of the same techniques. Early experiments used mainly horse plasma which has slightly different stability properties to human plasma. The purification procedure is difficult mainly due to the complexity of the ChE structure, the minute quantities present in plasma, and the poor yield.

In most of the purifications from horse plasma the initial step was to precipitate proteins using 33% saturation with ammonium sulphate. The ChE remained in solution and this gave a two fold purification. Strelitz (1944) perfected this technique using acidified 33% saturated serum which resulted in a reported 40-fold purification and 58% yield. This method was used as the basis of preparation commercially throughout the 1960s, giving approximately 100-fold purification, However, few workers were able to obtain the same degree of purification claimed by Strelitz (Jansz and Cohen, 1962; Heilbronn, 1962). Later work also indicated that the harsh acid conditions used by Strelitz were actually be destroying one or more of the active sites of ChE. (Main 1987).

After 1970, methods were still using commercially available horse serum BuchE purified by the Strelitz method. Two methods notably used the same starting material and the same techniques but to different effect. Lee and Harpst (1971) used differential sedimentation, zone electrophoresis (Jansz and Cohen, 1962), ion-exhange chromatography eluting with a linear sodium chloride gradient and then gel filtration on sephadex G.200. The overall yield was reported to be 10% and no isoenzymes were found to be present. This was possibly due to the initial

Strelitz step.

Main et al (1972) used preparative PAGE on commercially available ChE which had been purified 8-fold by filtering through Sephadex G.200 and then Sepharose 6B. This process was time consuming and involved three runs before finally removing gel contaminant by passing down Sepharose 6B. It did, however, successfully remove the remaining contaminants and several bands (isoenzymes) of activity were evident.

A method by Main et al (1974) aimed to reduce the large losses of cholinesterase activity in the Strelitz method. Particular attention was paid to the stability of ChE at pH 3.0 was found that ChE lost 50% of its activity in about 25 minutes at 24oC. This loss was reduced dramatically in 33% saturated ammonium sulphate. During the Strelitz method ChE is exposed to a more dilute ammonium sulphate at pH 3. This procedure may well have destroyed the ChE and Main et al (1974) overcame this by adjusting the pH of acidified ammonium sulphate solutions to pH 7 before ChE was exposed to more dilute concentration of ammonium sulphate. The procedure then followed an adaptation of Yoshida (1970) using ion exchange gel chromatography on three successive DEAE-Sephadex A 50 columns and eluting with linear gradients of choline chloride, sodium chloride and choline chloride respectively. The product was concentrated by precipitation with 70% saturated ammonium sulphate and subjected to preparative PAGE (Main et al, 1972). The gel contaminants were removed as before. The results were impressive:- cholinesterase was purified 19,000-fold and 15-18 mg product was obtained from 20 l serum.

The difficulties in purifying horse cholinesterase, although significant, are less

than those encountered by workers interested in human ChE, of which smaller quantities of plasma are available.

The Strelitz method has not been widely applied to the purification of human plasma because human ChE is less stable than horse ChE at low pH. The protective effect of ammonium sulphate on the stability of human ChE is less than one-tenth that of horse ChE (Main, 1987).

Interest in the isoenzymes of cholinesterase, the desire to characterize these multiple molecular forms and relate them to the atypical genetic cholinesterase variant has been the stimulus for most of the work on human serum.. Gaffney (1970) and Das and Liddell (1970) were among the first to work on the human enzyme. They both used various combinations of the methods mentioned above with considerable success. Das and Liddell avoided using the Strelitz method and the high loss of activity associated with it. They achieved a 13,000-fold purification and a yield of 54%. No contaminating proteins were discovered but the authors did not claim the product was 100% pure.

The most exciting development in recent years has been the use of affinity gels. These have resulted in purification methods with the capacity and efficiency to supply the quality of pure enzyme needed for structural studies.

Lockridge and La Du (1978) used only a two step procedure - ion exchange gel as described by Das and Liddell and modified by Muensch (1976) and procainamide affinity gel to obtain an yield of 70% pure enzyme.

Ralston et al (1983) also used procainamide affinity gels as a second step.

Ammonium sulphate precipitation was deemed more suitable for an initial 'clean-up' method prior to large scale preparation of pure human ChE.

La Du and Choi (1975) used the apparent lower affinity of the atypical ChE for choline ester substrates to advantage when separating a mixture of atypical and typical ChE. A different affinity gel was used in this case (N (6-aminocaproyl - 6' - aminocaproyl) - p - aminophenyl - trimethylammonium bromide hydrobromide). As predicted the atypical cholinesterase eluted before the usual form due to the lower affinity for the gel.

Despite the advent of much more sophisticated analytical and preparative techniques, most methods used today still use those procedures first introduced when enzyme purification was in its formative years.

Lockridge (1990) details routine preparation of purified cholinesterase, the yield being 8-12 mg from 10 l serum. He uses two ion-exchange columns and an affinity column to obtain enzyme of electrophoretically pure quality from which the amino acid sequence has been elucidated (Lockridge et al, 1987).

9.2.1 Ion-Exchange Gel Chromatography

DEAE - Sephacel is an ion-exhange gel consisting of microcrystalline cellulose reformed into beads (40-160 um). The small particle size ensures minimal flow disturbance and good resolution (James and Stanworth, 1964). The gel like structure of the beads is strengthened by cross-linking with epichlorohydrin but the main-structure forming bonds are hydrogen bonds. During ion-exchange, depending on conditions such as ionic strength and pH, it is possible to bind the

substance of interest and elute the contaminants or 'vice versa'. In the method detailed below it is the former property which was used. The chloride ions in equilibrium with the sephacel gel initially are replaced with acetate ions from the sodium acetate buffer. At this point the eluting buffer exhibits the same pH and conductivity as the applied buffer.

The iso electric point of cholinesterase has been estimated at 3.99 (Das and Liddell, 1970) and the optimal pH of the buffer for anion-exchange gels such as DEAE - Sephacel is one pH unit above the isoelectric point (anonymous,1987). This means at pH 5.5 cholinesterase carries a net negative charge and replaces the acetate ions on the gel. Other proteins may also be negatively charged at this pH and are similarly attracted to the anion exchange gel.

Chloride ions in the form of increasing concentrations of sodium chloride are introduced in a stepwise fashion at this point. Proteins which are less strongly attracted to the gel are eluted at lower concentrations of chloride ions. As the concentration increases, proteins which are more firmly attached are replaced by chloride ions. This results in an elution profile of the various proteins from the crude sample. Eventually the DEAE - Sephacel is regenerated when all the remaining protein and acetate ions are replaced by chloride ions. The gel can be re-equilibrated with acetate ions and re-used.

9.3 Method

9.3.1 Preparation of Ion-Exchange Gel

DEAE - Sephacel was allowed to equilibrate with 50 mM sodium acetate buffer pH 5.5 containing 5 mM calcium chloride and 0.1mM EDTA. The mixture was stirred gently, and the buffer decanted off and replaced with new at intervals.

When the supernatant attained a pH of 5.5, the Sephacel was ready for use. This was degassed under vacuum.

A glass column of dimensions 26 mm x 4000 mm was positioned vertically using a plumb line. The Sephacel slurry was stirred gently and a small quantity (approximately 1 cm depth) was allowed to settle at the base of the column. This prevents small cellulose beads forcing their way into the fine mesh of the net base and impeding the flow.

The remaining slurry was poured slowly and carefully down a glass rod to prevent trapping any air bubbles. At this point a flow of buffer through the column was initiated by releasing the outlet pipe. A bed volume of approxmately 80 ml was poured into the column.

Four or five bed volumes of degassed buffer were passed through the bed until the pH and conductivity of the buffer eluting from the base of the column was the same as that entering the column. The flow rate was kept at 1 ml/min.

9.3.2 Plasma Application to Column

Plasma was thawed at room temperature and centrifuged at 3,000 g for 5 minutes to remove the fibrin.

An accurate volume of plasma between 4 and 5 ml was applied to the top of the column. Sodium acetate buffer containing 5 mM calcium chloride, 0.1 mM EDTA and 50 mM sodium chloride was used to elute those contaminating proteins less strongly bound to the gel. 5 ml fractions were collected until the eluting proteins became minimal (low absorbance at 280 nm on a direct reading spectrophotometer ie less than 0.01 abs units). When this occurred the sodium chloride was increased to 100 mM and the process repeated. Finally buffer containing 200 mM sodium chloride was used to elute the most strongly bound proteins and clean the column.

Once the eluting buffer no longer contained proteinous matter (ie absorbance at 280 nm was zero) buffer containing no sodium chloride was used to regenerate the column.

When pure human albumin was applied to the column, the technique was identical to that for plasma.

9.4 SDS - Polyacrylamide Gel Electrophoresis (SDS - PAGE)

9.4.1 Theory of SDS - PAGE

Proteins carry a net charge when at any pH other than their isoelectric point and will migrate in an electric field. Viscous drag which is dependent on the size and shape of the protein impedes this movement (Hayes and Stockman, 1989a).

The support matrix provides a porous media in which the pore size is in the same order as the size of the proteins to be separated. This minimises the free diffusion of components. They also dissipate some of the heat generated by the electric current.

I chose polyacrylamide which is a synthetic polymer of the acrylamide monomer. It consists of long chains with a cross linking of free functional groups by bifunctional compounds such as N N1 - methylene bisacrylamide. The relative proportions of these determine the pore size of the matrix (Hames, 1985).

Discontinuous Buffer System

This uses a stacking gel which is a polyacrylamide gel of low ionic strength and pore size to concentrate the sample (Hayes and Stockman, 1989b).

Reservoir Buffer

The gel is submerged in a buffer which interacts with the gel to create a voltage gradient. This occurs as the result of pH differences in the stacking and resolving gels. The proteins become unstacked and separate according to intrinsic charge and the size of the protein subunits.

9.5 Method of SDS - PAGE (High pH Discontinuous System)

9.5.1 Preparation of Gels

i) Resolving Gel

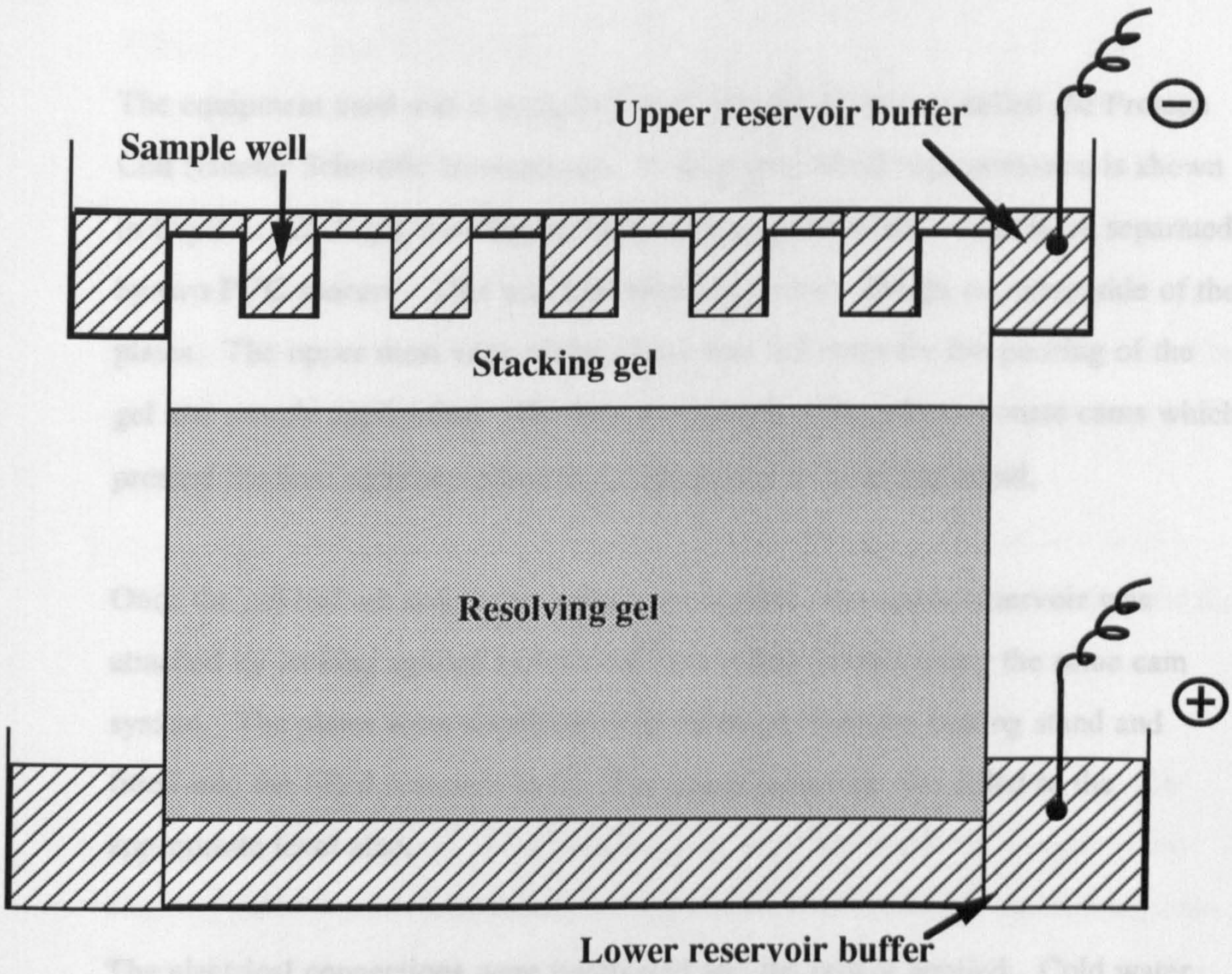
Reagents - all filtered through Whatman no. 1 filter paper as appropriate.

1	3OM Tris-HCl (pH 8.8) - Resolving gel buffer stock
2	Acrylamide - bisacrylamide in proportions 30:0.8
3	Ammonium persulphate 1.5% w/v
4	Sodium Dodecylsulphate (SDS) 10% w/v
5	(N N N' N' - Tetramethylethylene diamine) TEMED

In order to prepare a 7.5% gel :- 7.5 ml acrylamide - bisacrylamide (30 : 0.8); 3.75 ml resolving gel buffer stock; 0.3 ml 10% SDS; 1.5 ml 15% ammonium persulphate; 16.95 ml water and 15 µl TEMED.

The reagents were mixed thoroughly. TEMED was added last since this is the polymerization catalyst and the gel requires immediate pouring after this is added.

Figure 9.1 Discontinuous PAGE Apparatus



ii) Stacking Gel

Reagents

1 0.5 M Tris - HCl (pH 6.8) Stacking gel buffer stock.

It is then made by the same method as the resolving gel.

9.5.2 Apparatus

The equipment used was a cooled vertical slab gel apparatus called the Protean Cell (Hoefer Scientific Instruments). A diagrammatical representation is shown in Fig 9.1. Each gel was formed between two glass plates which were separated by two PVC spacers. This was held together by two clamps on either side of the plates. The upper most edge of the plates was left open for the pouring of the gel and sample application. The box was sealed using polycarbonate cams which pressed the base against a silicone rubber gasket in a casting stand.

Once the gel had set and the samples been applied, the upper reservoir was attached by locking against another silicone rubber gasket using the same cam system. The plates were simultaneously released from the casting stand and fitted into the filled reservoir tank. The upper reservoir was filled to the appropriate level also.

The electrical connections were completed and the power applied. Cold water from the tap was used as the coolant and circulated constantly through the perspex tube heat exchanger. The reservoir buffer was stirred continuously to

dissipate heat. A current of 50 mA was applied initially until the resolving gel was reached then it was increased to 100 mA. Too small a current would allow diffusion of the proteins to occur resulting in band spreading. Too large a current can result in poor resolution. This was run until the dye front was about 2cm from the base of the gel plate.

2.5 ml acrylamide: bisacrylamide (30 : 0.8); 5 ml stacking gel buffer stock; 0.2 ml 40% SDS, 1 ml 1.5% ammonium persulphate and 0.015 ml TEMED were mixed thoroughly and poured immediately.

9.5.3 Pouring the gel

The glass surfaces of the plates were thoroughly cleaned with ethanol. A 30 ml syringe with a silicone tubing attachment was filled with the freshly prepared gel solution. The solution was carefully dispensed through the nozzle between the glass plate sandwich. Care was taken not to generate too many air bubbles. The gel plates were filled to about 8 cm from the top. Any air bubbles were removed by careful aspiration using an IV cannula 12 G needle.

The surface was carefully covered with water saturated butanol and left to set for at least half an hour.

When the gel had set the butanol was poured off and the surface washed with water two or three times. This removed any unpolymerized acrylamide. The stacking gel solution was carefully syringed, in a similar manner as before, onto the surface of the resolving gel to about 0.5 cm from the top of the plates. The comb which forms the sample wells was carefully positioned to avoid trapping any air bubbles which distorts the sample wells.

The space between the plates was overfilled with stacking gel. As the stacking gel sets it contracts which reduces the sample well size. The stacking gel took about 20 minutes to set. The comb was carefully removed and the wells filled with reservoir buffer stock.

9.5.4 Sample preparation

a) Sample buffer

Reagents

1	0.0625 M Tris - HCl pH 6.8
2	10% v/v glycerol
3	2% w/v SDS
4	5% v/v Mercaptoethanol
5	0.02% v/v Bromophenol blue

The above were prepared. The reconstituted sample containing cholinesterase activity was mixed with an equal volume of sample buffer. This was boiled for 5 minutes.

Molecular weight markers were also applied to the gel. The prepared standards were reconstituted with 2 ml of sample buffer, and boiled as with the samples. Those chosen had molecular weights ranging from 12,300 - 78,000. The commercial cholinesterase was reconstituted with water to a concentration of approximately 1 mg protein/ml. An equal volume of this and sample buffer was mixed and treated as before.

b) Sample Application

A microliter syringe was found to be the easiest way of placing the prepared samples into the wells. 100 µl of the sample was carefully dispensed into the base of alternative sample wells taking care not to contaminate adjacent wells. Blank sample buffer was placed in the wells between samples.

9.5.5 Reservoir Buffer Stock

0.25 M Tris

1.92 M glycine

1% SDS (pH 8.3)

These were dissolved in and made up to 1l with water. This was diluted 1 in 10 prior to use and a 5l volume was required each time.

The apparatus was transferred into the tank containing reservoir buffer as described in 9.3.3.

9.5.6 Removal of the gel

The current was turned off and the gel plates removed from the reservoir buffer tank intact. The side clamps were removed leaving the gel sandwiched between the two glass plates supported by the spacers. One spacer was removed and was used to carefully lower the top glass plate away from the gel which bonds very tightly with the glass. The stacking gel was cut away using the edge of the spacer and disgarded in most cases. A small cut was made in one corner of the

gel to identify its position.

The gel was now ready for staining.

9.6 Non-SDS Gels For Native Proteins

These were used when esterase activity was monitored. In such cases the basic method and reagents are identical to those above except SDS was excluded in all reagents and the sample buffer not boiled.

During the electrophoresis of native proteins separation occurs due to size and charge differences of the molecules.

9.7 Staining the Gels

9.7.1 Protein Stain

Reagents

1	0.1% Coomassie Blue R250			
2	Water : methanol:	glacial acetic acid		
	5	5	2	by volume

The above stain filtered through a Whatman no. 1 filter paper to remove any insoluble material.

The gel was submerged in approximately 500 ml stain overnight.

Destaining was carried out using a large vessel (4l volume). The gel was wrapped in netting and protected in a cylindrical perspex tube through which destain can freely pass. This was placed in the vessel on a special support beneath which a magnetic stirring rod was used to continually agitate the destain solution.

This was left for approximately four hours until dark blue spots were visible and the gel had changed to a much paler blue background colour.

9.7.2 Staining Native Proteins

In order to stain for esterase activity, the temperatures used during the process must be optimal for enzyme activity. Samples were kept on ice until application to the gel. Incubations with substrate were carried out at room temperature or 37oC.

a) Napthylacetate Esterase Activity

This involved coupling of the napthol produced by hydrolysis of the naphthyl ester with the diazo salt Fast Blue 2R to form an azodye. It is a general esterase dye.

Reagents

Substrate

alpha napthylacetate	1 g
acetone	50 ml
water	50 ml

Stain

Fast Blue 2R	100 mg
Tris - HCl pH 7.0 (100 mM)	10 ml
alpha-napthylacetate 1%	3 ml
water	87 ml

The method is a modification of that by Hopkinson et al, 1973. The gel was incubated in the above stain for approximately half an hour or until the bands of esterase activity appeared. The gel was then removed to avoid overstaining and placed in water.

b) Butyrylthiocholine Esterase Activity

The method used to detect cholinesterase activity was that used by Coates et al (1975).

Reagents

1	Butyrylthiocholine 100 mg
2	0.07 M phosphate buffer pH 6.1 containing 2 mM 20% copper sulphate, 10mM glycine and 30mM sodium sulphate
3	30 mM sodium sulphate
4	0.01% dithioxamide solution in 0.02 M sodium acetate

Butyrylthiocholine (100 mg) was dissolved in Reagent (2) and the gel was incubated in this solution at 37^{o}C for 3 hours with gently shaking. The gel was

washed with water and then transferred to a solution of 20% sodium sulphate for overnight incubation. The bands of cholinesterase activity were evident as a dark green colour due to dithio-oxamide complexing with the copper. (Chubb and Smith, 1975).

The protein content of the fractions was analyzed using the method outlined below. This enabled the calculation of the appropriate volume of sample to apply to the polyacrylamide gel. In Chapter 10 it was used to the calculate Vmax which was expressed per mg protein in whole plasma and purified fraction, and the purification factors.

9.8 Protein Assay

9.8.1 Introduction

The method used was a modification of that developed by Lowry et al (1951) as suggested by Peterson (1977).

The basic reaction is the formation of a coloured complex when the phosphomolybdic-tungsten mixed acid in Folin and Coicalteu's phenol reagent reacts with the amino acids (Peterson, 1979).

Peterson consolidated several methods to remove interfering salts and optimise assay conditions. This resulted in a rapid, consistent protein assay for dilute samples.

9.8.2 Method

Dilute Fractions from Ion-Exchange Column

The initial concern was that the concentration of EDTA (approximately 0.7 mM) may interfere with the assay (Peterson, 1979). In order to test this, a known amount of protein was added to blank buffer, taken through the assay and read against a standard curve made up in water. This sample did not differ by more than 6% from the actual value and so the precipitation step was considered unnecessary in this case. The standard curves produced using water and buffer solvents were also comparable (Fig 9.2).

Plasma

This required a 1 in 100 dilution prior to analysis.

Stock Reagents

1 Copper-tartrate-carbonate (CTC)

20% sodium carbonate, copper sulphate to a final concentration of 0.1%, 0.2% potassium tartrate and 10% sodium carbonate.

2 10% SDS

3 0.8 M sodium hydroxide

4 Folin-Ciocalteu's phenol reagent

Working Solutions

Reagent A

Equal proportions of CTC, NaOH, SDS and water.

Reagent B

One volume of Folin-Ciocalteu's phenol reagent mixed with five volumes of distilled water.

A protein standard solution was prepared containing 600 ng/ l Bovine Serum Albumin (BSA) in reconstituted freeze dried buffer. A serial dilution of stock solution was made from 0 to 60 µg/ml protein in 1.0 ml water. 15 µl of each fraction or 15 µl of 1 in 100 dilution of plasma was made up to 1.0 ml with water. 1.0 ml of reagent A was added to each tube, mixed and left for exactly 10 minutes. 0.5 ml reagent B was added, mixed and left for at least 30 minutes. Each sample was repeated in triplicate.

The resulting blue complex was read at 750 nm on a uv spectrophotometer within two hours.

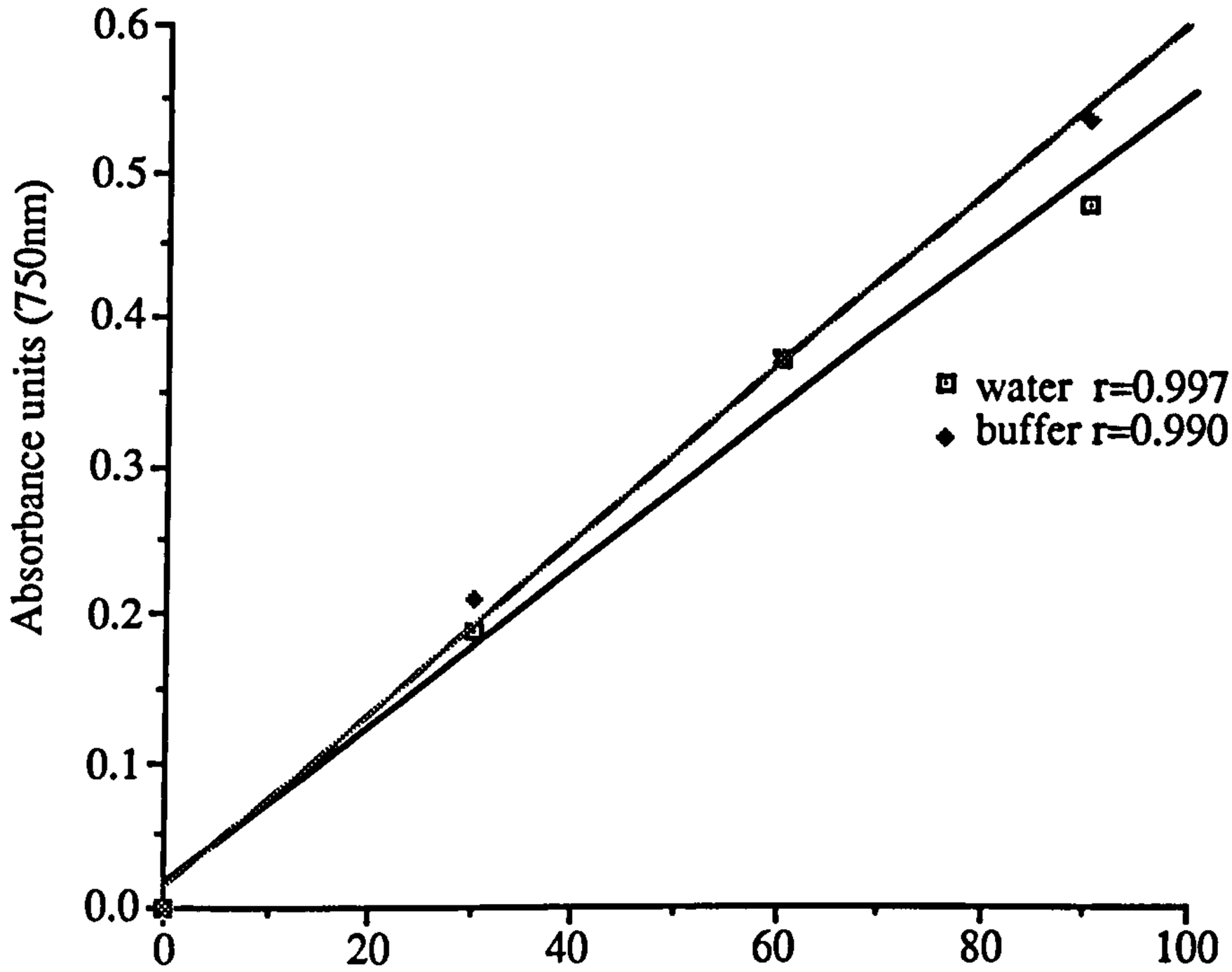


Figure 9.2

Title : Protein standard curve comparing the curve obtained when protein is prepared in water and reconstituted buffer

Ordinate: Absorbance units at 750nm

Abscissa: µg protein in incubation

Each value is the mean of triplicates. The solid line is the least square regression line

9.9 Preliminary Background Studies

This work involved confirming the removal of albumin from the purified cholinesterase fractions and identifying the peaks of protein content using various esterase substrates and the cholinesterase inhibitor, physostigmine.

9.9.1 Methods

Removal of Albumin Component

Human albumin solution 4.5% was used. 5.0 ml of albumin solution was applied to the top of the column and eluted as described in 9.3.2. This was compared with that for plasma.

Identification of Peaks

Peaks were located by monitoring for protein at 280 nm. They were identified by the methods described below.

a) Phenylacetate Esterase Activity (Arylesterase)

This was measured by a modification of the method of Kitchen et al (1973). 200 µl fraction, 795 µ l Tris-HCl buffer pH 8.0 and 3mM phenylacetate (final concentration) were mixed and incubated at 37oC.
The formation of phenol was monitored at 272 nm by uv spectrophotometry. Physostigmine (0.2 mM) was included as a specific inhibitor of cholinesterase activity.

b) Cholinesterase Activity

This was measured as previously described. The plasma was replaced with 100 µl fraction. Physostigmine (0.2 mM) was included in parallel incubations in order to confirm activity was due to cholinesterase.

c) Aspirin Esterase Activity

This was measured using a modification of the method in Chapter 8. 200 µl of fraction, 1.0 ml Tris-HCl buffer containing 200 mM calcium chloride pH 7.4 and aspirin at a final concentration of 2 mM, to a final volume of 1.5 ml were incubated at 37oC. Physostigmine was included at a final concentration of 0.2 mM to confirm aspirin esterase activity due to cholinesterase.

9.9.2 Results

The elution profile obtained for the purification of whole plasma on DEAE-Sephacel is shown in Fig 9.3. Table 9.1 shows the activity of peaks 1 and 2 (Fig 9.3) to three esterase substrates - aspirin, benzoylcholine chloride and phenylacetate. The specific cholinesterase inhibitor physostigmine inhibited all the aspirin esterase and cholinesterase activity in peak 1. Phenylacetate hydrolysis in peak 1 was also inhibited by physostigmine. This showed that esterase activity in peak 1 was due to cholinesterase.

Peak 2 contained no aspirin esterase or cholinesterase activity. It did, however, contain arylesterase activity which was unaffected by physostigmine. These results are shown in Table 9.1.

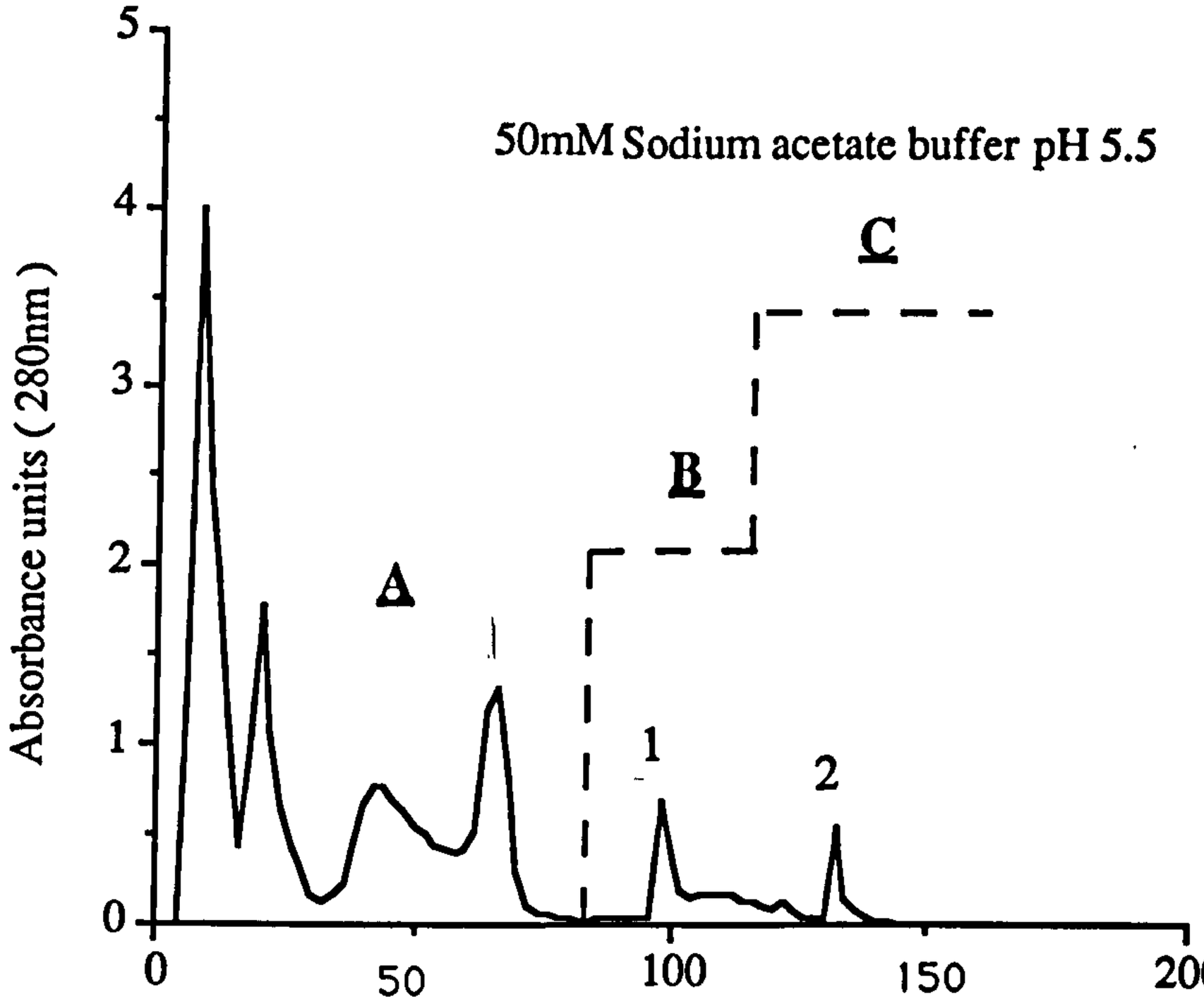


Figure 9.3

Title : The elution profile of whole plasma after passage down a DEAE- Sephacel ion exchange column

Ordinate : Protein content of each 5ml fraction : absorbance at 280 nm

Abscissa : Number of 5ml fractions collected

A 50mM sodium chloride
B 100mM sodium chloride
C 200mM sodium chloride

Table 9.1

Peak Identification ; Peaks 1 and 2 showing Esterase Activity

	Aspirin*		Benzoylcholine chloride#		Phenylacetate@	
	2mM	+ 0.2mM physos-tigmine	0.05 mM	+ 0.2mM physos-tigmine	3mM	+ 0.2mM physos-tigmine
1	2517	0	800	0	11.14	0
2	0	0	0	0	1.6	1.14

*nmol salicylate produced /ml fraction /min

#nmol benzoylcholine chloride hydrolysed / ml fraction / min

@nmol phenol produced/ ml fraction / min

Peak A was identified as albumin as shown in Fig 9.4. The fraction numbers do not correspond exactly which is probably due to slight variations in flow rate. A small percentage of albumin showed the same retention time as peak 1. This was quantified by plotting the two peak profiles on graph paper, cutting them out and weighing them on a microbalance. In this way the percentage of albumin not removed by the first increment of 50 mM sodium chloride could be calculated.

The cholinesterase activity in peak 1 expressed as activity per ml fraction closely followed the protein content outline of the peak (Fig 9.5). Cholinesterase activity with benzoylcholine as substrate was used instead of aspirin esterase as an indication of enzyme activity in the peak because it was much more sensitive.

The activity in peak 1 was sensitive to inhibition by 0.2 mM physostigmine (Table 9.1) confirming the results of Rainsford et al (1980) and Wilde and Kekwick (1964), thus the activity was indeed due to the cholinesterase enzyme. The activity in peak 2 was insensitive to physostigmine and was thus identified as arylesterase. Despite concentrating the fraction and substrate as much as possible an accurate outline of the enzyme activity in this peak was not possible due to a lack of sensitivity.

It was found that 97% of albumin was removed from the plasma by 50 mM sodium chloride.

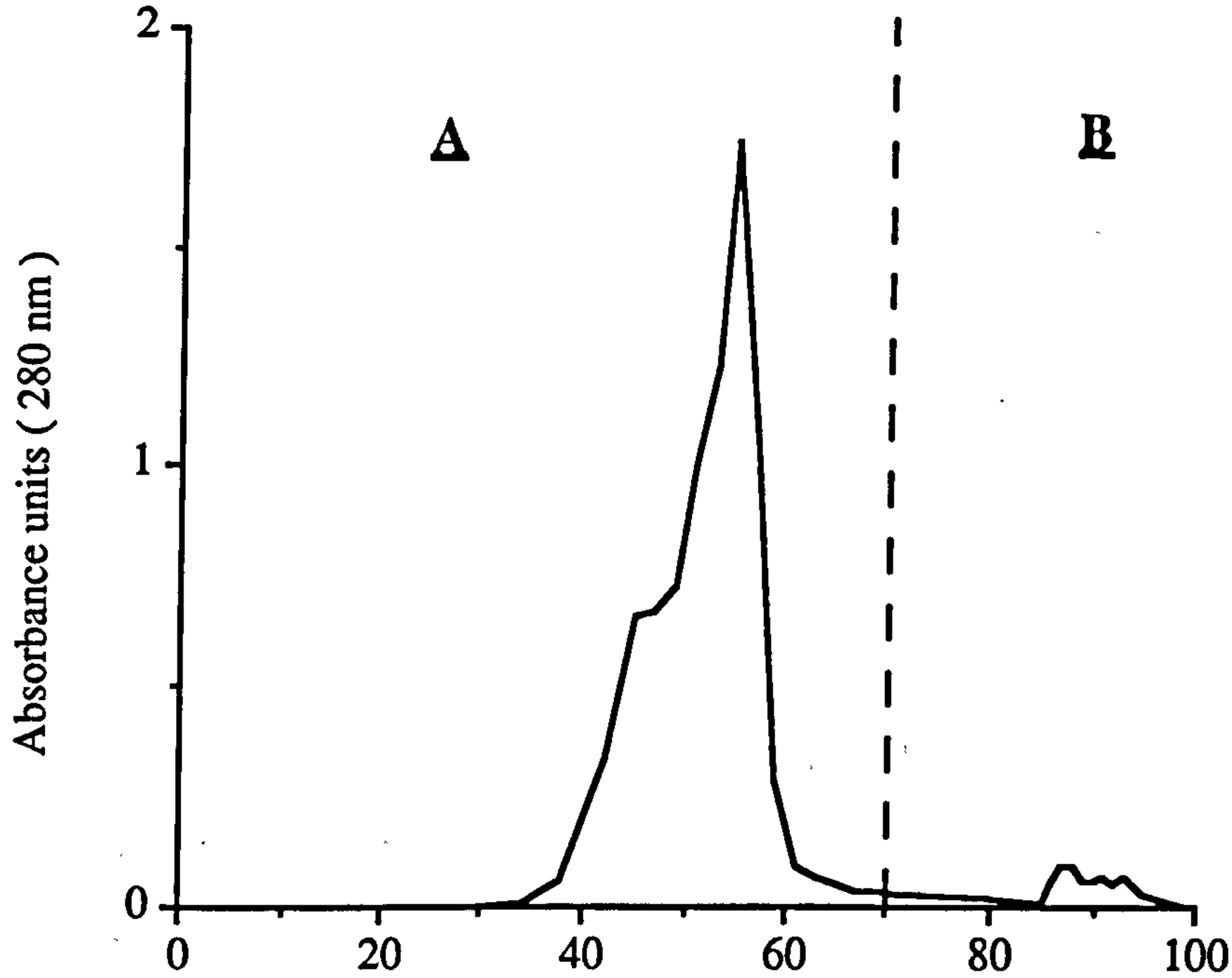


Figure 9.4

Title : The elution profile of human albumin 4.5 % after passage down a DEAE- Sephacel ion exchange column.

Ordinate : protein content of each 5ml fraction. Absorbance at 280 nm

Abscissa : Number of 5ml fractions

A 50mM sodium chloride

B 100mM sodium chloride

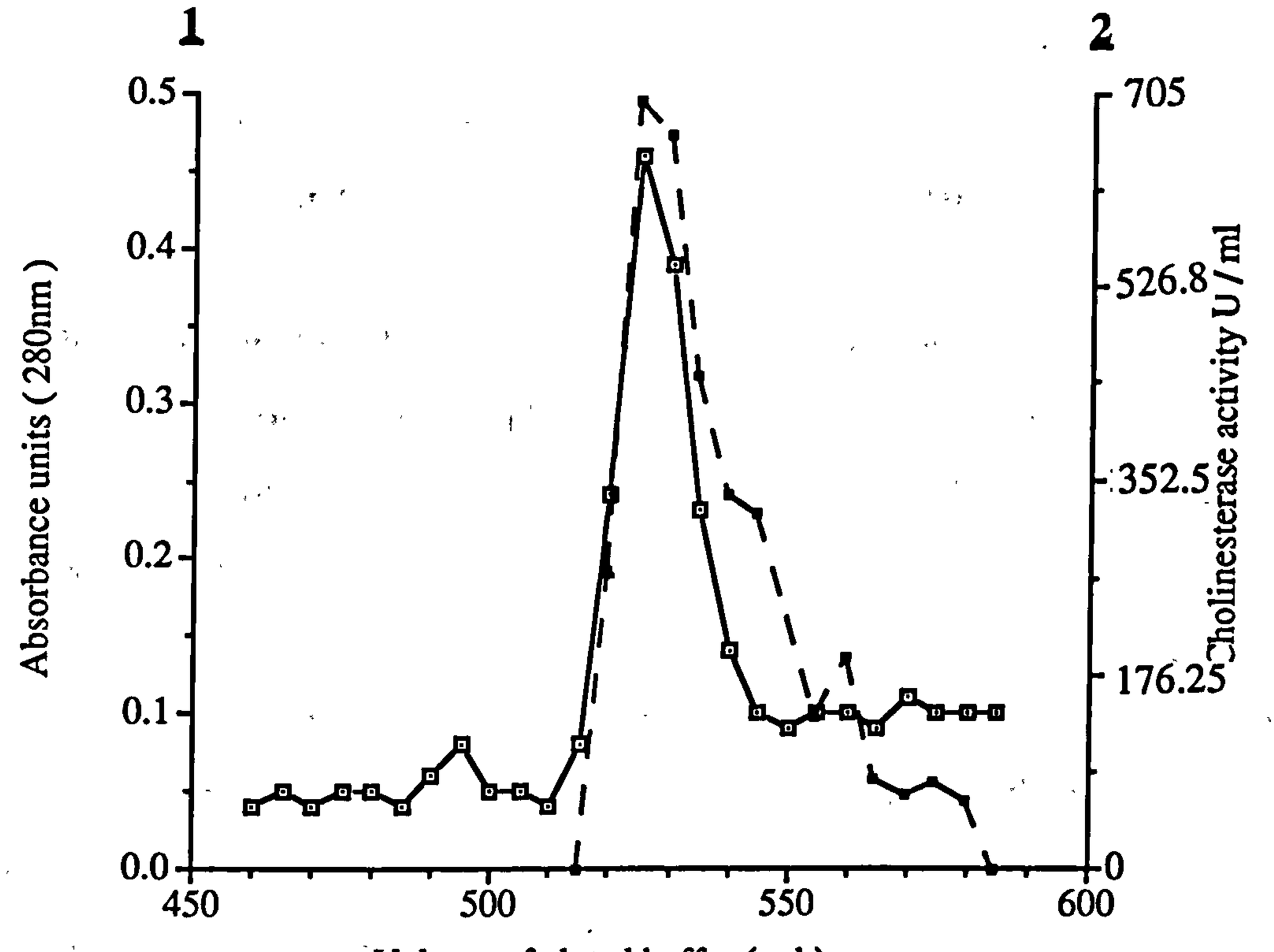


Figure 9.5

Title : The cholinesterase activity of the elution profile (peak1) obtained with 100mM sodium chloride in 50 mM sodium acetate buffer (figure 9.3).

Ordinate:

1 —▪— The absorbance of each 5 ml fraction at 280 nm

2 —▪ - The cholinesterase activity of each fraction expressed as U / ml where: U = nmol benzoylcholine chloride hydrolysed per minute at 37°C

Abscissa : The volume of eluting buffer collected as 5ml fractions

9.9.3 Discussion

The elution profile obtained in this method was very similar to that described by Rainsford et al (1980) and Wilde and Kekwick (1964). Das and Liddell (1970) also used a similar technique but instead employed a sodium chloride gradient following the initial elution with sodium acetate buffer alone.

Rainsford et al (1980) identified the albumin fraction as having 20% of the aspirin esterase activity of plasma, using a fluorimetic assay which involved incubation of the enzyme source with aspirin followed by extraction of salicylate with ether.

Although no aspirin esterase activity was found attributable to albumin using the spectrophotometric assay, it was possible to demonstrate the removal of 97% of the albumin content. Thus 3% or less remained in the protein fraction containing cholinesterase activity (Fig 9.4).

Both Rainsford et al (1980) and Wilde and Kekwick (1964) claimed that the albumin peak contained naphthylacetate esterase activity when separated on a non-SDS gel. Incubation of the albumin fraction separated in this study with phenylacetate showed no arylesterase activity.

By applying the separation technique described above I could separate purified enzyme from a series of plasmas to determine the kinetics of aspirin esterase.

CHAPTER 10

Determination of Aspirin Esterase Kinetics(Km) using the Cholinesterase Fraction Recovered from DEAE-Sephacel in Young; Fit and Frail Elderly

Chapter 10

Determination of Aspirin Esterase Kinetics (Km) using the Cholinesterase Fraction Recovered from DEAE-Sephacel in Young, Fit and Frail Elderly.

10.1 Introduction

In order to determine the Km values of plasma aspirin esterase activity, due to the cholinesterase enzyme, it was necessary to remove the albumin component. This study involved the use of purified cholinesterase fractions prepared from young, fit and frail elderly individuals by ion-exchange gel chromatography as previously described. Determination of Km values would indicate whether the lower Vmax of plasma aspirin esterase (Chapter 8) exhibited by the frail elderly was due to a reduced affinity of the enzyme.

10.2 Method

Patients

Frail elderly, fit elderly and young individuals were recruited into the study as discussed in Appendix I by Dr H Wynne. Table 10.1 shows individual patient details. 8 frail elderly individuals (mean age 82$\pm$2.5) with a low plasma aspirin esterase activity [< 100 nmol salicylate/ml plasma/min (Williams et al, 1989)] were selected; 8 fit elderly individuals (mean age 73$\pm$2.6) and 9 young individuals (mean age 25$\pm$4.0)were also chosen.

Table 10.1

Volunteer Details

Volunteer (sex) **Frail Elderly**	Age (years)	Plasma aspirin esterase (nmol salicylate/ml plasma/min)	Albumin (g/l)	Drug (if any)
1(F)	88	61.8	30	chlorpheniramine, docusate
2(F)	85	44.2	32	digoxin frusemide
3(F)	71	44.2	31	
4(M)	70	35 .5	38	
5(F)	84	53.0	28	
6(F)	89	39.7	38	
7(F)	83	48.6	24	bendrofluazide
8(F)	87	48.6	29	terodiline, amiloride frusemide
Mean ±SEM	82±2.5	48.6±0.0*	31±1.6	
Fit Elderly				
9	69	88.3	43	
10	88	97.2	48	
11	66	110.4	43	
12	71	106.0	41	
13	70	-	41	
14	67	110.4	41	
15	83	101.6	-	
16	71	123.6	37	
Mean ± SEM	73±2.6	106.0±0.0	42±1.2	

Table 10.1 continued

Volunteer (sex) **Young**	Age (years)	Plasma aspirin esterase (nmol salicylate/ml plasma/min)	Albumin (g/l)	Drug (if any)
17 (F)	29	136.9	41	
18 (F)	27	101.6	42	
19 (F)	28	97.2	42	
20 (F)	25	-	43	
21 (F)	24	79.5	43	
22 (F)	20	97.2	48	
23 (F)	25	66.2	48	
24 (M)	21	97.2	44	
25 (F)	25	88.3	50	
Mean $\pm$SEM	25$\pm$4	93$\pm$0.0	45$\pm$1.0	

*$p < 0.005$,therefore plasma aspirin esterase significantly lower in the frail elderly

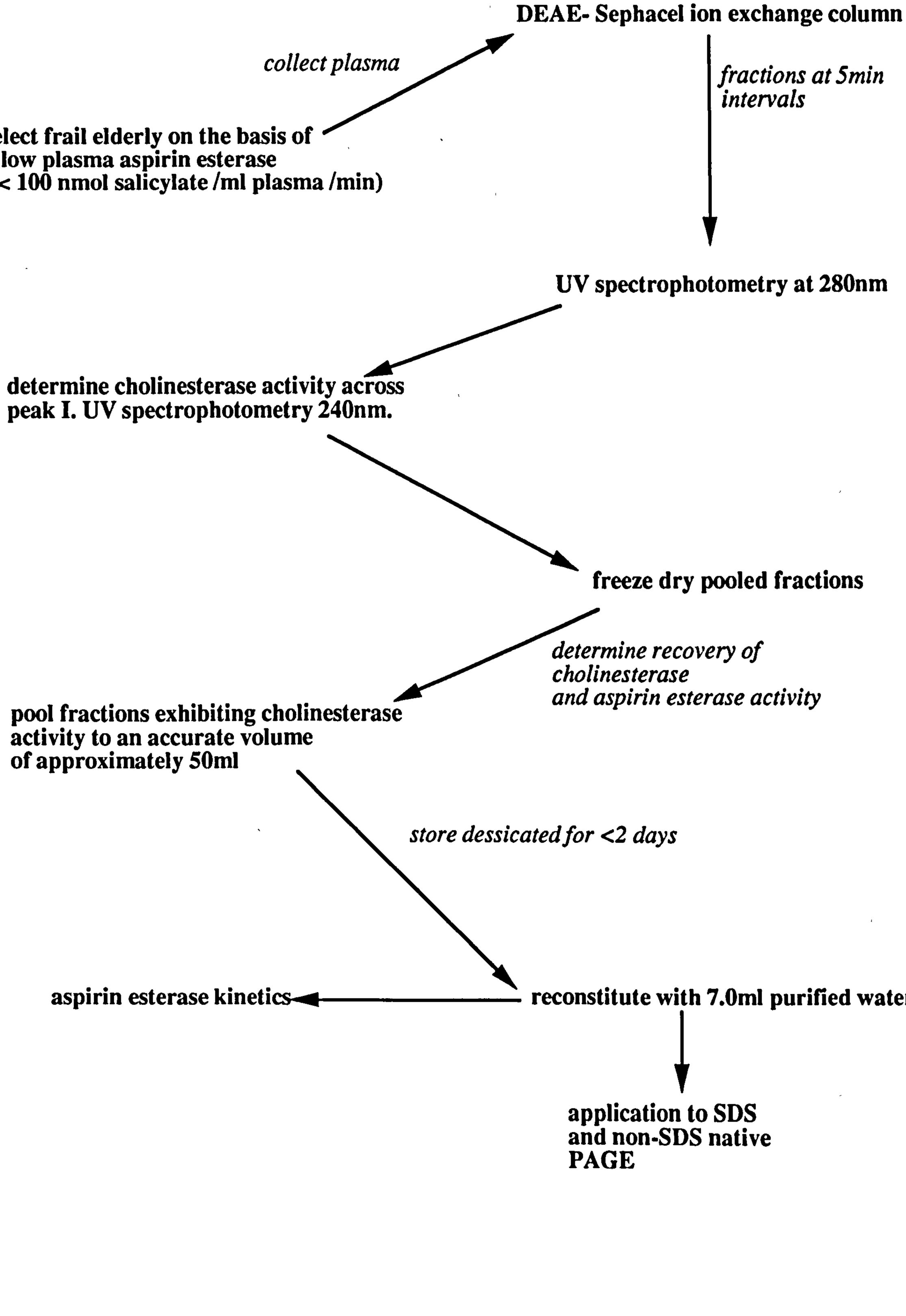
Aspirin Esterase Assay as applied to Purified Cholinesterase

The outline of the procedure followed for each sample is shown in the flow chart (Fig 10.1).

The protein fraction was separated as described in Chapter 9. A modified aspirin esterase assay was then applied to the purified protein fraction. 200 µl of the freshly reconstituted fraction (kept on ice) was incubated with a freshly prepared aspirin solution. This was made up to a final volume of 1.5 ml with Tris-HCl buffer as before.

The incubation mixture was prewarmed to 37oC for one minute, aspirin substrate was added and the salicylate formation was monitored continuously for 1.5 min at 340 nm on a uv spectrophotometer. The aspirin concentrations used were 1 to 4 mM. The maximum concentration was limited by the degree of spontaneous bydrolysis. Corrections were made for spontaneous hydrolysis by the parallel incubation of blank buffer containing aspirin.

Figure 10.1 Summary of Method for Determining the Kinetics of Aspirin Esterase from a purified fraction



10.2.1 Linearity of the Reaction

The assay involved a continual monitoring of salicylate formation so linearity with time can by visually judged. The linearity at high substrate concentration had to be assumed due to a shortage of protein material. Linearity with protein was tested for by incubating 50 - 300 µl fraction with low substrate concentration (0.25mM) over 1.5 min (Fig 10.2).

10.2.2 Commercial Cholinesterase

An analysis of commercial cholinesterase was made in order to compare the Km value and purification factor obtained with that from purified fraction.

The freeze-dried powder was reconstituted to 2 mg protein/ml in water. A final incubation volume of 1.5 ml Tris-HCl buffer contained 40 µl protein preparations and aspirin solution ranging from 1 to 4 mM. This was incubated and monitored as before. Figures 10.3 and 10.4 show linearity of the reaction with protein at low (0.5mM) and high (8mM) aspirin concentrations respectively.

The following parameters were calculated:

1. Recovery of activity- the total number of units of cholinesterase activity recovered as a percentage of that applied .
2. Purification factor- the activity of plasma aspirin esterase and cholinesterase expressed per mg protein/min in purified fraction divided by that in whole plasma.
3. Protein content (mg/ml)
4. Plasma aspirin esterase Km and Vmax- both calculated using the direct-linear plot method (Appendix II). Vmax was expressed as nmol salicylate formed/ mg protein/min

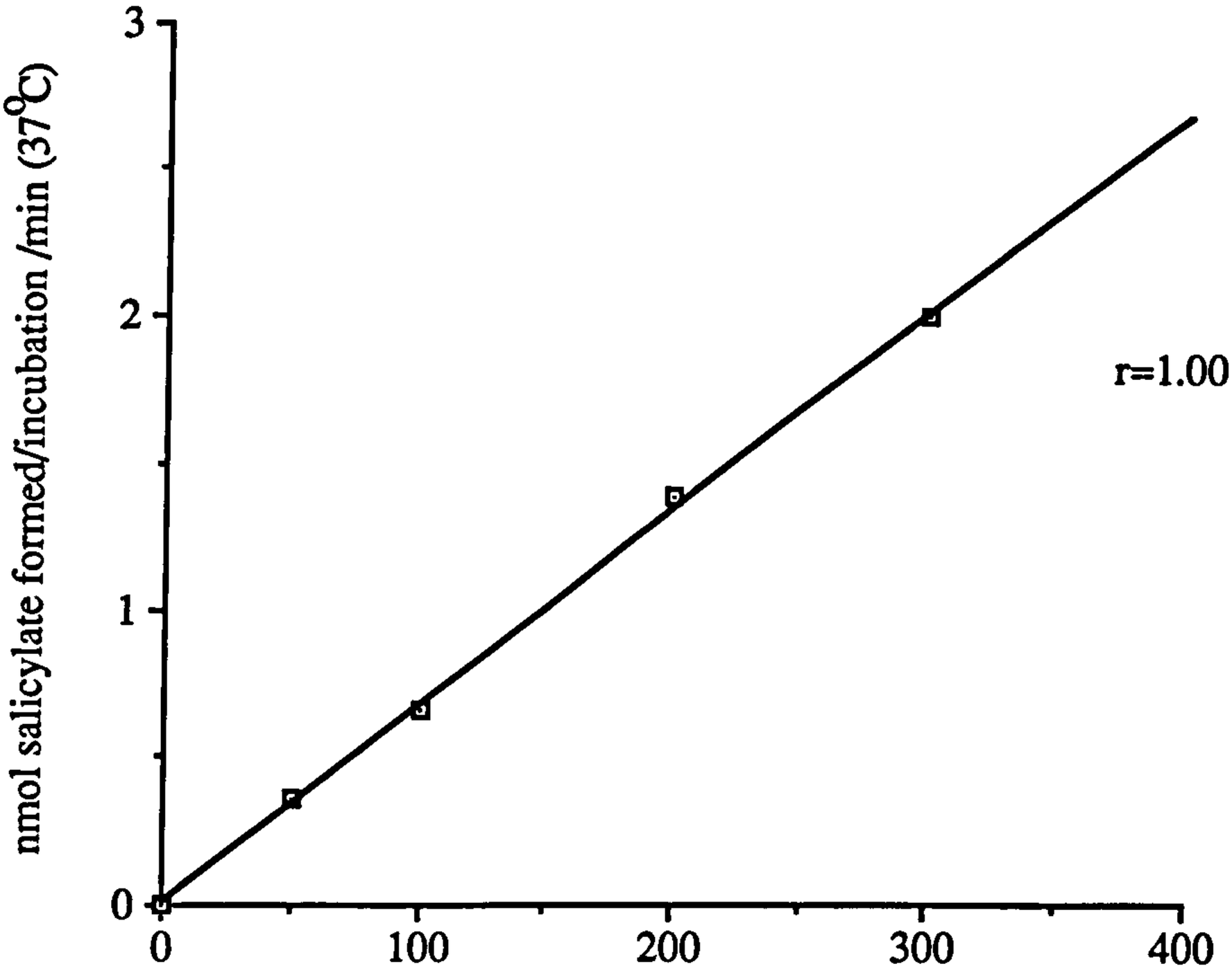


Figure 10.2

Title: The formation of salicylate by a semi- purified fraction of cholinesterase ; the effect of increasing protein concentration

Ordinate : nmol salicyate formed over a 1min. time period at an aspirin concentration of 0.25mM and at 37°C.

Abscissa : Amount of protein reconstituted fraction in incubation

Values are the mean of two determinations. The solid line is the least square regression line.

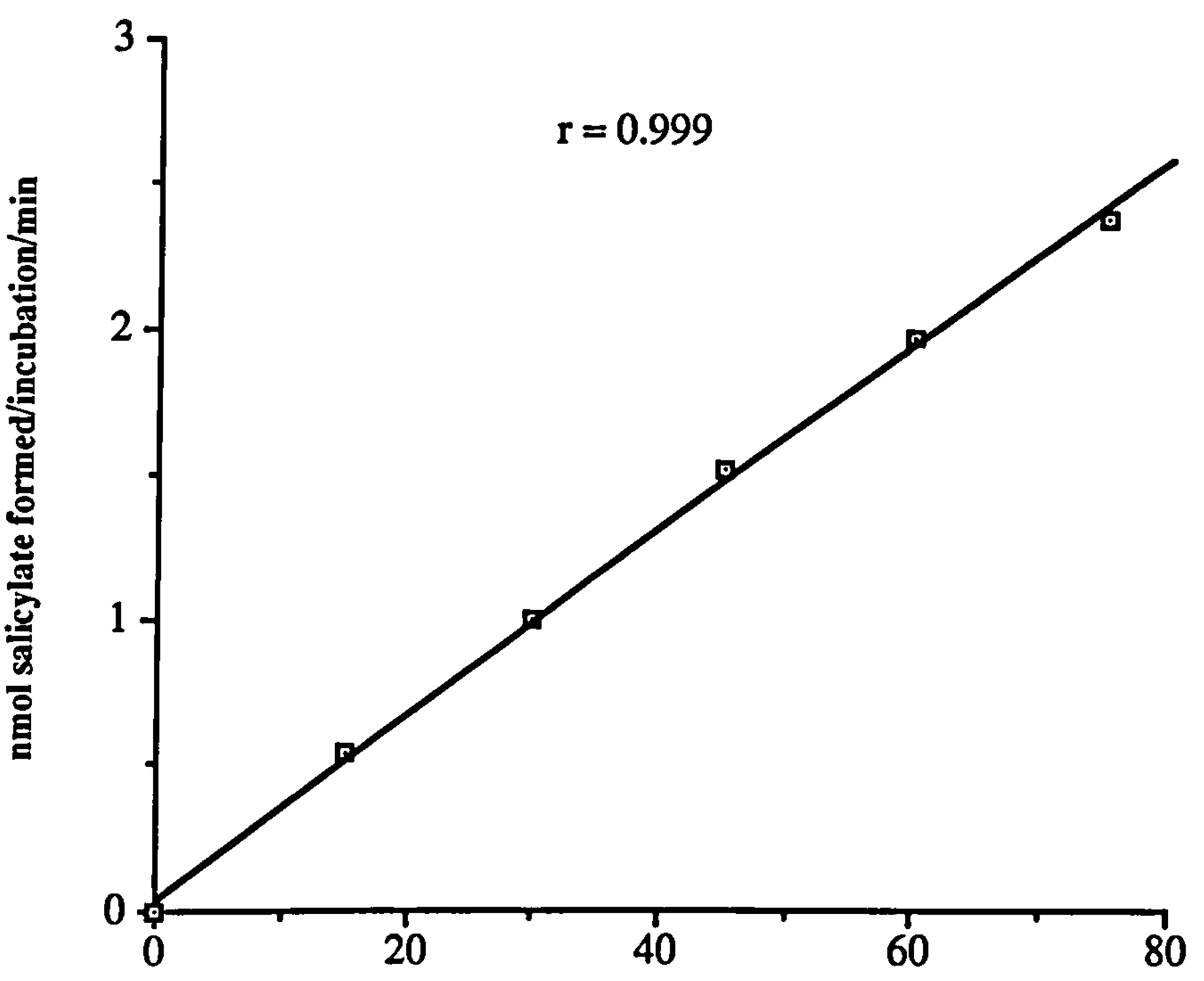


Figure 10.3

Title: The formation of salicylate by a commercial preparation of cholinesterase (Sigma): the effect of increasing protein concentration at an aspirin concentration of 0.5mM.

Ordinate: nmol salicylate formed over a 1min. time period at an aspirin concentration of 0.5mM and 37°C.

Abscissa: protein content of incubation, added as cholinesterase (Sigma) freeze dried preparation in water.

The values are the mean of two determinations. The solid line is the least square regression line.

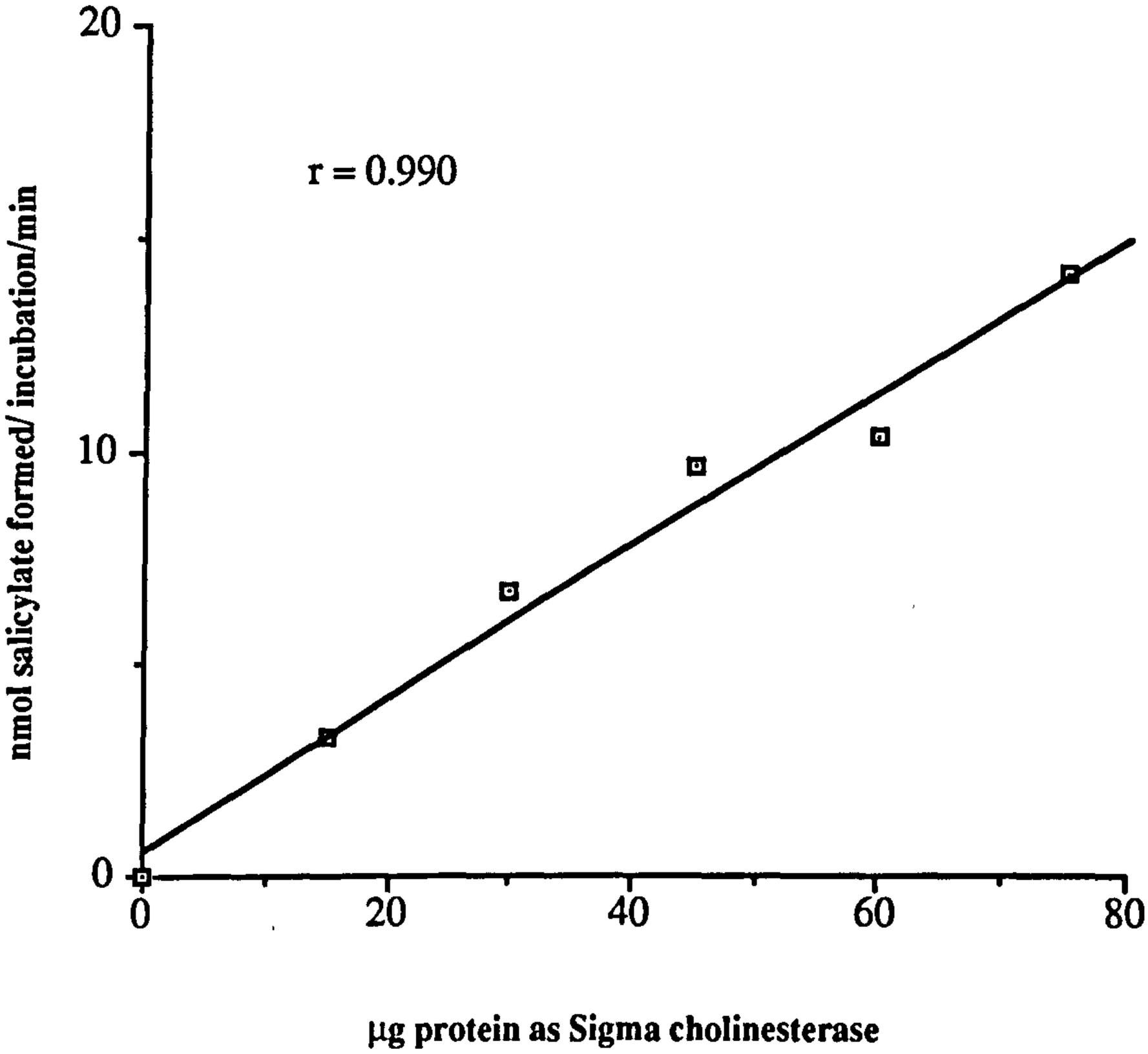


Figure 10.4

Title: The formation of salicylate by a commercial preparation of cholinesterase (Sigma): the effect of increasing protein concentration at an aspirin concentration of 8mM.

Ordinate: nmol salicylate formed over a 1min. time period at an aspirin concentration of 8mM and 37°C.

Abscissa: protein content of incubation, added as cholinesterase (Sigma) freeze dried preparation in water.

The values are the mean of two determinations. The solid line is the least square regression line.

10.3 Results

10.3.1 Purification

The plasma aspirin esterase activity in the frail elderly (mean 48.6 $\pm$ 0.0) was significantly lower than the mean value in the fit elderly (106.0 $\pm$ 0) and the young (93 $\pm$ 0) at the 0.5% significance level (Table 10.1).

The percentage recovery of cholinesterase and aspirin esterase activity was not significantly different in any of the three groups as shown in Table 10.2 (frail elderly: 58.5 $\pm$ 3.3 and 55.7 $\pm$ 5.6; fit elderly: 66.3 $\pm$ 3.3 and 59.6 $\pm$ 4.0; young 59.5 $\pm$ 2.4 and 49.8 $\pm$ 3.0) at the 5% significance level. There was a significant correlation between aspirin esterase and cholinesterase recovery ($r=0.606$; $p < 0.005$; $t = 3.57$; Fig 10.5).

The mean purification factors for aspirin esterase (frail: 29 $\pm$ 2; fit: 30 $\pm$ 2; young: 26 $\pm$ 3) and cholinesterase activity (frail: 20 $\pm$ 3; fit: 23 $\pm$ 3; young: 30 $\pm$ 4) are shown in Table 10.3 and summarized in Table 10.6. They were not significantly different.

The correlation between the albumin content of plasma and purification factor was tested for in order to determine whether this had any bearing on a successful separation. No correlation was found in either case (aspirin esterase: $r = 0.04$; $t = 0.19$ and cholinesterase: $r = 0.316$; $t = 1.6$).

Table 10.2

Recovery of Esterase Activity

Volunteer	% Aspirin esterase recovery	% Cholinesterase recovery
Frail Elderly		
1	45	44
2	-	63
3	76	70
4	51	71
5	41	53
6	81	76
7	45	52
8	51	58
Mean ± SEM	55.7± 5.6	58.5±3.3
Fit Elderly		
9	70	61
10	51	63
11	50	71
12	53	61
13	48	53
14	-	86
15	70	72
16	75	63
Mean ±SEM	59.6+4.0	66.3±3.3

Table 10.2 continued

Volunteer	% Aspirin esterase recovery	% Cholinesterase recovery
Young		
17	40	72
18	41	60
19	54	64
20	-	63
21	61	65
22	36	40
23	38	53
24	64	66
25a	54	56
25b	53	57
25c	47	58
Mean $\pm$ SEM	49.8 $\pm$3.0	59.5$\pm$2.4

No significant difference between the three groups at $p<0.05$

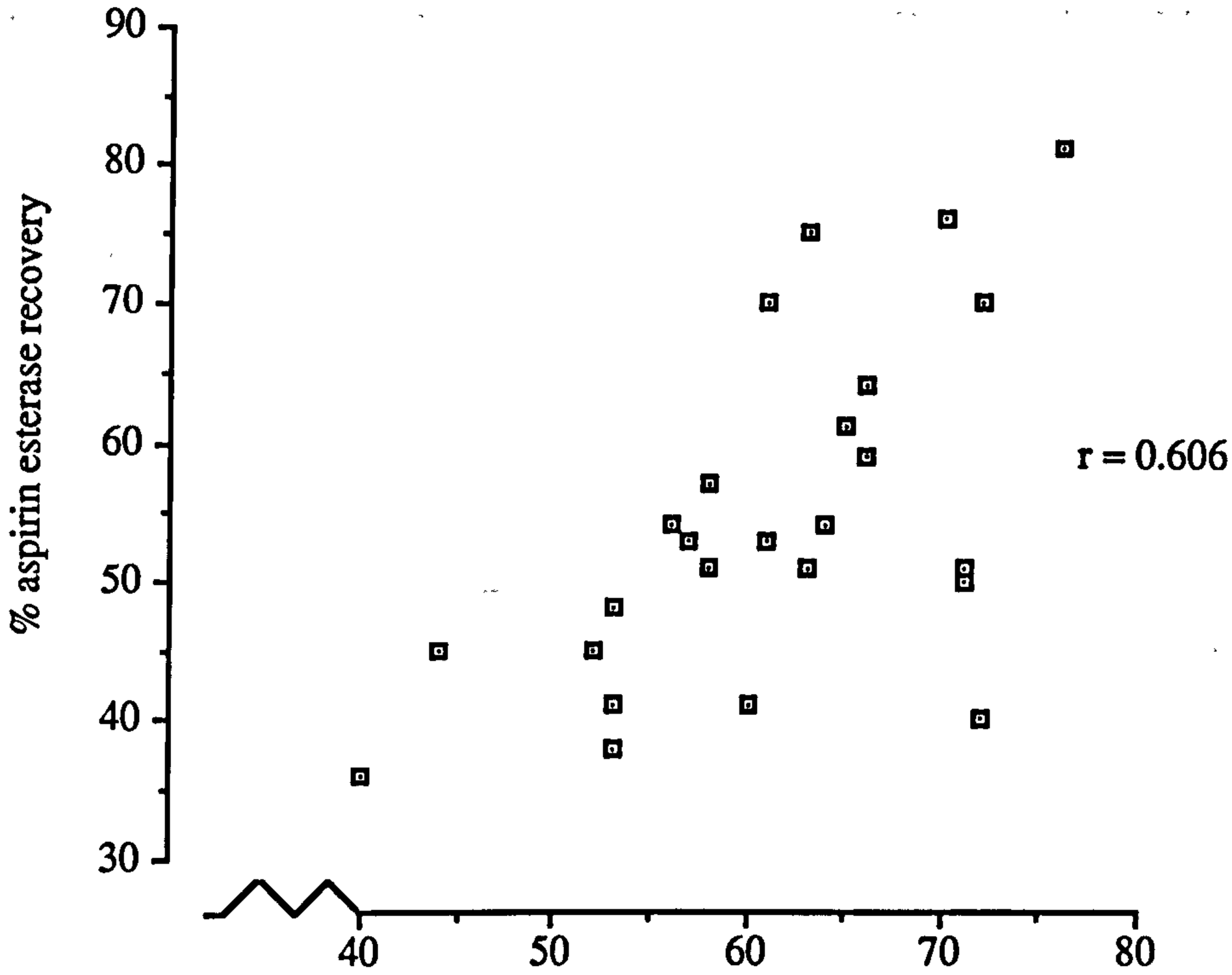


Figure 10.5

Title ; Correlation between the recovery of activity for aspirin esterase and cholinesterase following the elution of plasma down a DEAE- Sephacel ion exchange column.

Ordinate : Aspirin esterase recovery (%)

Abscissa : Cholinesterase recovery (%)

$p < 0.005$ correlation significantly different from zero

Table 10.3

Purification Results from Plasma Cholinesterase

subject	[1]Aspirin Esterase Activity (1mM)			[2] Cholinesterase Activity *Vmax*		
	Plasma	purified fraction	purification factor	plasma	Purified fraction	purification factor
Frail Elderly						
1	0.54	18.81	35	13.5	317	24
2	0.92	27.2	30	15.3	365	24
3	-	33.4	-	-	158	-
4	0.45	12.5	28	6.99	47	7
5	0.70	14.6	21	13.8	444	33
6	0.54	21	39	13.3	283	16
7	0.58	14.6	25	11.7	141	12
8	0.74	16.7	23	13.8	374	27
Mean $\pm$SEM			29$\pm$2			20$\pm$3

Table 10.3 continued

subject	[1]Aspirin Esterase Activity (1mM)			[2]*Vmax* (Cholinesterase)		
	Plasma	purified fraction	purification factor	plasma	Purified fraction	purification factor
Young						
17	1.42	35.5	25	22.5	491	22
18	1.32	27.2	21	25.1	606	24
19	1.20	35.5	29	20.5	630	31
20	1.02	25.1	25	15.5	436	28
21	-	22.9	-	-	344	-
22	0.81	23	28	21.3	1310	62
23	0.64	17.1	27	12.6	520	42
24	1.34	41.8	31	27	1010	37
*25a	1.32	31.4	23	24	455	19
25b	1.35	31.4	23	24	505	22
25c	1.28	33.4	26	24	398	17
Mean ± SEM			26±3			30±4

Table 10.3 continued

subject	Aspirin Esterase Activity (1mM)			*Vmax* (Cholinesterase)		
Fit Elderly	Plasma	purified fraction	purification factor	plasma	Purified fraction	purification factor
9	1.22	39.7	33	21.8	44.6	20
10	1.56	33.4	21	27	482	19
11	1.32	31.4	24	21.4	508	26
12	1.38	51.6	37	25.9	1026	40
13	1.28	35.5	28	26.6	398	15
14	1.85	48.1	26	23.0	503	22
15	1.42	48.1	34	21	508	24
16	1.44	48.1	33	25.7	493	18
Mean $\pm$ SEM			30$\pm$2			23$\pm$3

1 nmol salicylate formed / mg protein /min at 37°C

2 nmol benzoylcholine chloride hydrolysed/mg protein/ min at 37°C

*CV=6% (n=3; x=3.6;SD=0.24)

10.3.2 Kinetics

The protein content of the plasma and purified fractions is shown in Table 10.4. These were used in order to establish the Vmax values for the enzymes which were expressed as units of enzyme activity per mg protein. Vmax for aspirin esterase and cholinesterase were significantly different for the three groups at $p < 0.05$ ($F = 3.9$ and $F = 5.2$ respectively) as shown in Table 10.5 (frail: 80 $\pm$ 17 and 266 $\pm$ 45; fit: 124 $\pm$ 17 and 546 $\pm$ 65; young: 183 $\pm$ 30 and 645 $\pm$ 105 respectively). This is to be expected since the frail elderly were selected on the basis of a low plasma aspirin esterase. There was a significant correlation between Vmax for aspirin esterase and Vmax for cholinesterase ($p < 0.001$; $t = 34.4$) as shown in Fig 10.6 and Table 10.5.

The Km values were calculated using the direct linear plot method (Fig 10.7). This is explained in Appendix II. The calculated values are shown in Table 10.5. The mean value for the young individuals was 4.7 $\pm$ 0.4; for the fit elderly: 3.6 $\pm$ 0.2 and for the frail elderly: 4.3 $\pm$ 0.5 (mean in mM $\pm$SEM), shown in Fig 10.8.

There were no significant differences between the three groups at $p < 0.05$, Table 10.6 summarizes this data.

Table 10.4

Protein Content of Plasma and of Purified Fraction

volunteer	protein concentration mg/ml	
Frail Elderly	**plasma**	**purified fraction**
1	69.5	0.86
2	77.4	1.41
3	-	2.1
4	65.0	4.5
5	66.0	0.75
6	65.8	1.71
7	-	1.93
8	61.2	0.89
Fit Elderly		
9	82.0	1.63
10	63.9	1.51
11	85.0	1.37
12	65.5	0.65
13	75.2	1.75
14	76.5	2.05
15	79.6	1.61
16	81.2	1.72

Table 10.4 continued

volunteer	protein concentration mg/ml	
Young	plasma	purified fraction
17	69.9	1.48
18	66.3	1.05
19	72.3	1.01
20	74.2	1.25
21	-	1.32
22	82.5	0.37
23	71.8	0.63
24	77.2	0.75
25a	68.1	1.40
25b	68.1	1.17
25c	68.1	1.61

Table 10.5

Kinetic Results from Purified Cholinesterase

Subject	*Vmax* Purified Enzyme		*Km*(mM)
	Aspirin Esterase[1]	Cholinesterase[2]	Aspirin Esterase
Frail Elderly			
1	109	317	4.2
2	116	365	4.5
3	44	158	3.2
4	135	47	3.9
5	92	444	3.4
6	46	283	2.7
7	52	141	5.9
8	170	374	6.9
Mean ± SEM	80±17*	266±45*	4.3±0.5
Fit Elderly			
9	111	446	3.3
10	53	482	3.8
11	108	508	2.5
12	233	1026	3.5
13	109	398	4.0
14	129	503	4.7
15	113	508	3.0
16	134	493	3.6
Mean ±SEM	124±17	546±65	3.6±0.2

Table 10.5 continued

Subject	***Vmax* Purified Enzyme**		***Km*(mM)**
	Aspirin Esterase[1]	**Cholinesterase[2]**	**Aspirin Esterase**
Young			
17	107	491	3.8
18	179	606	6.3
19	155	630	3.3
20	107	436	4.0
21	125	34.4	6.0
22	370	1310	4.2
23	190	520	5.9
24	305	1010	4.6
25a	110+	455++	3.8
25b	132	505	3.8
25c	88	398	3.3
Mean ±SEM	183+30	645+105	4.7+0.4

Key :

1. nmol salicylate/mg protein /min. Calculated from the direct-linear plot.
2. nmol benzoylcholine chloride hydrolysed/mg protein/min

\+ CV=16%

++CV=11% only the first value is used in the calculation of the means.

* Significantly different from the young group $p<0.05$; $F=4.85$ (aspirin esterase) ; $F=3.87$ (cholinesterase)

Regression analysis between aspirin esterase and cholinesterase: $r=0.93$; $t=34.4$; $p<0.0001$

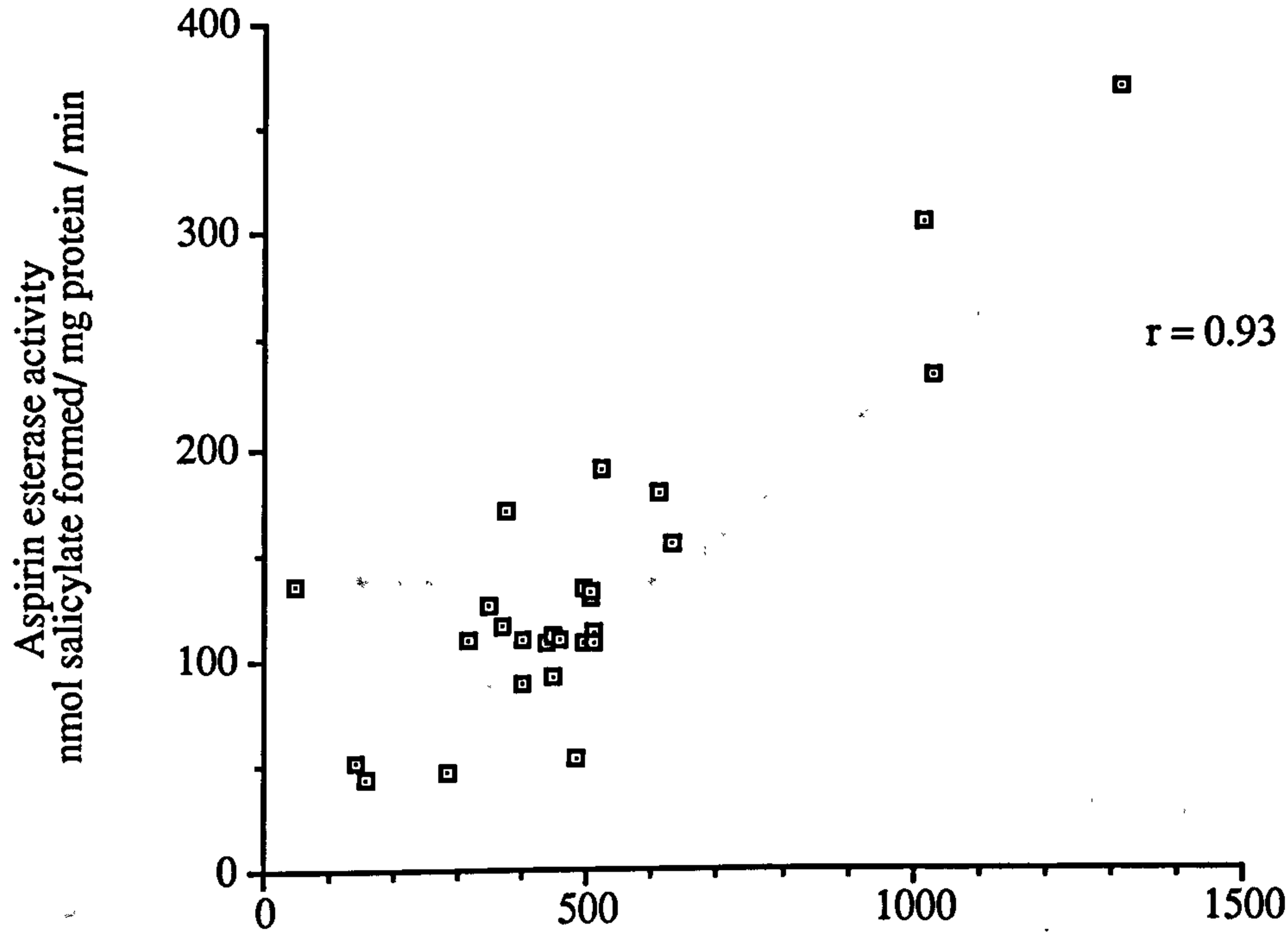


Figure 10.6

Title : The correlation between aspirin esterase activity and cholinesterase activity in purified fractions of human plasma from young; fit and frail individuals.

Ordinate : Aspirin esterase activity at $37^{o}C$

Abscissa : Cholinesterase activity at $37^{o}C$

$p < 0.001$ correlation significantly different from zero

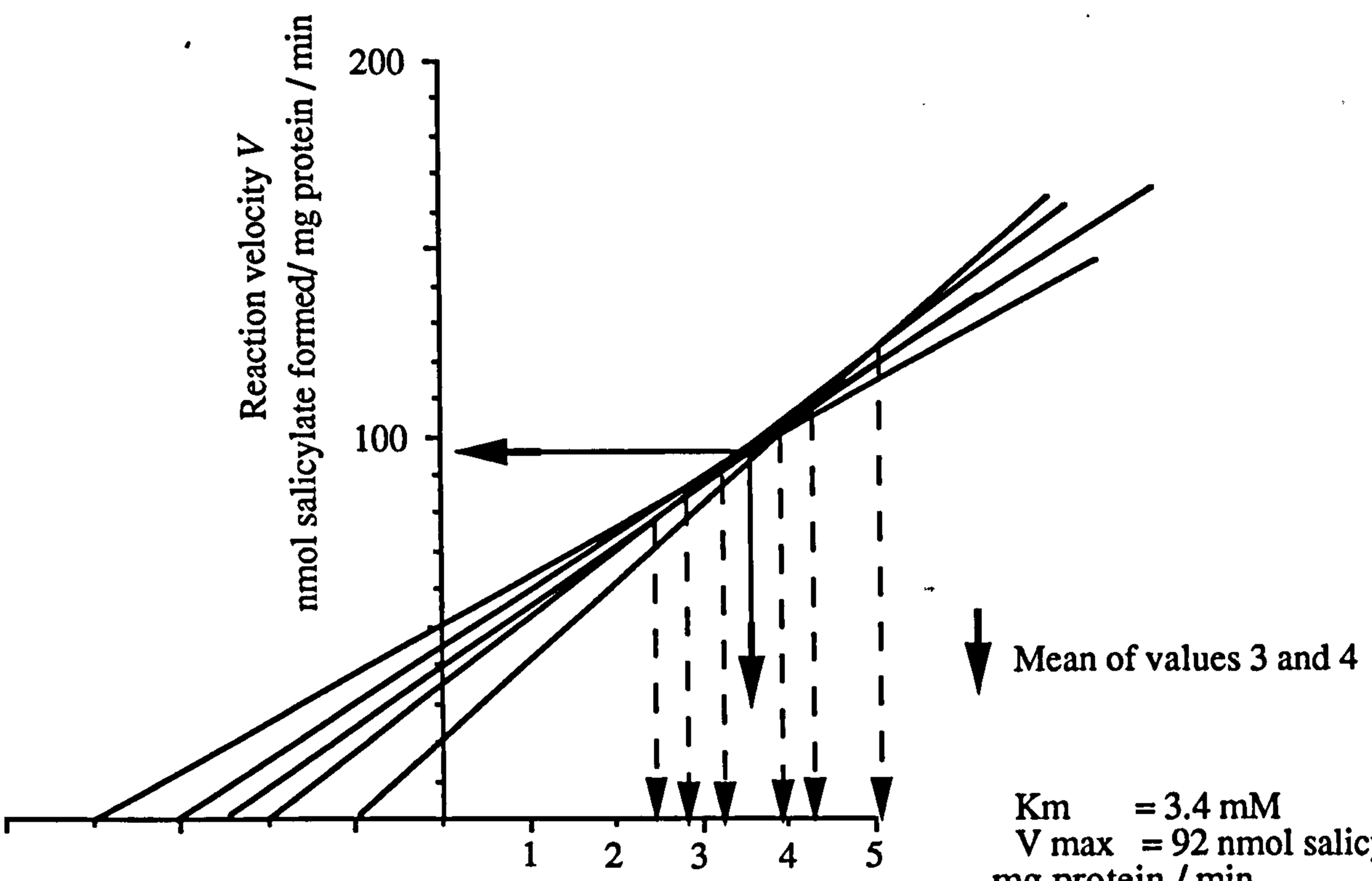


Figure 10.7

Title : A direct - linear plot showing the calculation of *Km* and *Vmax* for aspirin esterase from a preparation of partly purified plasma (volunteer no. 5)

Ordinate : The reaction velocity V at 37^0C

Abscissa : Aspirin concentration (mM)

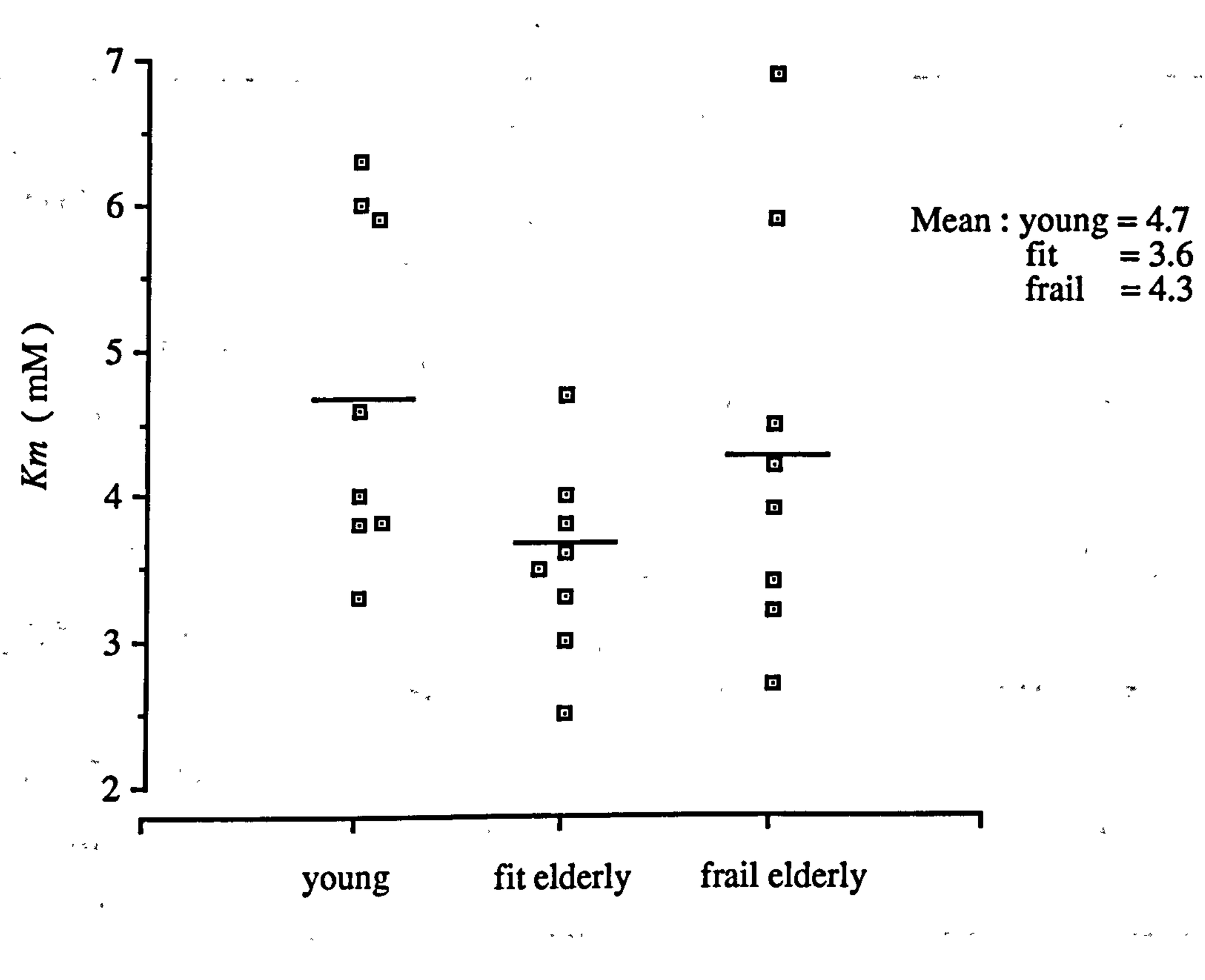


Figure 10.8

Title : *Km* values for plasma aspirin esterase using a semi- purified cholinesterase preparation in young, fit and frail elderly

Ordinate : *Km* value (mM). Values are the mean of duplicates.

Abscissa : Values in young; fit and frail elderly

Table 10.6

Summary of Data from Tables 10.3 and 10.5

	Frail Elderly	Fit Elderly	Young
Aspirin Esterase	Mean ±SEM		
*Vmax purified enzyme	+80±0.17	124±17	183±30
purification factor	29±2	30±2	26±3
Km	4.3±0.5	3.6±0.2	4.7±0.4
Cholinesterase			
**Vmax purified enzyme	+266±45	564±16	645±105
purification factor	20±3	23±3	30±4

* nmol salicylate produced/mg protein/min
**nmol benzoylcholine chloride hydrolysed/mg protein/min
+$p < 0.05$ compared with the young

10.3.3 Polyacrylamide Gels

The SDS gels, when stained for protein, (Plate 10a) showed the bands of protein present in pooled fractions of purified plasma with cholinesterase activity. These showed a very similar profile to the Sigma preparation of cholinesterase (gel 2; tracks 2 and 4). Comparison with molecular weight markers (12,300 - 78,000) indicated a band which had a similar Rf value to ovalbumin (Rf = 0.32). This protein also appeared to be present in a higher concentration in the Sigma preparation than purified plasma enzymes. Using the Sigma cholinesterase preparation as standard it was not possible to distinguish a protein band due to cholinesterase. However albumin (Rf = 0.54) was evident in each sample.

The general esterase stain using naphthylacetate substrate is shown with a 5% non SDS gel (Plate 10b; gel 4). The general esterase activity is expressed as the nmol phenol produced per mg protein following incubation of an aliquot of purified fraction with phenylacetate, another general esterase substrate.

The more specific cholinesterase stain is shown on a 7.5% gel and there is only one band of activity (Plate 10b; gel3)

Plate 10a

Purified Fractions Separated on SDS gels and stained for Protein. (7.5% gel)

Gel 1. From left to right

Track 1: Volunteer no 11 protein on gel: 68 µg

Track 2: Volunteer no 24 protein on gel: 37 µg

Track 3: Volunteer no 6 protein on gel: 87 µg

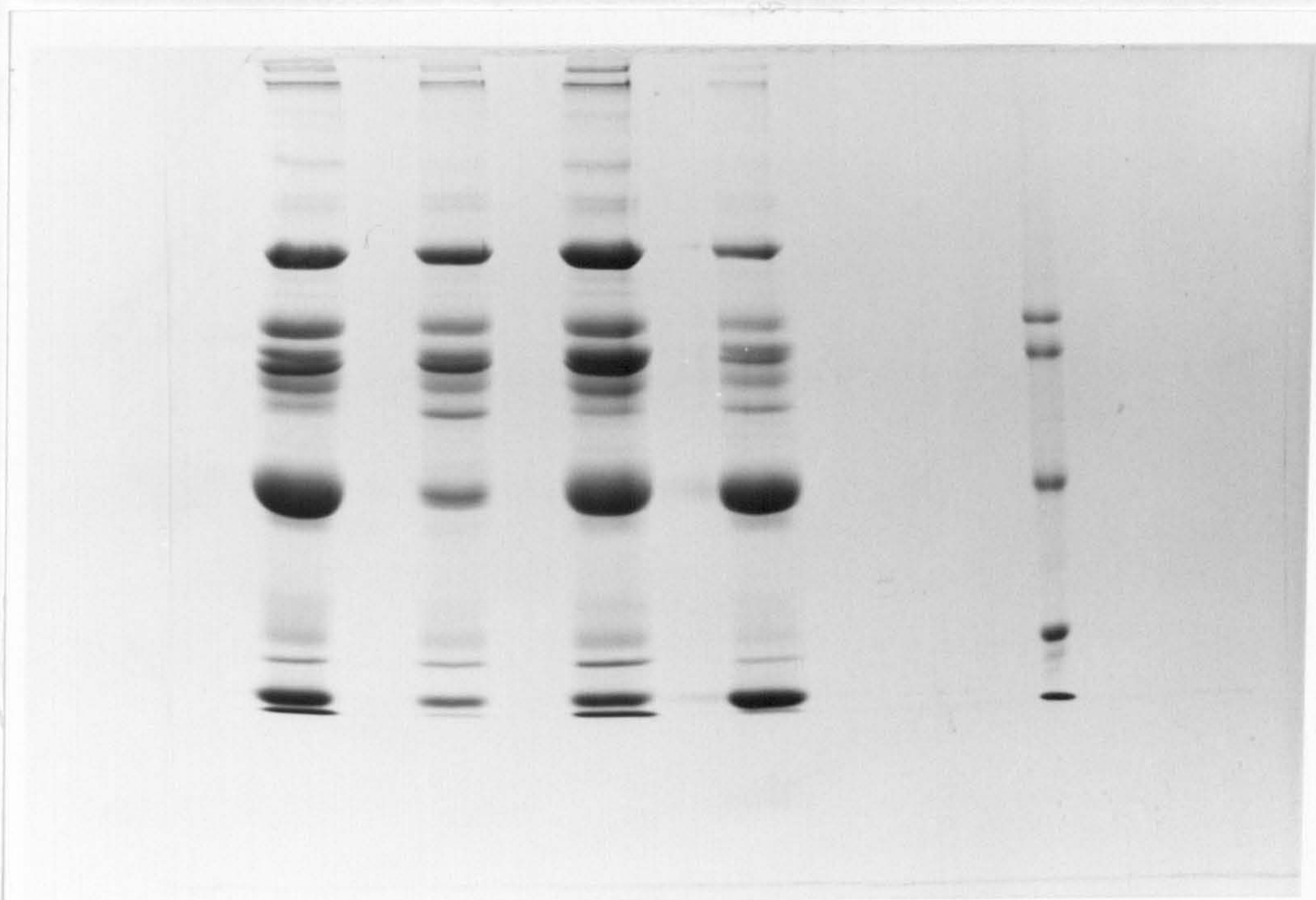
Track 4: Volunteer no 13 protein on gel: 88 µg

Track 5: molecular weight markers range 12,300-78,000

Gel 2. From left to right

Tracks 2 and 4: Sigma cholinesterase preparation

Protein on gel: 35 µg



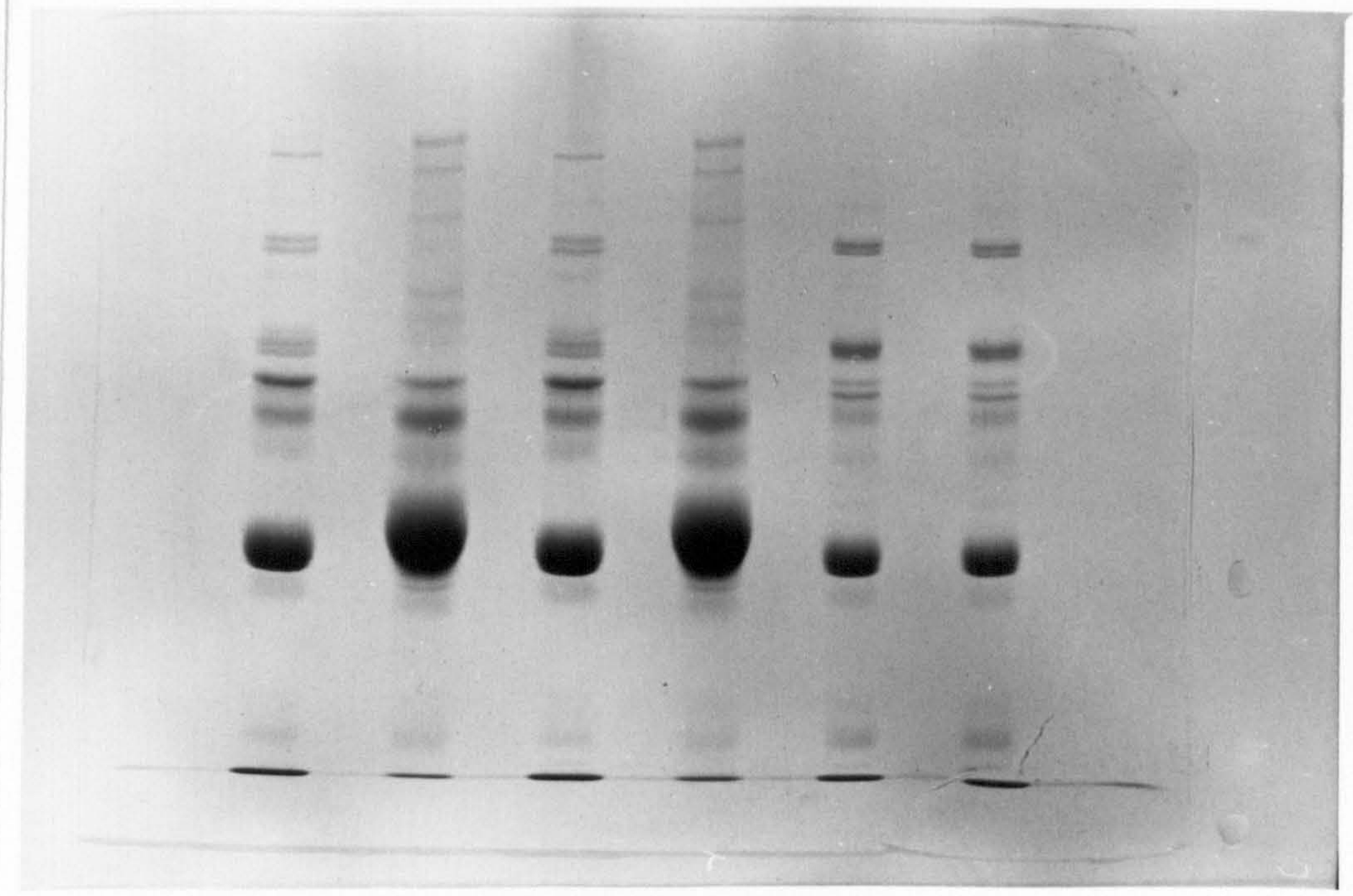


Plate 10b

Purified Fractions Separated on Non-SDS Gel for Native Proteins

Gel 3. Stained for Cholinesterase Activity (7.5% gel)

Track 1: Volunteer no 20, 15.2 u * of cholinesterase activity on gel.

Track 2: Sigma cholinesterase preparation, 42 u of cholinesterase activity on gel.

Track 3: Volunteer no 12, 23 u of cholinesterase activity on gel.

Track 4: Same as track 2.

* u = μmol benzylcholine chloride hydrolysed/min at 37oC.

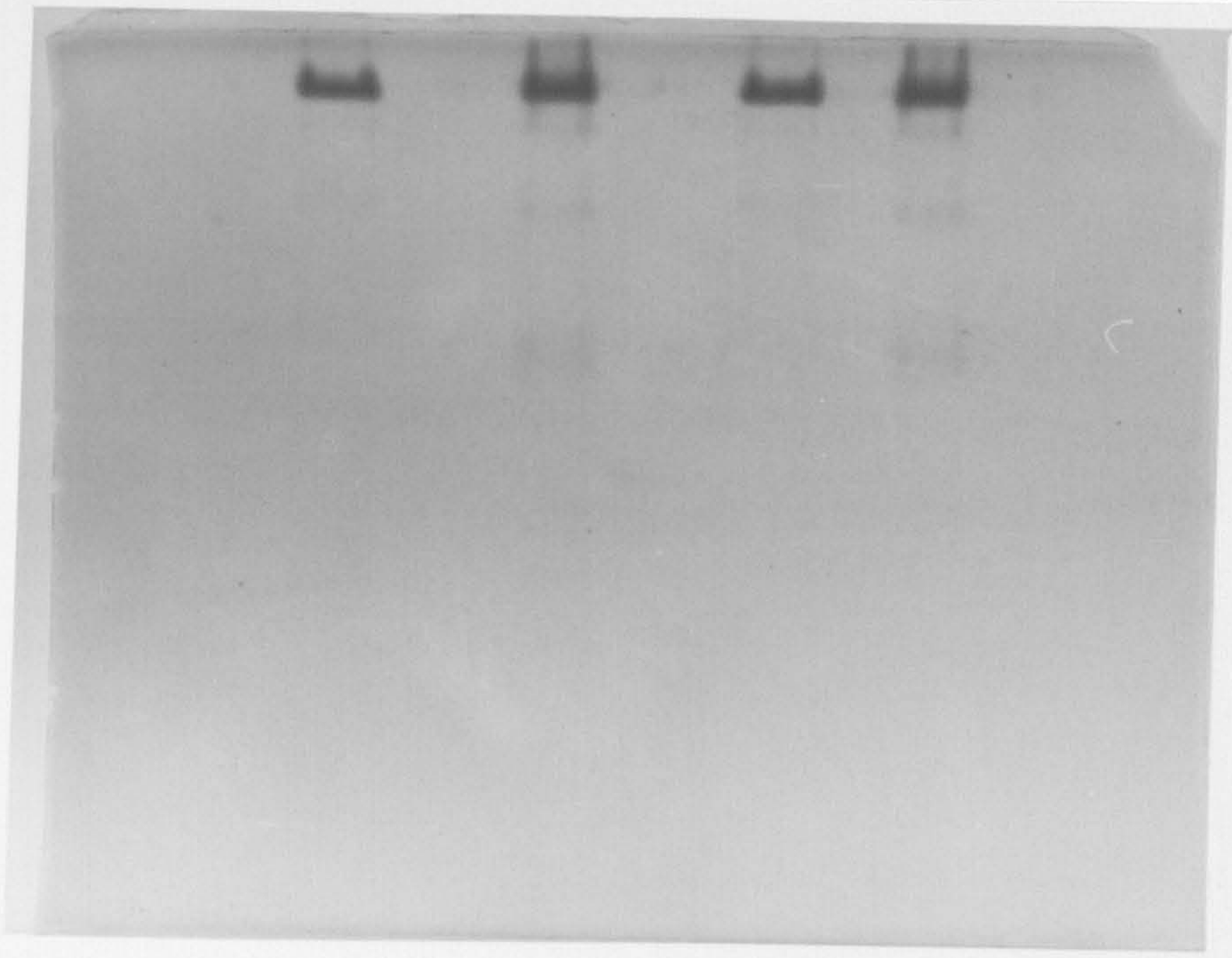
Gel 4. Stained for Naphthylacetate Esterase Activity (5% gel).

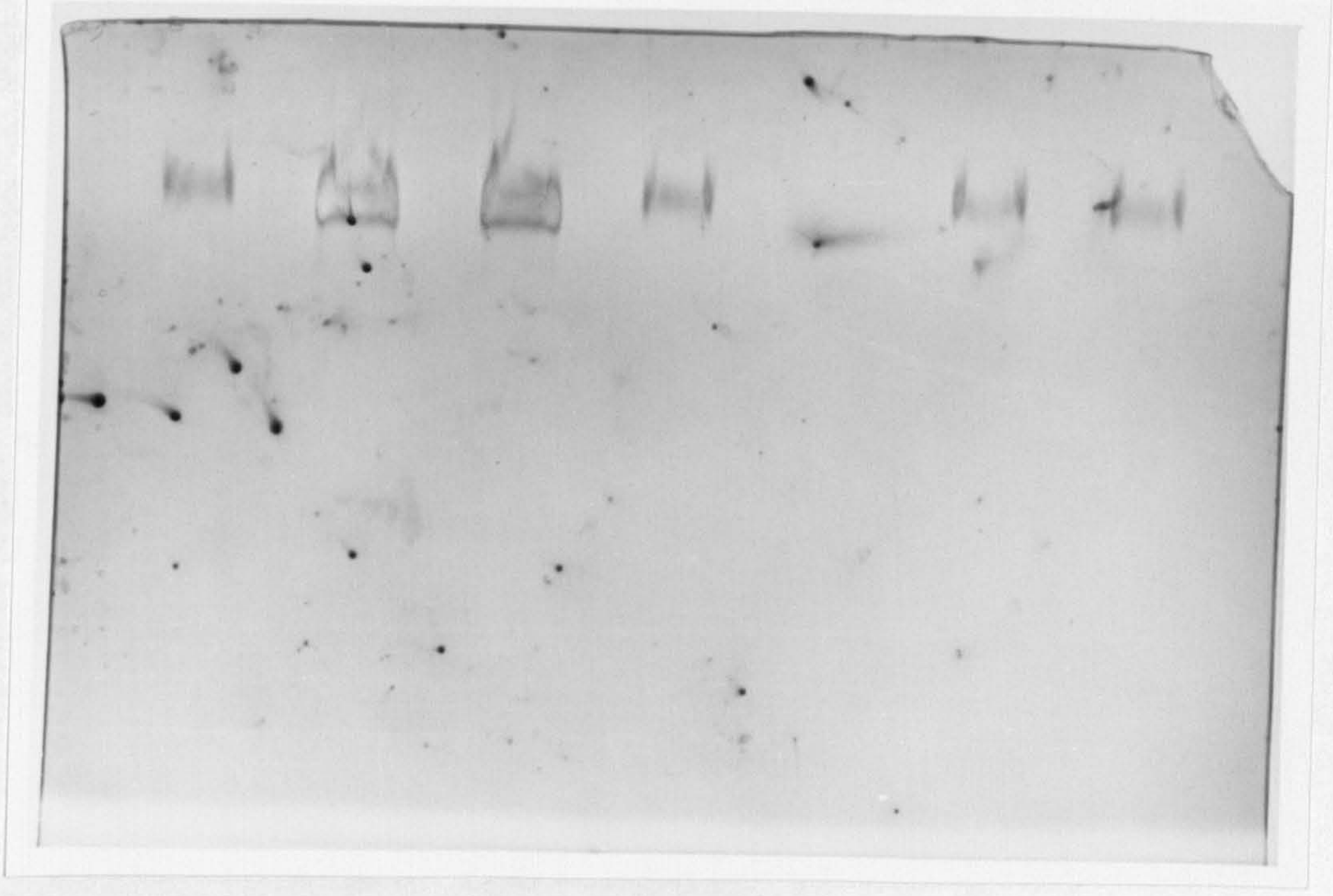
Tracks 1 and 4: Volunteer no 20, 12.5 u ** of phenylacetate esterase activity on gel.

Tracks 2 and 3: Sigma cholinesterase preparation, 32 u of phenylacetate esterase activity on gel.

Tracks 5 and 6: Volunteer no 12, 11.1 u of phenylacetate esterase activity on gel.

** u= nmol phenol produced/min at 37oC.





10.3.4 Commercial Cholinesterase

The Km and Vmax values for the cholinesterase preparation from human plasma produced by Sigma Chemical Co. were also calculated (Fig 10.9). The Km value was 5.0 mM and the Vmax was250nmol salicylate/ mg protein/min. The purification factor for this preparation was approximately 50 - fold (Table 10.7) when compared with a representative plasma.

Table 10.7

Purification Factor of Sigma Chemical Co Preparation of Human Plasma Cholinesterase.

Preparation	units of activity (U) *	Protein mg / ml	Specific activity U / mg	Purification factor
commercial cholinesterase	864	0.73	1183	50
plasma (volunteer 25)	1606	68.1	21	-

*nmol benzoylcholine chloride hydrolyzed per ml per minute at 37°C and 0.05mM substrate

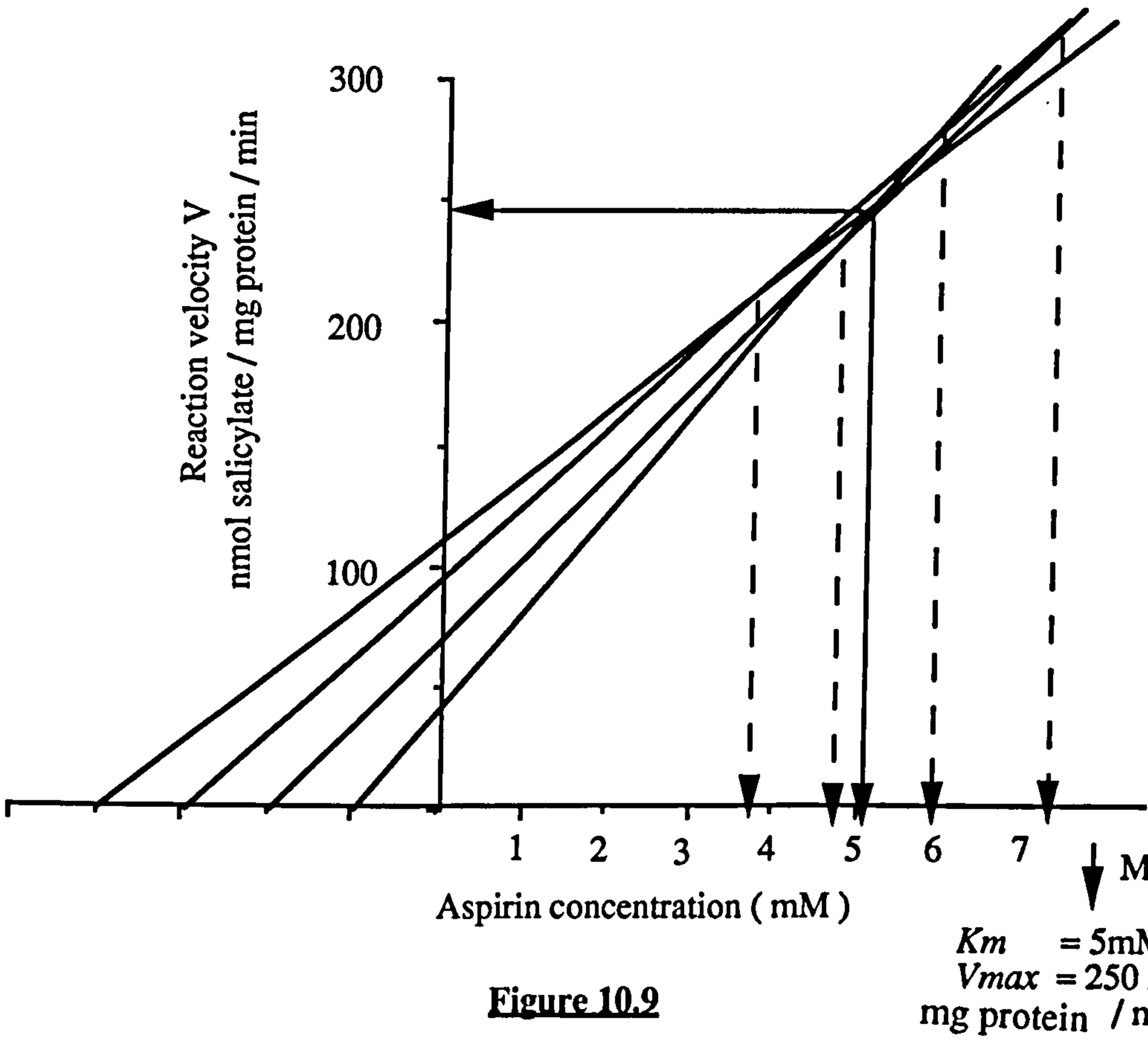


Figure 10.9

Title : A direct - linear plot showing the calculation of *Km* and *V max* for aspirin esterase from a commercially available preparation of cholinesterase

Ordinate : The reaction velocity at 37 °C

Abscissa : Aspirin concentration (mM)

10.4 Discussion

10.4.1 Kinetics

The frail elderly were selected on the basis of a low aspirin esterase activity which enabled me to determine whether the Km values in the fit and frail elderly and young individuals altered as a consequence. The Km values obtained for three groups were not significantly different and were similar to those obtained in whole plasma in Chapter 8 and also to that of Valentino et al (1981) who calculated the Km value as 4.2 mM.

If albumin contributed a significant degree of activity to aspirin esterase, removal of this protein would alter the affinity of cholinesterase enzyme for aspirin substrate. An increase in affinity would be apparent as a reduced Km value. This Km value would therefore be a measurement of the affinity of cholinesterase *only* for aspirin.

The calculated Vmax values were expressed as nmol salicylate/mg protein/min. Because of the selection process the Vmax value of the purified fraction was significantly lower in the frail elderly ($p < 0.05$), as was the Vmax of plasma cholinesterase. These values were also dependent on the recovery of enzyme activity after purification. The mean recovery of activity for both enzymes between the three groups was not significantly different. The two Vmax values were also significantly correlated, again confirming the relationship between the two enzymes.

10.4.2 Purification Procedure

Lockridge and La Du (1982) used 10 l of human plasma in order to obtain 12 mg yield of electrophoretically pure cholinesterase. This had a specific activity of 200 µmol benzoylcholine chloride hydrolysed/mg/min at 25oC. This was determined using the same assay as I used (Kalow and Lindsay, 1955). Thus, these results can be related to my results and used in order to obtain an approximation of the amount of cholinesterase in my purified protein fractions.

If we consider the results from subject 13 whose purified cholinesterase fraction had a specific activity of 398 nmol/mg/ml at 37oC. This is equivalent to 217 nmol/mg/min at 25oC using the temperature correction factors supplied by King (1965). It is therefore approximately 900 times less pure than the samples obtained by using affinity chromatography (200,000 ÷ 217 nmol/mg/ml).

Lack of protein material from clinical sample restricted subsequent analytical procedures. SDS-PAGE was useful in order to show other proteins present, however. Using a coomassie blue stain for protein it was possible to detect 0.2 - 0.5 µg protein in each band (Hames, 1985). 50 ul of sample was the maximum volume which could be applied to each sample well of the gel. In the case of volunteer 13 this volume would obtain 87.5 ug total protein (1.75 mg/ml; Table 10.4) of which 97.2 ng would be pure cholinesterase (87.5 µg ÷ 900). This was insufficient to be detectable with coomassie blue stain on this gel.

Using Electron molecular weight markers, the albumin band was indicated (Plate 10a; gel 1). Even though approximately 97% of the albumin is removed, about 1.2 g/l (ie 3% x 40 g/l) remains. Again, considering volunteer 13, if 3% of the fraction protein content was albumin, then this was equivalent to

52 µg/ml (ie 1.75 mg/ml x 3%). In a sample volume of 50 µl this would mean that approximately 2.6 ug albumin may be present which will be strongly evident stained on an SDS gel, but as a proportion of the original concentration is very small.

Non-SDS native gels were a sensitive visual means of showing esterase activity (Plate 10b, gels 3 and 4). Unfortunately, because the cholinesterase enzyme is a tetramic structure of molecular weight 340,000, it remains as a very large molecular form which does not resolve successfully even on a 5% polyacrylamide gel (Plate 10b; gel 4). This was also confirmed by Lockridge et al (1979).

Lockridge et al (1987) proposed that serum cholinesterase consisted of four identical subunits held together by covalent bonds. On SDS gels the resultant bands from pure cholinesterase appeared to be due to monomers and dimers. This degree of analytical process would require amounts of plasma vastly in excess of that available in a clinical situation.

The small amount of cholinesterase in human plasma may also be one of the reasons for the unsuccessful attempts by other workers in this laboratory to stain for aspirin esterase activity (Wood and Williams, personal communication) using thioaspirin substrate (White and Hope, 1981).

The purified human cholinesterase from Sigma Chemical Co had a purification factor of approximately 50-fold based on a plasma protein content of 68 mg/ml. This was almost twice the value I achieved (Table 10.3), although not nearly as pure as that acheived by affinity chromatography (Lockridge and La Du, 1978).

CHAPTER 11

Blood Esterase Activity Measurements in Young; Fit and Frail Elderly

Chapter 11

Blood Esterase Activity Measurements in Young, Fit and Frail Elderly

11.1 Introduction

The aim of this section of work was to establish normal ranges of several blood esterase activities in the young; fit and frail elderly. The esterases of interest included: red blood cell acetylcholinesterase and arylesterase and the plasma esterases: aspirin esterase, cholinesterase, phenylacetate esterase and paraoxonase. There are existing literature levels for aspirin esterase and cholinesterase (Williams et al, 1989) but other established ranges using the same methods have been only in fit, young people (Mutch et al, in press).

11.1.1 Plasma Aspirin Esterase and Cholinesterase Activity

The range of activity in young; fit and frail elderly has already been established earlier in this thesis. The fit elderly had a range of plasma aspirin esterase activity (nmol salicylate/ml plasma/min): 93.7 - 172.8, mean $\pm$ SEM 119.2 $\pm$ 10.3, n = 7; frail elderly: 66.6 - 112.4, mean $\pm$SEM 88.3 $\pm$ 5.5, n = 10 and young: 93.7 - 193.8, mean $\pm$ SEM 114.5 $\pm$ 9.2, n = 10. Williams et al (1989) also established ranges using larger numbers of individuals and these results are summarized in Table 11.4.

The assay for cholinesterase activity described in Chapter 8 of this thesis was carried out at 37oC, that by Williams et al (1989) at 25oC and the measurements in this section at 30oC. For this reason it was necessary to use the temperature correction factor advocated by King (1965) in order to allow a direct comparison

of all three groups of data. The results I obtained for all three groups at 37oC are as follows: (nmol benzoylcholine chloride/ml plasma/min. fit elderly) 1345 - 2410, mean ± SEM 1731 ± 139, n = 7; frail elderly: 480 - 1570, mean 1183 ± 145, n = 7 and young: 990 - 2435, mean ± SEM 1666 ± 140, n = 9.

11.2 General Methods

11.2.1 Selection of Subjects

These were recruited by Dr H Wynne (Consultant in Geriatric Medicine). Frail elderly were selected from local long-stay geriatric hospitals, the fit elderly from local social clubs and the young from hospital and University colleagues, as described in Chapter 8.

All the samples were treated as soon as possible after collection as described in Chapter 8. In addition, for these series of experiments, the red blood cells were also used. This is described in 11.3.1 .

11.2.2 Storage of Samples

The samples required storage for a reasonably short period of time (maximum of two months). During this time it is essential that the original esterase activity was maintained. Turner et al (1984) found plasma cholinesterase was retained for more than twelve months when stored at -20oC, and thus plasma aspirin esterase activity will also be stable for this period.

Mueller et al (1983) found plasma paraoxonase activity was retained for up to

one year at -70oC. Reiner et al (1989) showed plasma phenylacetate activity was stable at -15oC for at least seven weeks.

The stability of the red blood cell esterases needed to be established and this was done by comparing the activity of fresh cell preparation with aliquots stored at -80oC over a few days.

11.2.3 Linearity of the Reactions

For enzyme mediated reactions it is necessary to confirm that optimal conditions are maintained throughout the time period of product formation and correlation of the regression line is continually monitored.

Linearity with time could be visually judged in those assays which involved the continual monitoring of product formation. Red blood cell esterase activity was measured by an end point method and thus a time curve was necessary to confirm linearity.

Protein concentration in the incubation was varied as indicated in the corresponding graphs and all other conditions were kept constant.

Substrate activity curves were also executed. These confirmed that activity measurements were taking place at the maximal enzyme rate. Thus product formation would minimally reduce substrate concentration.

11.3 Red Blood Cell Acetylcholinesterase

The method used is a modification of the Ellman reaction (Ellman et al, 1961) by Kaplan (1964).

The technique uses the specificity of acetylcholinesterase for acetylthiocholine which is an analogue of the natural substrate. Thiocholine is released when acetylthiocholine is hydrolysed. This continually reacts with the colour reagent 5:5-dithobis-2-nitrobenzoate. The yellow colour is due to the action 5-thio-2-nitrobenzoic acid, the rate of formation of which is measured at 412 nm on a uv spectrophotometer.

11.3.1 Treatment of Blood Sample

The blood was collected and treated as usual.

The washed red cells were resuspended in an equal volume of saline. This was mixed well and a small sample removed for determination of the red cell count and haemoglobin concentration (Department of Haematology, Royal Victoria Infirmary, Newcastle Upon Tyne). From the remainder, 20 µl was removed using a positive displacement pipette and added to 4.98 ml distilled water. This was vortexed and frozen at -80oC until solid. On removal it was allowed to thaw at room temperature and then 5.0 ml of 0.2 M phosphate buffer pH 8.0 was added. This was vortexed again and stored at -80oC until analysis within 24 hours. (Mutch et al, in press).

11.3.2 Method

Reagents

0.2 M phosphate buffer pH 8.0
0.1 M phosphate buffer pH 8.0
0.1 M phosphate buffer pH 7.0

6.5 mg/10 ml physostigmine sulphate

Colour Reagents

5:5-dithiobis-2-nitrobenzoic acid (DTNB)

DTNB 39.6 mg } 10 ml phosphate buffer pH 7.0
NaHCO3 15 mg }

Substrate:

Acetylthiocholine iodide (ACTH) 130.2 mg in 6 ml water containing 60 l HCl

Plasma esterase inhibitor:

Quinidine sulphate 1 mg/ml

Combined Reagent:

ACTH 6 ml
DTNB 7.5 ml
Quinidine Sulphate 3.0 ml

Mixed well, aliquoted and stored at -20oC

Assay

The blood preparation was allowed to thaw at room temperature. The incubation mixture in the spectrophotometer cuvette consisted of 1.95 ml 0.1 M phosphate buffer pH 8.0 and 1 ml of lyzed, diluted red blood cells. A parallel blank

consisted of 1.85 ml buffer, 1 ml lyzed, diluted red blood cells and 100 µ l of physostigmine sulphate solution. 50 ul of combined reagent was added to each cuvette which were inverted to mix.

The rate of formation of 5-thio-2-nitrobenzoic acid was measured at 412 nm on a uv spectrophotometer at 30oC. The results were expressed as nmol thiocholine/mg haemoglobin /min. The reaction was shown to be linear with protein (Fig 11.1) and the activity is measured at maximum enzyme activity (Fig 11.2).

11.4 Plasma Paraoxonase

Paraoxonase, which hydrolyses the organophosphate paraoxon and has been thought to be the cysteine containing A esterase which hydrolyses phenylacetate and similar aromatic esters (Eckerson et al, 1983b). However Mackness et al (1987a) maintain that paraoxonase and arylesterase activities can be separated and have done so using avian plasma.

11.4.1 Assay

The substrate is paraoxon which, as an organophosphate requires very careful handling. It was essential to wear gloves at all times. The reagent was prepared for use by aliquoting an appropriate amount into dry acetone to a final concentration of 103 µmol/ml. 2.92 ml 50 mM glycine buffer containing 1 mM calcium chloride, 50 µ l plasma and 30 µl of stock paraoxon was added. This was incubated at 30oC and the rate of formation of yellow paranitrophenol

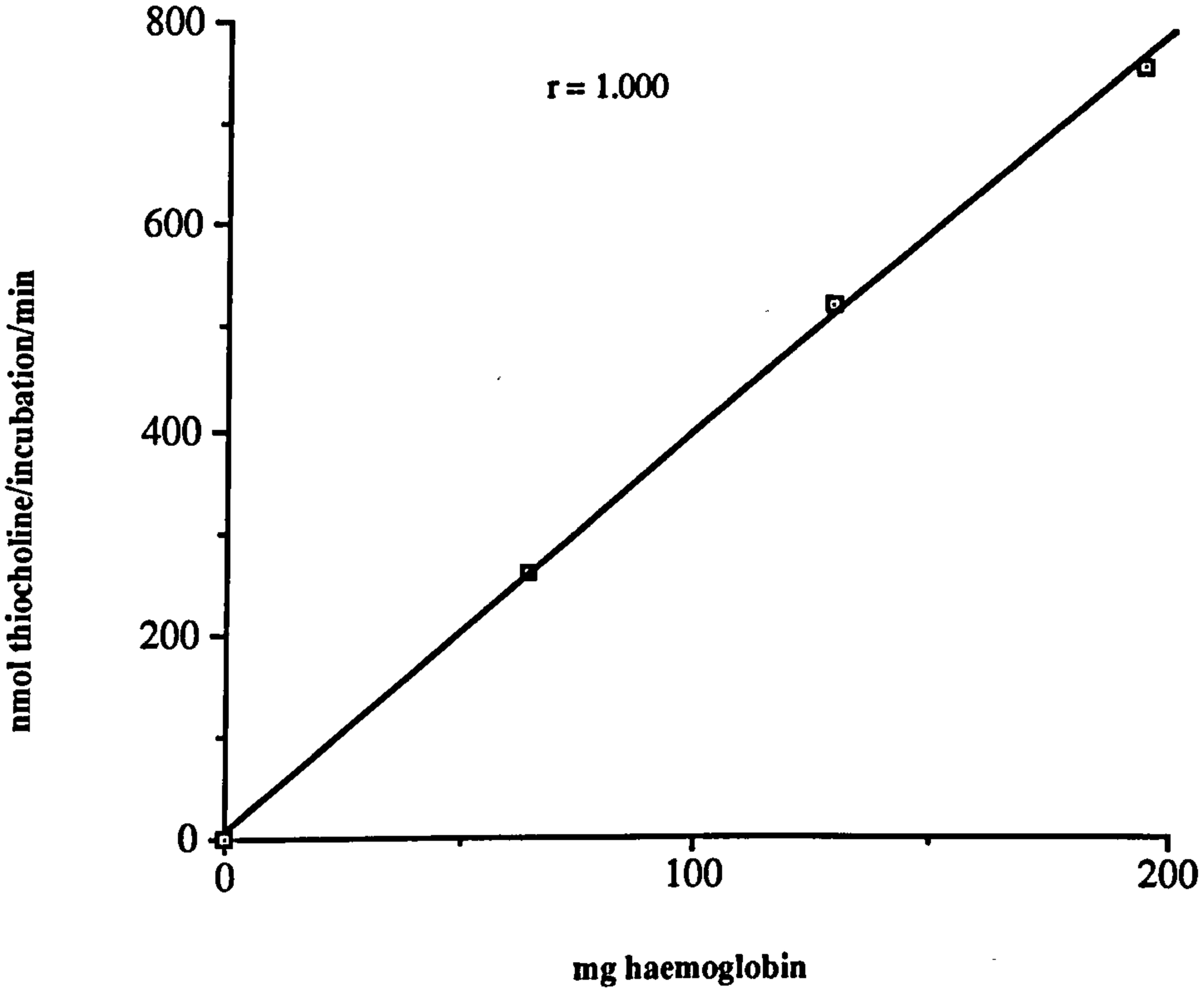


Figure 11.1

Title: The rate of formation of thiocholine as measured by the appearance of the yellow 5-thio-2-nitrobenzoic acid complex; the effect of increasing the amount of haemoglobin in the final incubation

Ordinate: nmol thiocholine produced per incubation over a 1 min. time period at 30°C.

Abscissa: amount of haemoglobin added as washed and lyzed red blood cells

The values are the mean of two determinations. The solid line is the least square regression line.

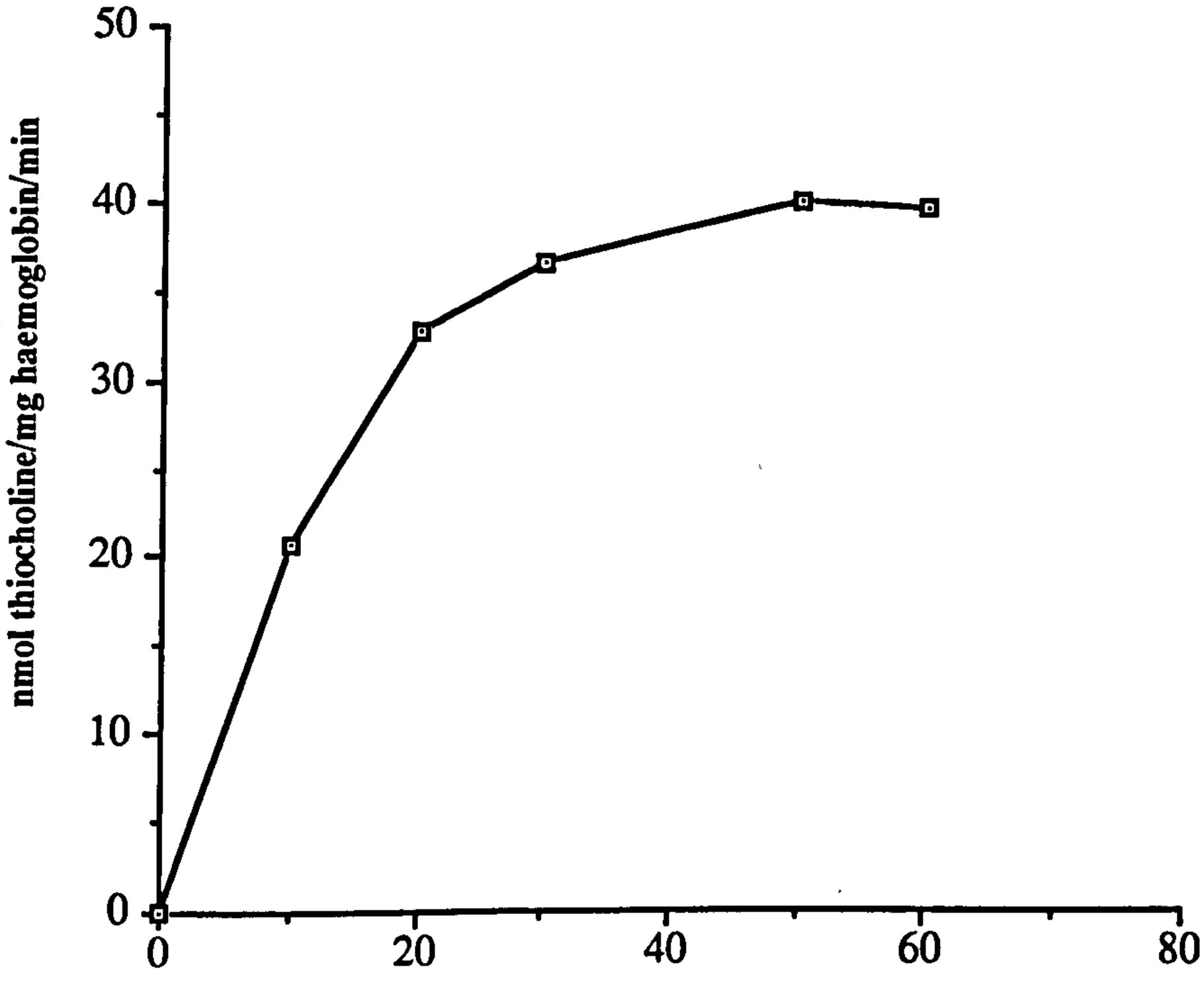


Figure 11.2

Title: The rate of formation of thiocholine as measured by the appearance of the yellow 5-thio-2-nitrobenzoic acid complex; the effect of increasing substrate concentration in the final incubation

Ordinate: nmol thiocholine produced per mg haemoglobin over a 1 min. time period at 30°C.

Abscissa: amount of combined agent (substrate and colour reagent) added.

The values are the mean of two determinations.

was continually monitored for a 2.5 minute period at 412 nm on a uv spectrophotometer. This was carried out in duplicate and a blank was incubated in parallel to correct for the spontaneous hydrolysis of paraoxon. This did not exceed 20% of enzymatic hydrolysis. The results were expressed as nmol paranitrophenol produced/ml plasma/min:

The reaction was shown to be linear with protein (Fig 11.3) and the activity was measured at maximum enzyme activity (Fig 11.4).

The interassay CV was 1% (n = 3; at a plasma paraoxonase activity of 97.4 nmol paranitrophenol produced/ml plasma/min).

11.5 Plasma Phenylacetate Esterase

Phenylacetate is a substrate for plasma arylesterase activity in human plasma. The method used is a modification of that described by Williams et al (1986) and involves the continuous monitoring of phenol formation.

11.5.1 Assay

3.0 ml of Tris - HCl buffer pH 8.0 containing 10 mM Calcium chloride, 5 µl plasma and 10 µl 0.2 mM physostigmine were mixed and incubated in triplicate at 30oC in a spectrophotometer cuvette. 15 µl of phenylacetate (600 mM) in dimethylsulphoxide was added to start the reaction. The formation of phenol was monitored at 272 nm on a uv spectrophotometer over a one minute time period.

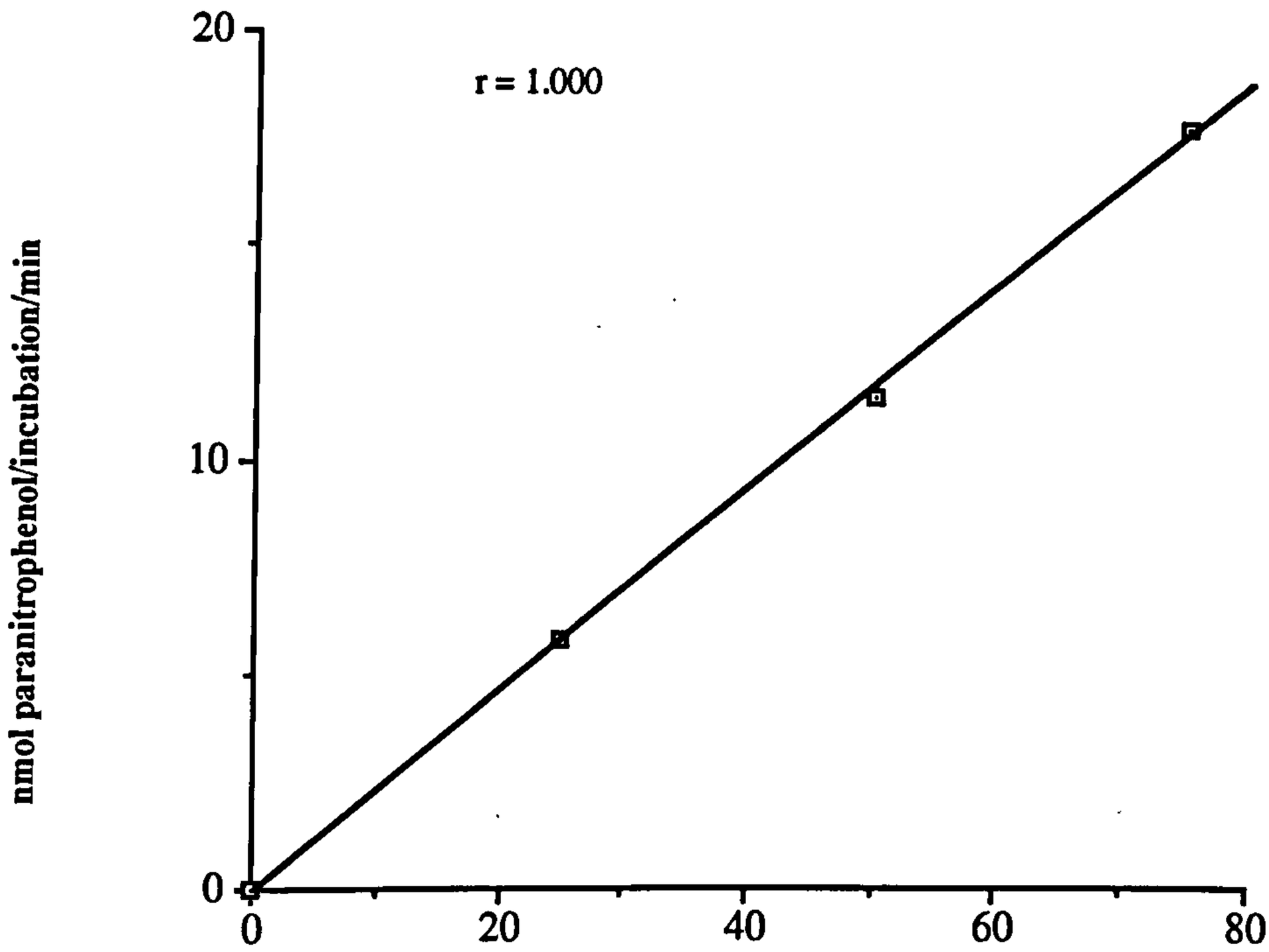


Figure 11.3

Title: The formation of paranitrophenol: the effect of increasing plasma concentration in the final incubation volume at a paraoxon concentration of 1mM.

Ordinate: the amount of paranitrophenol formed over a 1 min. time period at a paraoxon concentration of 1mM and at 30°C.

Abscissa: volume of plasma in incubation

The values are the mean of two determinations. The solid line is the least square regression line

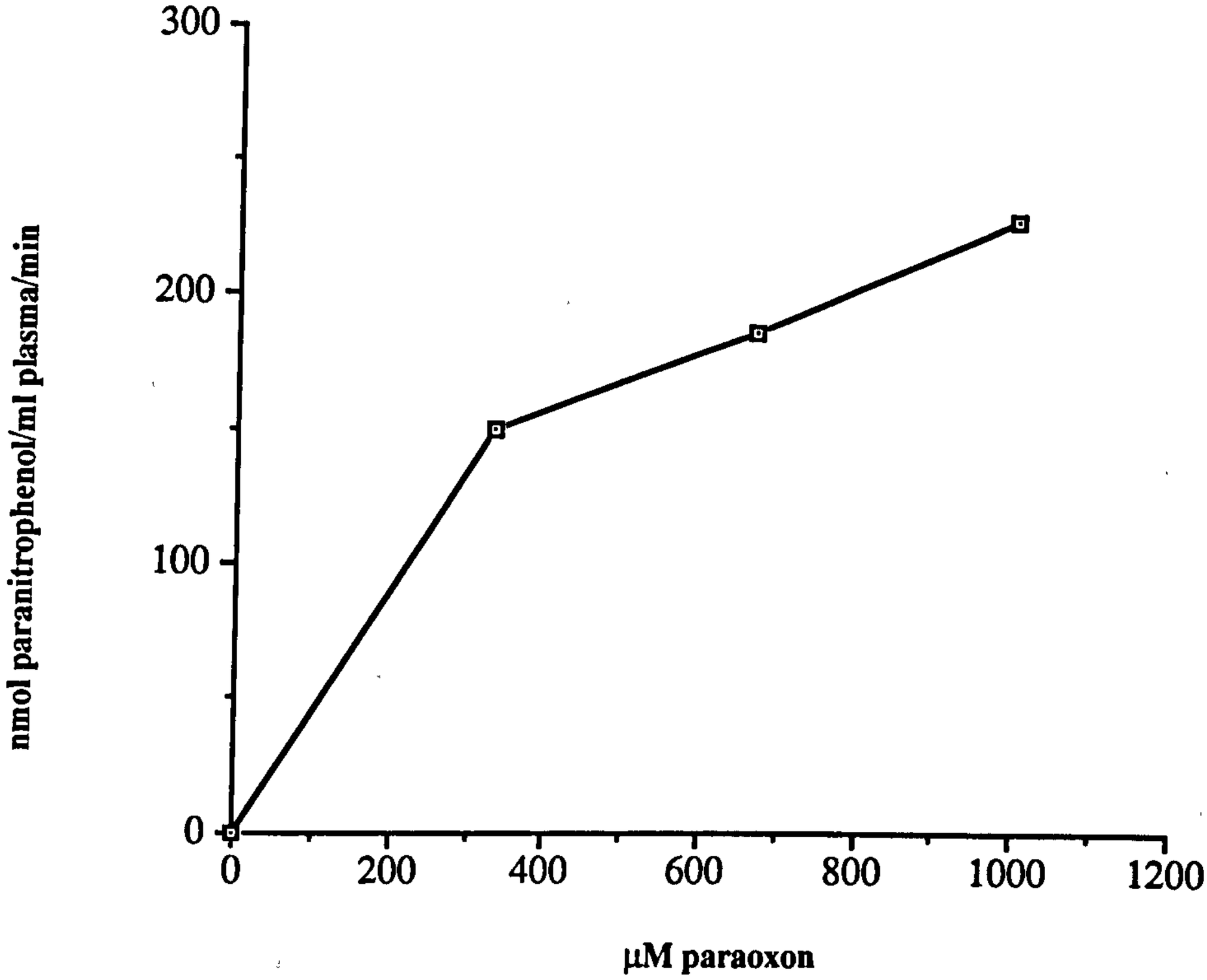


Figure 11.4

Title: The formation of paranitrophenol when incubated with 50μl plasma at 30°C: the effect of varying substrate concentration.

Ordinate: nmol paranitrophenol produced over a 1 min. time period by 50μl plasma and at 30°C.

Abscissa: concentration of paraoxon (μM)

The values are the mean of two determinations

Physostigmine was added in order to inhibit any hydrolysis of phenylacetate by cholinesterase which I found occured in purified plasma fractions. In fact there was no convincing measurable decline in activity.

The presence of calcium ions ensures maximal activity of the esterase (La Du and Snady, 1971). The reaction was linear with protein (Fig 11.5) and was measured at maximum enzyme activity (Fig 11.6). The results were expressed as nmol phenol produced/ml plasma/min. The interassay CV was 2.2% (n = 4; at a plasma phenylacetate esterase activity of 106.8 nmol phenol produced/ml plasma/min).

11.6 Red Blood Cell Esterase Activity

Unlike acetylcholinesterase, red blood cell aspirin esterase is an intracellular enzyme. Rylance and Wallace (1981) demonstrated the high aspirin esterase activity of intact red blood cells with red cell stroma.

Costello and Green (1983) identified a RBC hydrolase as the major determinant of *in vitro* aspirin survival in human blood and postulated that it was a non-specific arylesterase. Later work involving the correlation of *in vivo* aspirin survival with haemocrit in dogs confirmed the *in vitro* work (Costello and Green, 1984).

The method employed in this study uses phenylvalerate as substrate for the red blood cell hydrolase. Costello and Green (1983), using various inhibitors (sodium fluoride) and activators (metal ions), suggested that this arylesterase actually had some properties which belonged more to carboxylesterases. Thus it is highly probable that esterase activity measured by the hydrolysis

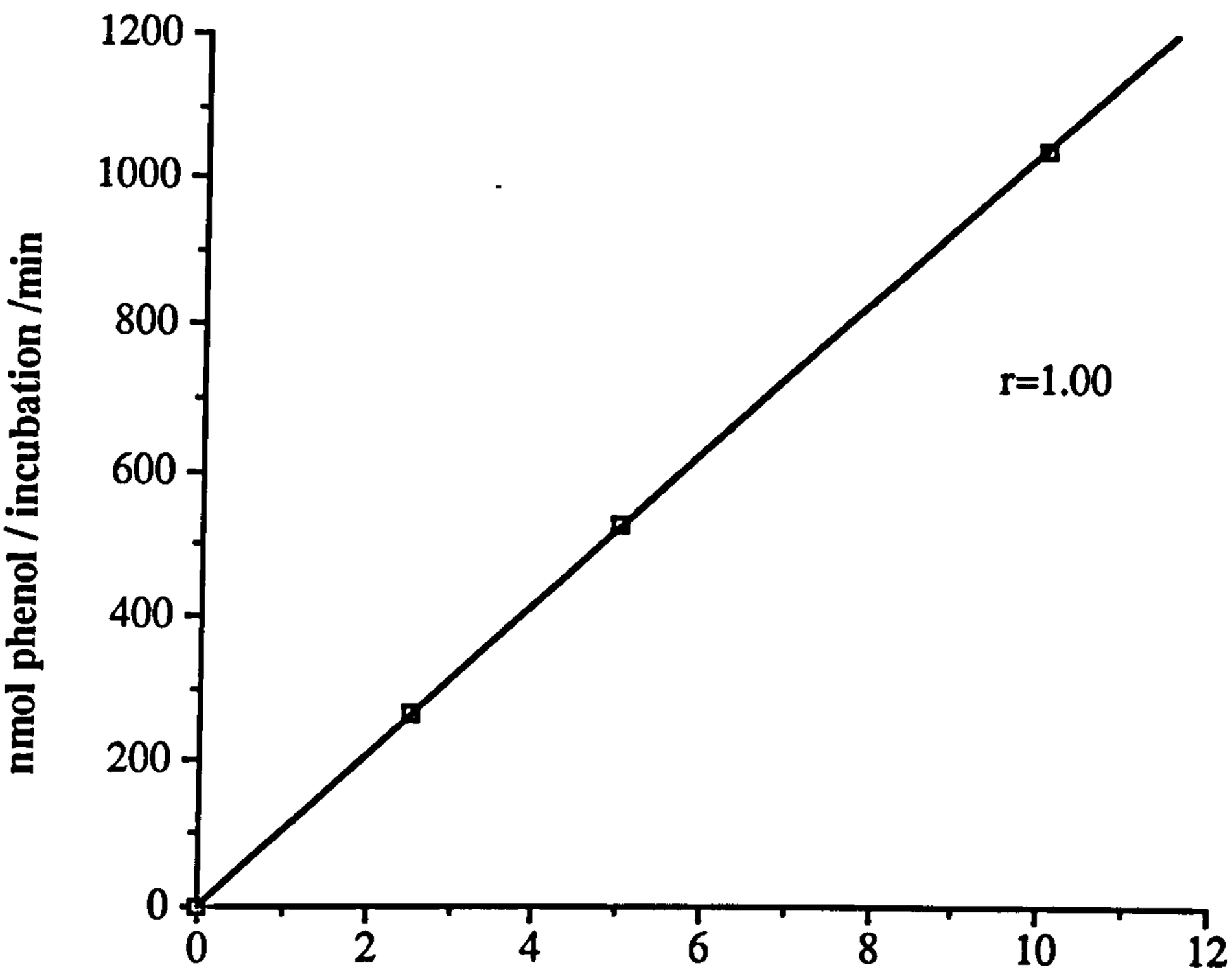


Figure 11.5

Title: The rate of formation of phenol: the effect of increasing plasma concentration in final incubation at a phenylacetate concentration of 3mM.

Ordinate: nmol phenol produced over a one minute time period at a phenylacetate concentration of 3mM and at 30°C.

Abscissa: amount of plasma in incubation.

The values are the mean of two determinations. The solid line is the least squares regression line

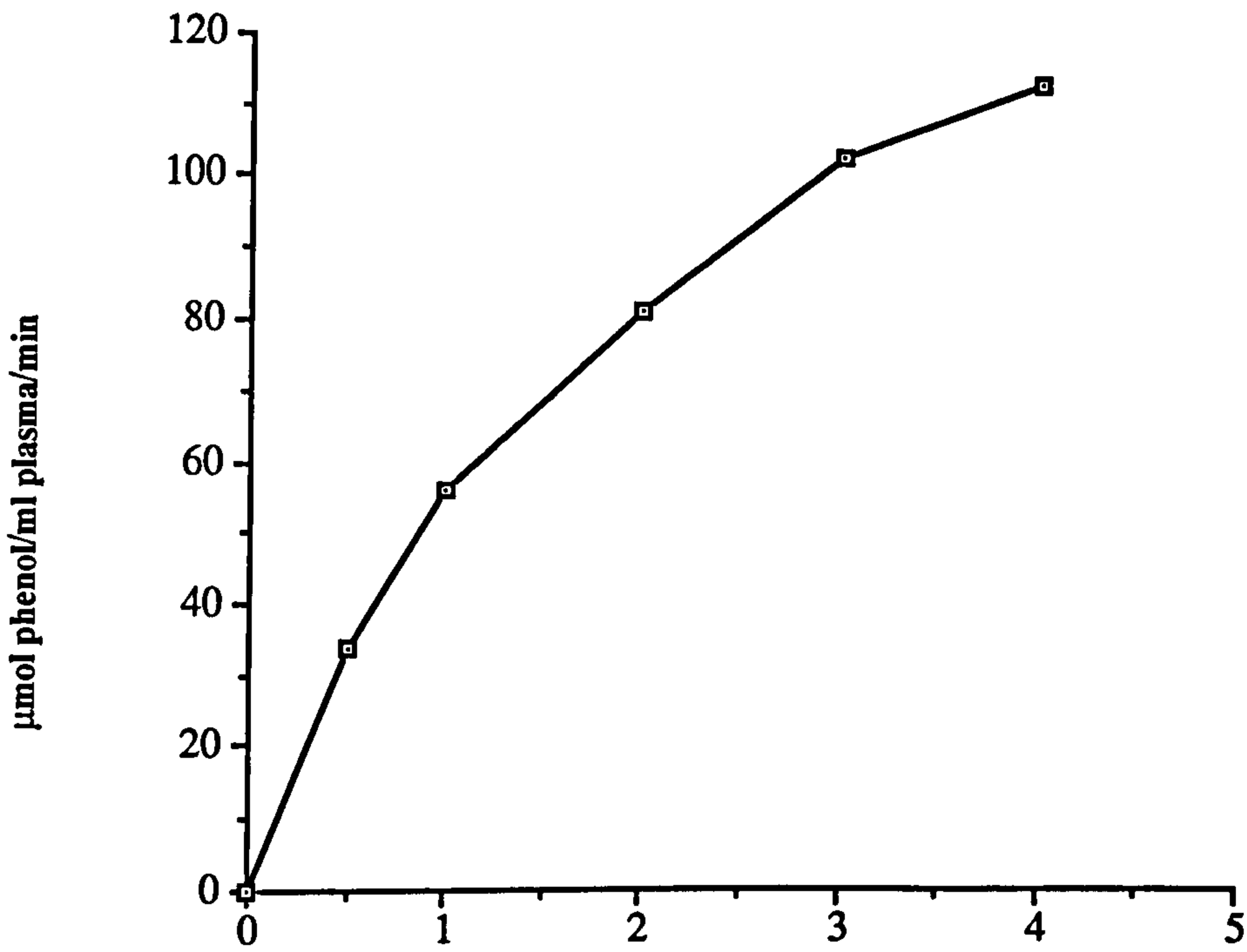


Figure 11.6

Title: The rate of formation of phenol when incubated with 5µl plasma; the effect of varying phenylacetate concentration

Ordinate: nmol phenol produced over a 1 min. time period by 5µl plasma at 30°C.

Abscissa: concentration of phenylacetate.

The values are the mean of two determinations

hydrolysis of phenylvalerate is an indirect measure of intracellular red blood cell aspirin esterase.

The assay uses the observation by Emerson (1943) that 4-aminoantipyrine condenses with aromatic aminos in the presence of acid oxidizing agents and with phenols (Gottlieb and Marsh, 1946). Thus a colour test for phenols was developed. The proposed structure of the dye formed during this reaction is shown in Fig 11.7. Substitution is thought to occur in the para-position to the phenolic - OH group (Emerson, 1943).

In this assay acetylcholinesterase activity was inhibited with physostigmine. Attempts were made to measure aspirin esterase activity directly using aspirin substrate and analysis of the supernatant following incubation, precipitation of the protein and High Perfomance Liquid Chromatography of the sample. However this proved unsuccessful due to poor duplication.

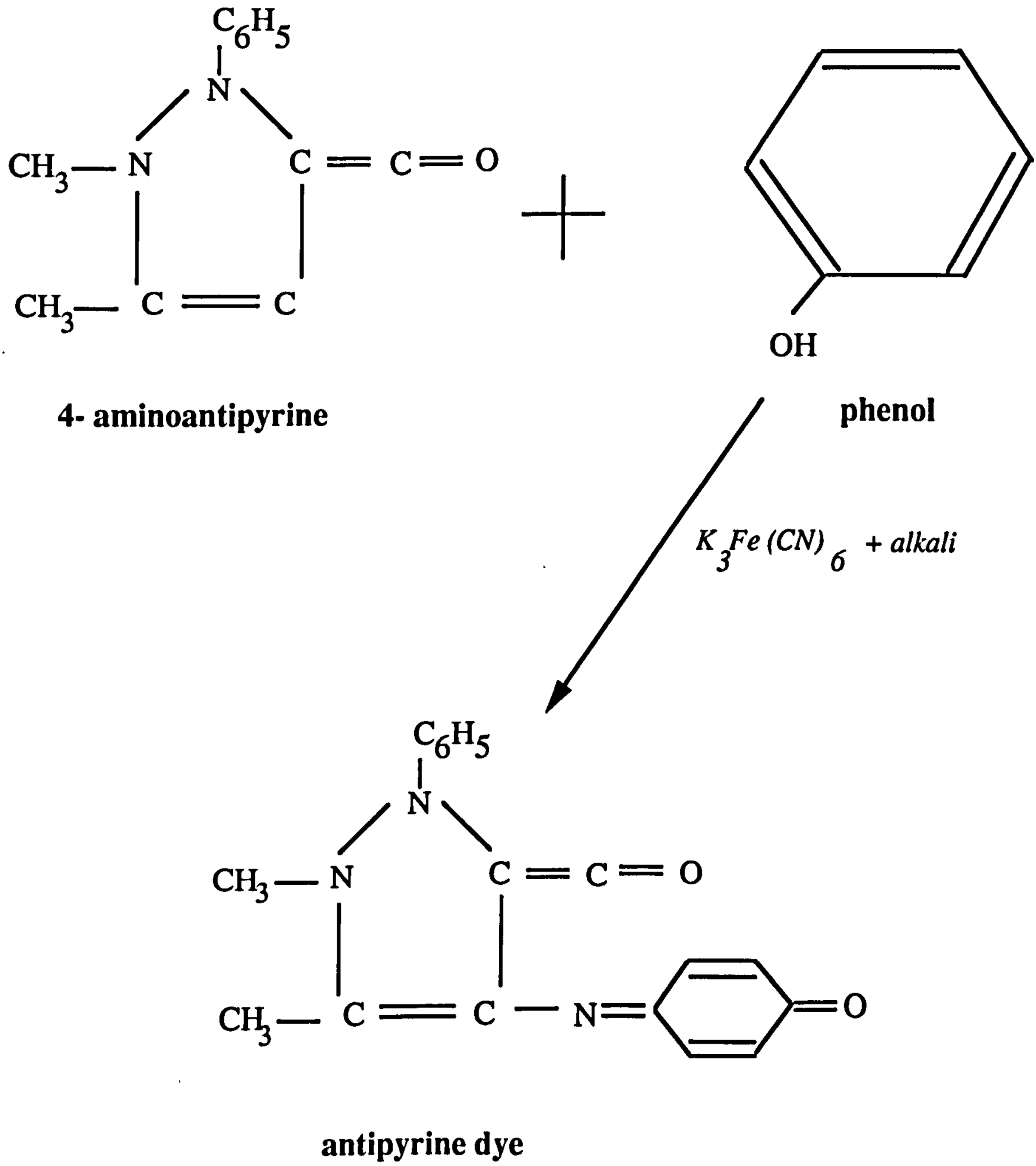
11.6.1 Method

Preparation of Patient Samples

The red cells were prepared and washed as in the method for Red Blood Cell Acetylcholinesterase (see 11.3). The washed red cells were aliquoted and frozen at -80oC until analysis within 24 hours. An aliquot was also sent to The Department of Haematology, RVI to obtain the red cell count.

Freezing lyzes the cells and releases the intracellular esterase.

Figure 11.7 Formation of the Red Antipyrine Dye



Reagents

Phenylvalerate 15 mg/ml in redistilled dimethylformamide

0.3% Triton X-100 in water

50 mM Tris-HCl with 0.2 mM EDTA buffer pH 8.0

1.05% sodium dodecylsulphate (SDS) in the above

0.5% 4-aminoantipyrine (AAP) in 0.5 M Tris buffer pH 9.0

0.4% Potassium ferricyanide (K_3 Fe (CN)$_6$)

Working Solutions

(Freshly prepared for each assay).

Substrate

2 ml of phenylvalerate (PhV) is added to 29 ml triton stock solution. Final concentration is 1 mg in 3.5 ml incubation volume.

Reaction Inhibitor

2 ml AAP is added to 18 ml Tris-SDS buffer.

Colour Developer

1 ml of 0.4% K_3 Fe (CN)$_6$ is added to 9 ml H_2O.

Assay

The red cell aliquot was diluted 1 in 2 with 50 mM Tris-HCl containing 0.2 mM EDTA pH 8.0. 25 µl of this preparation was dispensed into a tube using a positive displacement pipette, 10 µl of physostigmine in saline to give a final concentration of 2 mM was also added and the volume made up to 1 ml with Tris-HCl and EDTA buffer. This was repeated in duplicate (A). In a second pair of tubes all but the red cell aliquot was added (B), and the third pair of tubes (the blanks) contained the same as the first two at this stage (C). These six tubes were all required for measuring enzyme activity in one blood sample only.

These tubes were vortexed and preincubated at 37oC for 5 minutes with gentle shaking. 1.0 ml of 30% Triton-X was added to the blank tubes (C). 1.0 ml of substrate was added to tubes (A) and (B), these were vortexed and returned to the water bath at 37oC for gently shaking for 25 minutes.

After 25 minutes, the tubes were removed and placed on ice. The reaction was stopped by adding 1.0 ml reaction inhibitor. The 25 µl of 1 in 2 red cell preparation was added to tube (B). This measured the spontaneous hydrolysis of phenylvalerate at 37oC for 25 minutes and the addition of the red cell aliquot at this stage ensured the reaction had stopped before enzyme in the red cells was added. This was vortexed thoroughly.

The colour developer was added as a 0.5 ml aliquot and the tubes were vortexed a final time.

The colour change was measured in a direct reading uv spectrophotometer at

510 nm against the blank tubes. A QC for enzyme activity is not possible in this particular assay because the enzyme activity appears to deteriorate with time.

A typical standard curve is shown in Fig 11.8. The results were expressed as nmol phenol produced/10^6 RBC/min.

The reaction was shown to be linear with time at the lowest substrate concentration (Fig 11.9) and linear with protein at phenylvalerate concentrations of 0.05 mg (Fig 11.10), 1 mg (Fig 11.11) and 2 mg (Fig 11.12). A typical substrate curve is shown in Fig 11.13.

Standard Curve

A stock solution of 1 mM phenol in water was prepared. 125 µl, 250 µl, 500 µl of phenol stock solution was pipetted into a series of tubes to contain 125, 250 and 500 nmol of phenol respectively. This was made up to volume with water 1.5 ml 50 mM Tris-HCl with EDTA was added to each tube and mixed, then 1.0 ml of reaction inhibitor was added and mixed. Finally 0.5 ml colour developer was added and mixed. The colour intensity was measured against a blank containing no phenol at 510 nm in a uv spectrophotometer.

11.7 Results

The patient details are summarized in Table 11.1. The frail elderly were receiving more regular medication than the fit elderly or young.

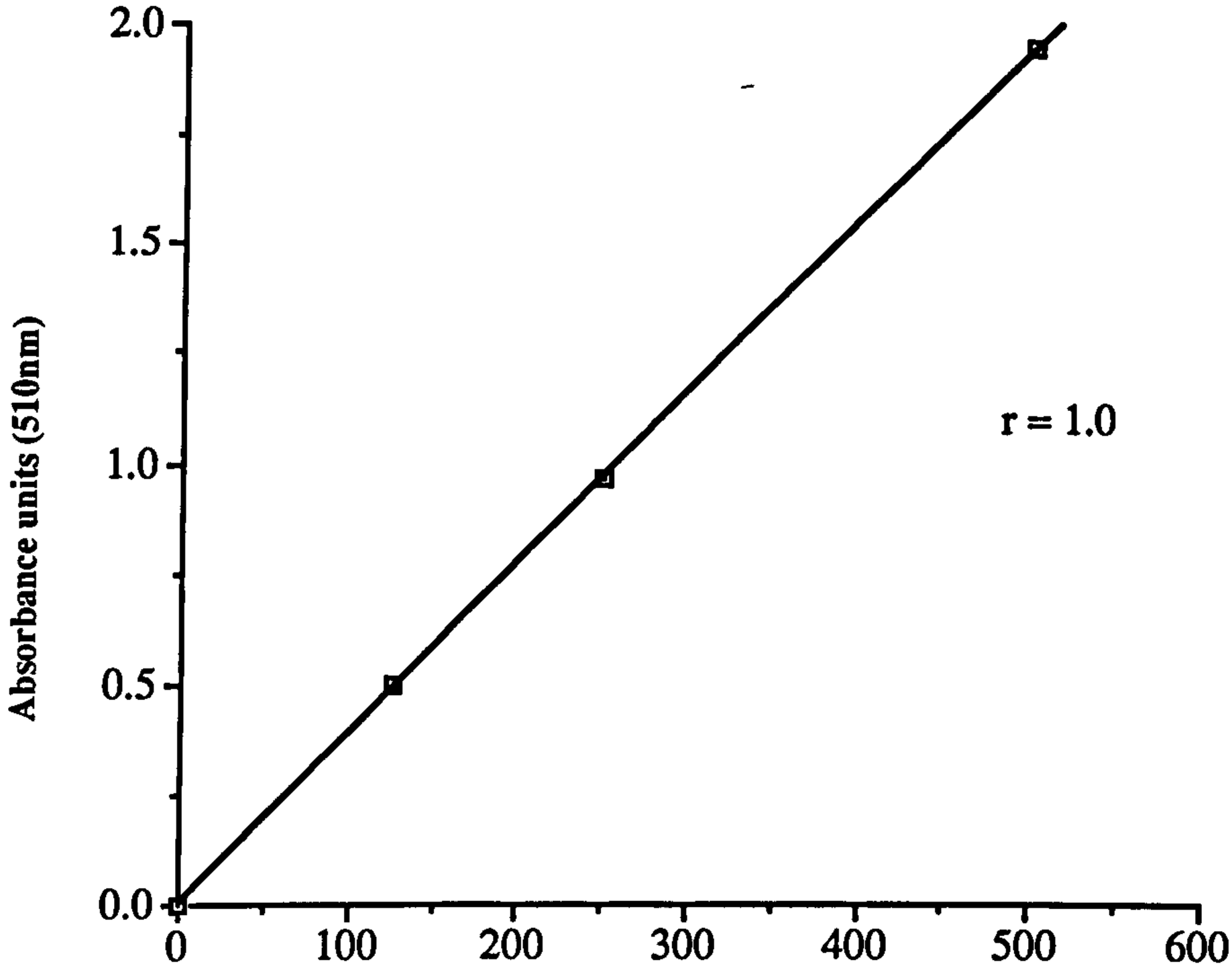


Figure 11.8

Title: Phenol standard curve

Ordinate: Absorbance units at 510nm.

Abscissa: nmol phenol/incubation volume (3.5ml)

The values are the mean of two determinations.
The solid line is the least square regression line.

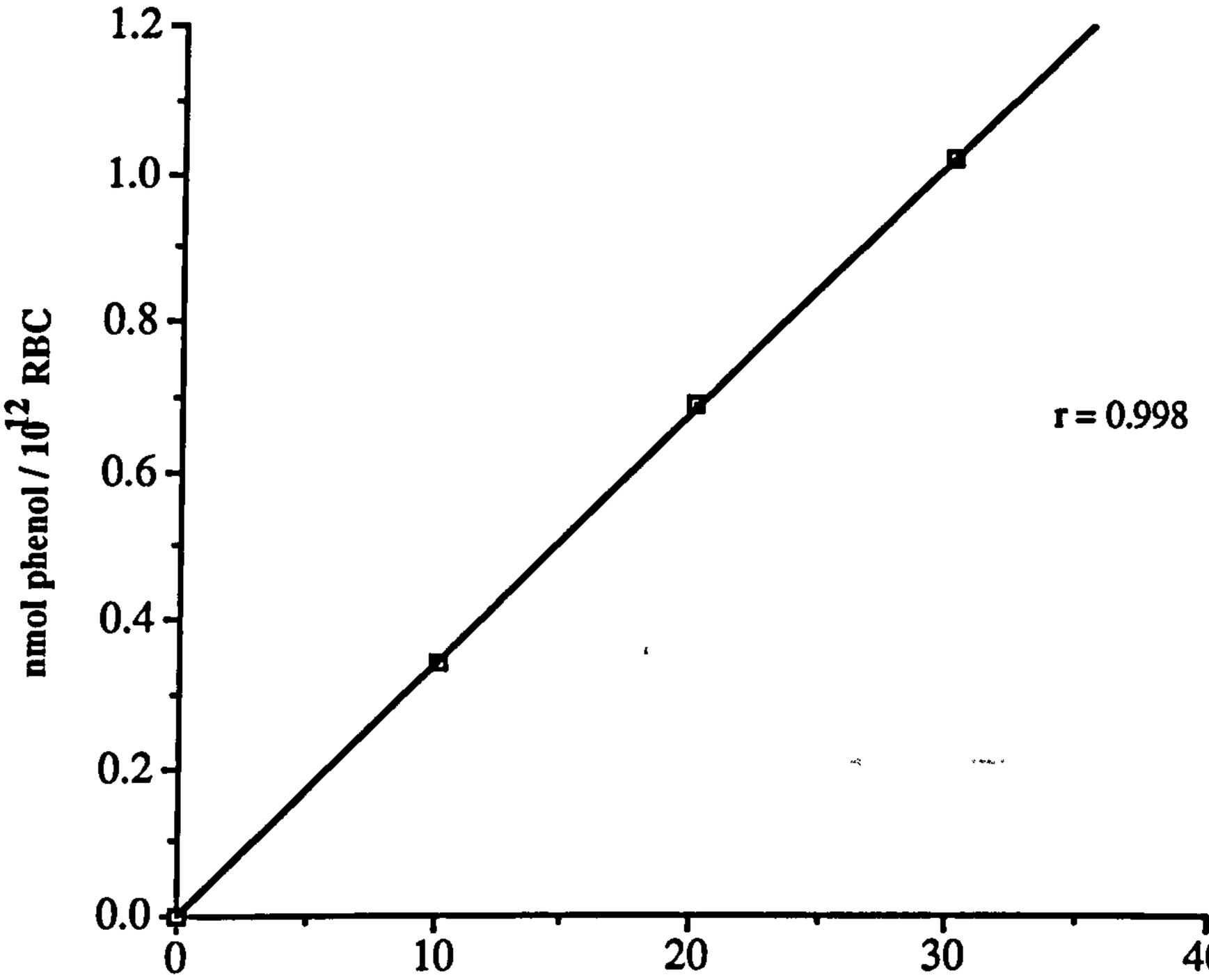


Figure 11.9

Title: The formation of phenol by red blood cells: the effect of increasing incubation time at a phenylvalerate concentration of 0.05mg/3.5ml and at 37°C

Ordinate: the amount of phenol formed by 10^{12} red blood cells at a phenylvalerate concentration of 0.05mg and at 37°C.

Abscissa: incubation time in minutes

The values are the mean of two determinations. The solid line is the least square regression line.

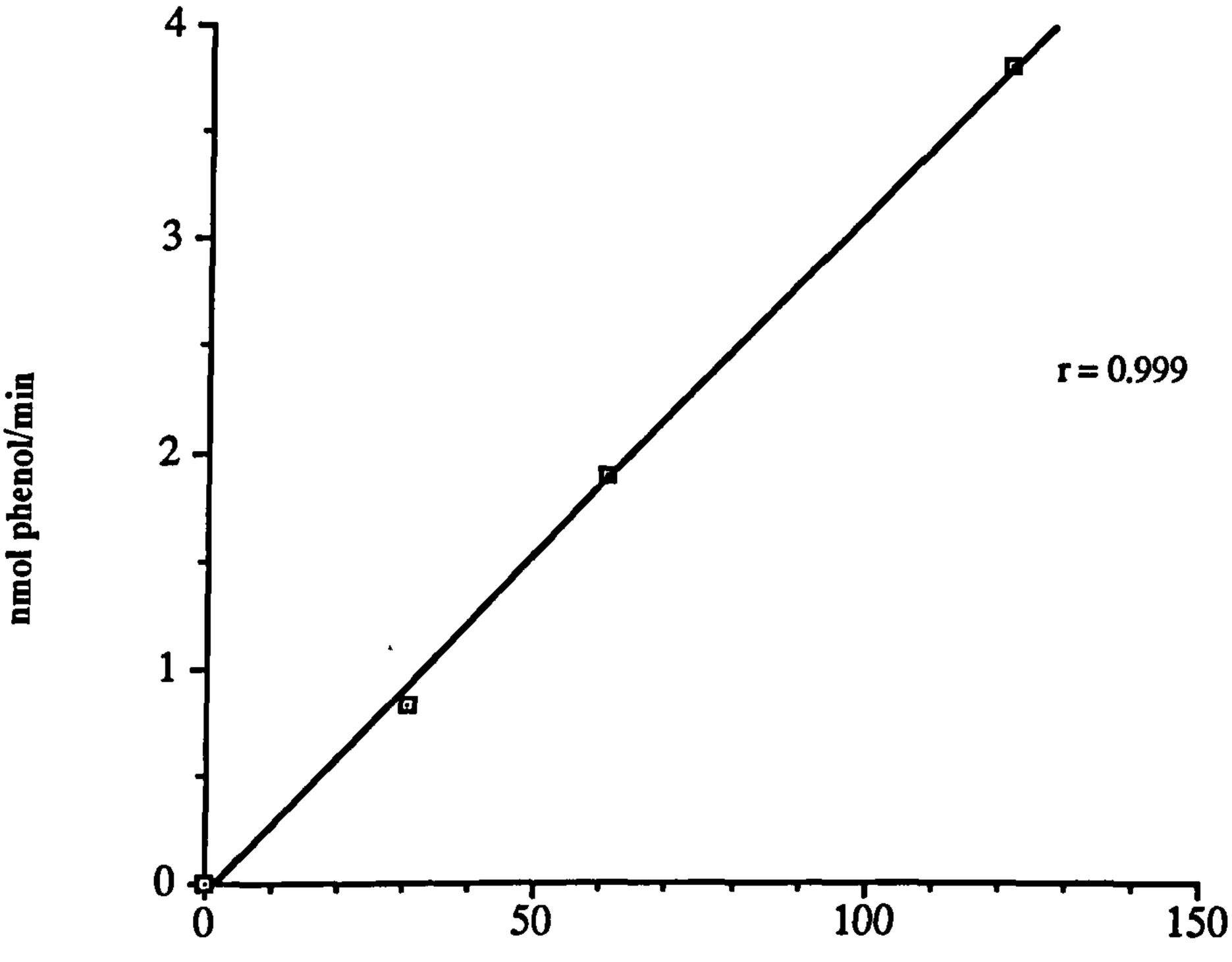


Figure 11.10

Title: The formation of phenol by red blood cells: the effect of increasing the number of red blood cells in the final incubation at a phenylvalerate concentration of 0.05mg/3.5ml

Ordinate: the amount of phenol formed per incubation over a 1 min. time period and at 37°C.

Abscissa: the number of red blood cells per incubation

The values are the mean of two determinations. The solid line is the least square regression line.

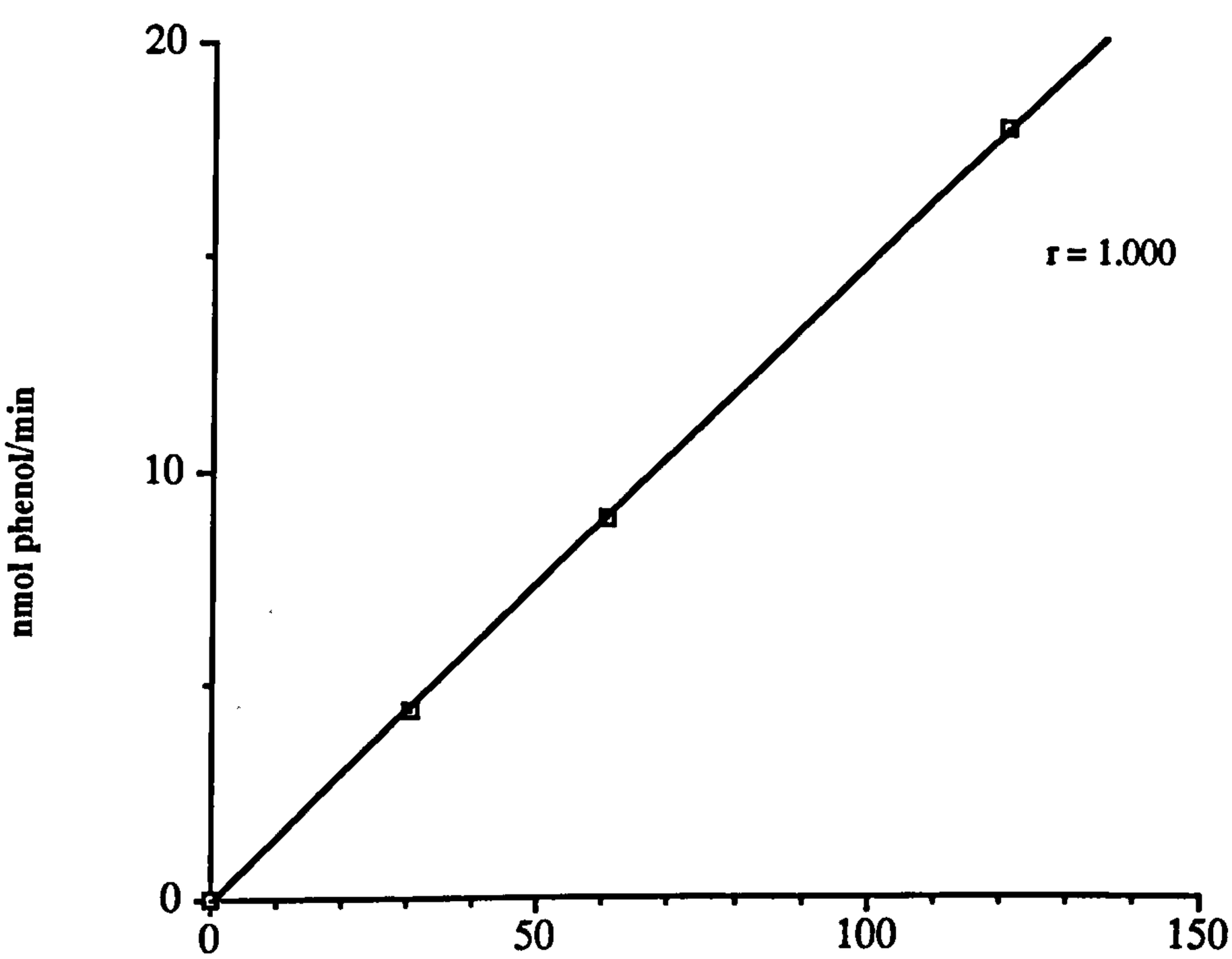


Figure 11.11

Title: The formation of phenol by red blood cells: the effect of increasing the number of red blood cells in the final incubation at a phenylvalerate concentration of 1mg/3.5ml

Ordinate: the amount of phenol formed per incubation over a 1 min. time period at 37°C.

Abscissa: the number of red blood cells per incubation

The values are the mean of two determinations. The solid line is the least square regression line.

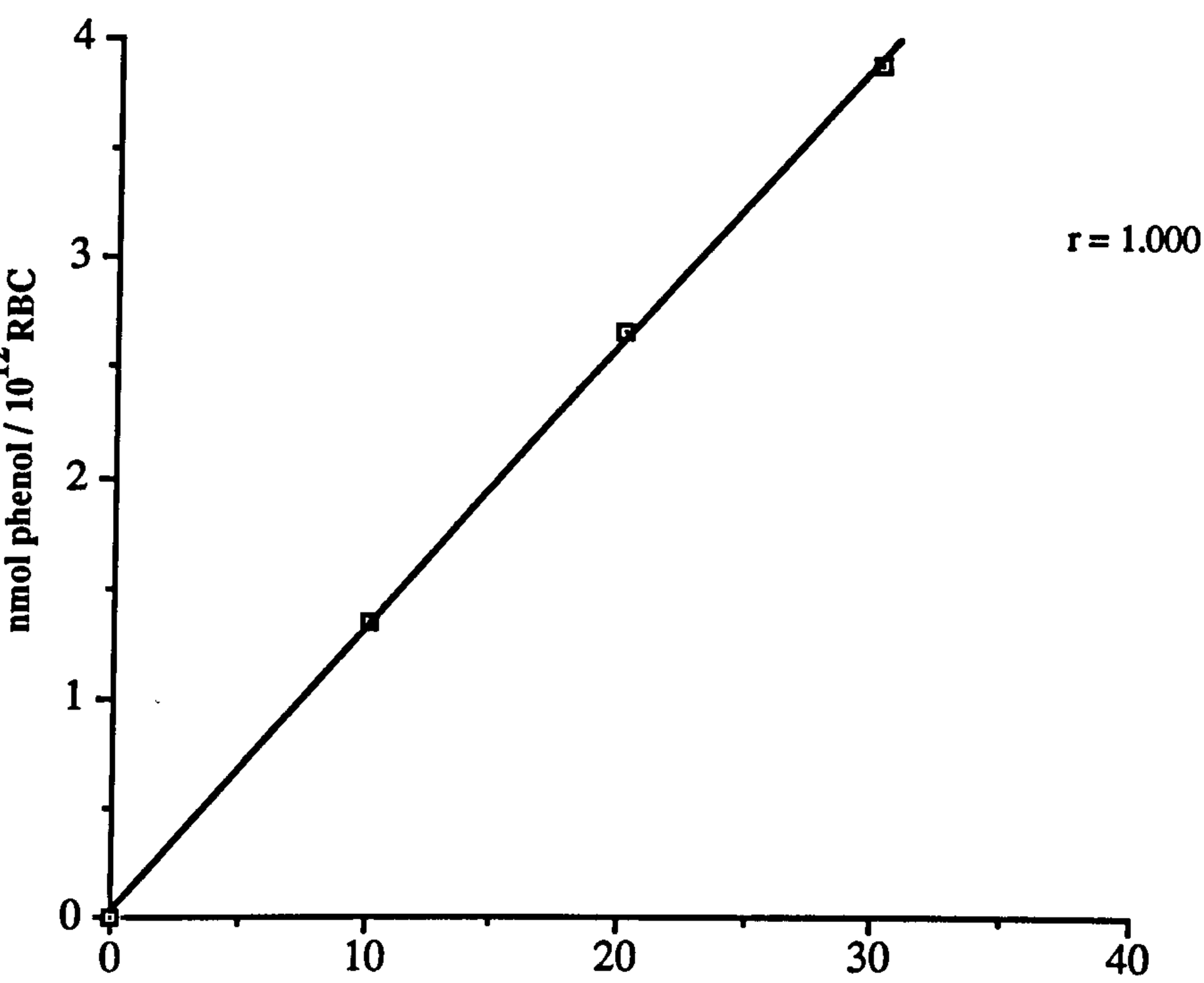


Figure 11.12

Title: The formation of phenol by red blood cells: the effect of increasing incubation time at a phenylvalerate concentration of 2mg /3.5ml and at 37°C

Ordinate: the amount of phenol formed by 10^{12} red blood cells at a phenylvalerate concentration of 2mg and at 37°C.

Abscissa: incubation time.

The values are the mean of two determinations. The solid line is the least square regression line.

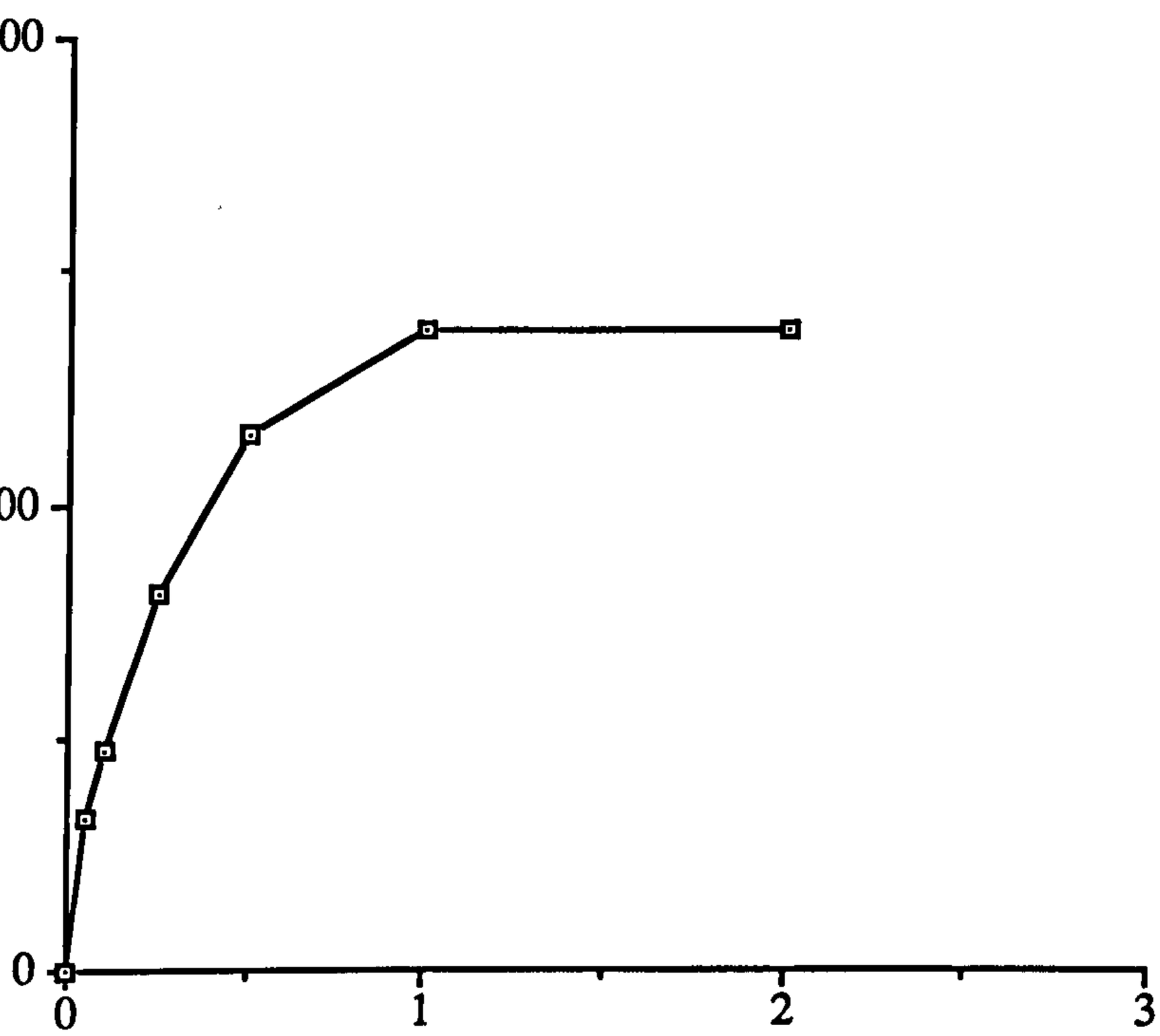


Figure 11.13

Title: The formation of phenol when incubated with an aliquot of washed and lyzed red blood cells at 37°C; the effect of varying substrate concentration.

Ordinate: nmol of phenol produced over a 1 min. time period by an aliquot of washed red cells at 37°C.

Abscissa: concentration of phenylvalerate (mg/3.5ml)

The values are the mean of two determinations.

Table 11.1

Volunteer Details

Volunteer (sex) Frail Elderly	Age (years)	Albumin (g/l)	Drugs (if any)
1(F)	93	34	dothiepin, codeine phosphate,
2(M)	88	34	
3(F)	84	35	frusemide ,salbutamol, tamoxifen, lactulose, haloperidol,chlormethiazole, carbamazepine, thioridazine
4(F)	81	33	-
5(F)	85	39	-
6(F)	88	29	docusate, bendrofluazide,chlormethiazole, morphine sulphate
7(F)	88	33	-
8(F)	86	37	chlormethiazole
9(F)	81	35	-
10(F)	88	30	paracetamol, codeine phosphate
11(F)	85	33	dothiepin, diazepam
12(F)	86	29	codeine phosphate , micralax enema
13(M)	70	36	-
14(M)	86	41	haloperidol, ranitidine, paracetamol, senna
15(M)	90	38	paracetamol ,senna
16(F)	91	37	dothiepin, paracetamol, temazepam
17(F)	94	35	digoxin, pericycline, chlormethiazole
18(F)	95	36	ranitidine , paracetamol, ,senna
19(F)	85	38	ranitidine , paracetamol, senna
20(F)	96	37	-
Mean ± SEM	87±1.3	35.0±0.7	

Table 11.1 continued

Volunteer (sex) **Fit Elderly**	Age (years)	Albumin (g/l)	Drugs (if any)
21(M)	65	45	warfarin
22 (F)	91	35	
23(F)	86	-	digoxin, thyroxine
24(M)	73	-	
25(F)	68	-	
26(F)	74	39	
27(F)	80	44	
28(F)	71	38	
29(F)	84	44	
30(M)	60	44	amilodipine
31(F)	87	44	
32(F)	81	42	
33(F)	73	40	
34(F)	79	39	
35(F)	72	39	
36(F)	78	47	
37(M)	79	41	
38 (F)	84	43	
39(M)	70	46	
40(F)	75	40	
Mean $\pm$ SEM	76.5$\pm$1.7	41.8$\pm$0.8	

Table 11.1 continued

Volunteer (sex) **Young**	Age (years)
41(M)	23
42(F)	22
43(F)	22
44 (F)	23
45(F)	25
46(F)	21
47(F)	21
48(M)	21
49(M)	22
50(F)	24
51(M)	24
52(F)	22
53(F)	29
54(F)	28
55(F)	22
56(F)	28
57(F)	28
58(F)	27
59(F)	24
60(F)	26
61(F)	26
62(F)	28
Mean $\pm$SEM	24.2$\pm$0.6

Table 11.2 and 11.3 show individual esterase activities in detail and Table 11.4 is a summary of these results. The final column in Table 11.4 shows a comparison of the results I obtained for plasma aspirin esterase and cholinesterase in young and fit elderly and in frail elderly (Chapter 8) and the results of Williams et al (1989) using the temperature correction factors of King et al (1965) as appropriate. I obtained a much greater range of activities in a smaller group for both these enzymes than Williams et al (1989). My range of activity for plasma aspirin esterase in the frail elderly was 27.1 - 97.9 (mean = 64 nmol salicylate/ml plasma/min, n = 20) compared with 75.1 - 83.0 (mean = 78.9 nmol salicylate/ml plasma/min, n = 43; Williams et al, 1989). The cholinesterase activities showed a similar profile where my range was 609-1863 nmol benzoylcholine chloride hydrolysed/ml plasma/min (mean= 1174, n=20) compared with that of Williams et al: 1523 - 1940 nmol benzoylcholine chloride hydrolysed/ml plasma/min (mean: 1731, n = 43). The lower mean value had major implications which will be discussed in Chapter 12. The plasma aspirin esterase and cholinesterase activities showed a significant correlation at $p < 0.001$ ($t = 6.8$) as shown in Table 11.3 and Fig 11.14 .

The paraoxonase and phenylacetate esterase activities in the frail elderly were significantly reduced at the 5% significance level (mean: 100 ± 7 and 65 ± 4 respectively) compared with the fit elderly (mean: 146 ± 9 and 83 ± 6 respectively) and the young (mean: 144 ± 10 and 99 ± 4 respectively).

Phenylacetate hydrolysis was significantly lower in the frail elderly compared with the young at $p < 0.001$ and at $p < 0.01$ paraoxonase activity in the frail elderly was significantly lower than that in that in the young and fit elderly subject groups. Esterase activities in the three groups is shown in Fig 11.15 .

Table 11.2

Normal Ranges for Blood Esterases in Young; Fit and Frail Elderly

Volunteer	[1]Paraoxonase *	[2]PhAc *	RBC x 10^{12}/l	[3]RBC Esterase+	[4]RBC Ache+
Frail					
1	152.0	95.7	4.26	189	42.9
2	47.2	56.8	3.22	103	42.6
3	106.9	73.7	4.29	138	56.3
4	50.1	53.7	4.72	105	38.9
5	138.3	85.4	3.83	119	44.7
6	121.1	80.6	4.14	110	34.2
7	61.1	43.5	3.75	134	31.0
8	160.5	98.8	4.43	214	34.7
9	93.6	76.7	4.10	134	35.8
10	86.9	74.1	3.84	122	38.2
11	107.0	69.6	3.15	82	42.6
12	66.8	37.3	4.07	119	34.9
13	109.0	58.4	4.90	156	-
14	137.0	75.9	3.5	134	-
15	98.6	52.8	4.43	92	-
16	106	53.9	4.64	89	-
17	96.9	78.9	4.22	170	-
18	113.6	54.6	3.63	122	-
19	66.8	57.9	3.25	109	-
20	71.8	35.9	3.63	-	-
Mean $\pm$SEM	100+7	65$\pm$4	4.0$\pm$0.1	128$\pm$8	40$\pm$2

Table 11.2 continued

Volunteer	[1]Paraoxonase **	[2]PhAc **	[3]RBC Esterase+ +	[4]RBC Ache ++
Fit Elderly				
21	191.2	85.9	130	-
22	92.5	55.7	128	-
23	190.2	107.1	124	-
24	113.1	66.6	99	-
25	137.0	84.7	140	-
26	135.4	92.9	132	-
27	170.3	110.6	115	-
28	97.6	54.3	138	-
29	145.1	95.7	137	-
30	117.1	68.0	131	-
31	243.8	136.7	116	-
32	107.2	88.2	140	-
33	97.0	54.9	128	-
34	114.8	65.4	133	-
35	110.5	29.5	98	-
36	200.6	116.8	108	40.2
37	195.1	84.2	139	42.7
38	166.3	108.2	144	38.1
39	124.9	68.9	103	33.9
40	160.3	93.2	144	38.3
Mean±SEM	146±9	83±6	126±3	39±1

Table 11.2 continued

Volunteer	[1]Paraoxonase ***	[2]PhAc ***	[3]RBC +++ Esterase	[4]RBC Ache +++
Young				
41	116.4	86.8	94	36.6
42	134.0	117.1	135	34.3
43	178.9	118.1	116	36.2
44	138.7	94.0	152	35.5
45	167.7	131.1	108	32.8
46	131.8	108.2	115	32.4
47	142.3	116.2	92	31.8
48	131.5	71.4	132	42.8
49	127.0	102.8	97	39.2
50	90.0	98.5	145	42.1
51	167.1	85.5	94	38.3
52	113.8	85.3	-	-
53	78.5	79.0	119	51.6
54	194.5	117.5	136	39.5
55	129.0	96.1	131	38.9
56	106.9	68.3	85	-
57	167.6	104.5	131	-
58	223.3	107.8	82	-
59	265.7	87.2	-	31.2
60	164.0	120.9	143	38.3
61	93.6	76.6	126	-
62	96.9	111.6	-	-
Mean±SEM	144±10	99±4	117±5	38±1

Key for Table 11.2

* r= 0.77; t=5.1 Significant correlation at p<0.001

+r=-0.08; t=-0.25

**r=0.83; t=6.3 Significant correlation at p<0.001

++r=0.45; t=0.87

***r=0.34; t=1.6

+++r=0.23; t=0.88

[1] nmol pnp/ml plasma /min

[2]μmol phenol/ml plasma/min

[3]nmol phenol/10^6 RBC/min

[4]nmol thiocholine /mg haemoglobin/min

Table 11.3

Plasma Aspirin Esterase and Cholinesterase Activities in the Frail Elderly Subjects

Frail Volunteer	Aspirin Esterase nmolsalicylate /ml plasma/min	Cholinesterase nmol benzoylcholine chloride hydrolyzed/ml plasma/min	
1	60.4	788	1119 *
2	27.1	429	609
3	70.8	620	880
4	74.9	933	1325
5	75.0	1030	1463
6	56.2	685	973
7	56.2	588	835
8	87.4	1227	1742
9	75.0	832	1181
10	85.4	865	1228
11	35.2	538	764
12	45.6	636	903
13	87.4	1135	1612
14	68.7	1061	1507
15	66.6	923	1311
16	97.9	1312	1865
17	77.0	1106	1571
18	50.0	780	1108
19	37.5	445	632
20	43.7	602	855
Mean $\pm$SEM	64$\pm$4	827$\pm$57	1174$\pm$80

r= 0.84 ; t= 6.8. Significant correlation at p< 0.001.* corrected to an assay temperature of 37^{o}C from 30^{o}C using the correction factor 1.42 (King et al,1965)

Table 11.4

Summary of the Normal Ranges for Esterases shown in Tables 11.2 and 11.3 including Literature values

	1.Paraoxonase	2.PhAc Esterase	3.RBC Esterase	4.RBC AChE Esterase	5.ASA Esterase	6.ChE Esterase
Frail						
range	*47.2-152.0	**35.9-98.8***	82-214	31-56.3	27.1-97.9 (75.1-83.0)	609-1863 (1523-1940)
mean	100	65	126	40	64 (78.9)	1174 (1731)
n=	20	20	19	12	20 (43)	20 (43)
Fit						
range	92.5-243.8	54.3-136.7	98-144	33.9-42.7	93.7-1728 (100-116)	1410-2410 (1918-2313)
mean	146	83	126		119.2 (108)	1731 (2115)
n=	20	20	20	5	7	7
Young						
range	78.5-265.7	71.4-131.1	82-152	31.2-51.6	93.7-193.8 (98-116)	990-2435 (1812-2163)
mean	144	99	117	38	114.5 (107.0)	1666 (1987)
n=	22	22	19	16	10 (30)	9 (30)

1. nmol paranitrophenol produced/ml plasma/min
2. µmol phenol produced/ml plasma/min
3. nmol phenol produced/10^6RBC/min
4. nmol thiocholine/mg haemoglobin/min

( ) Values from Williams et al (1989) adjusted using the temperature correction factor 1.83 (25' C-37'C)

from King et al (1965)

* significantly reduced activity compared with fit elderly and young subjects at $p<0.01$

** significantly reduced activity compared with fit elderly and young subjects at $p<0.05$

*** significantly reduced activity compared with young subjects at $p<0.001$

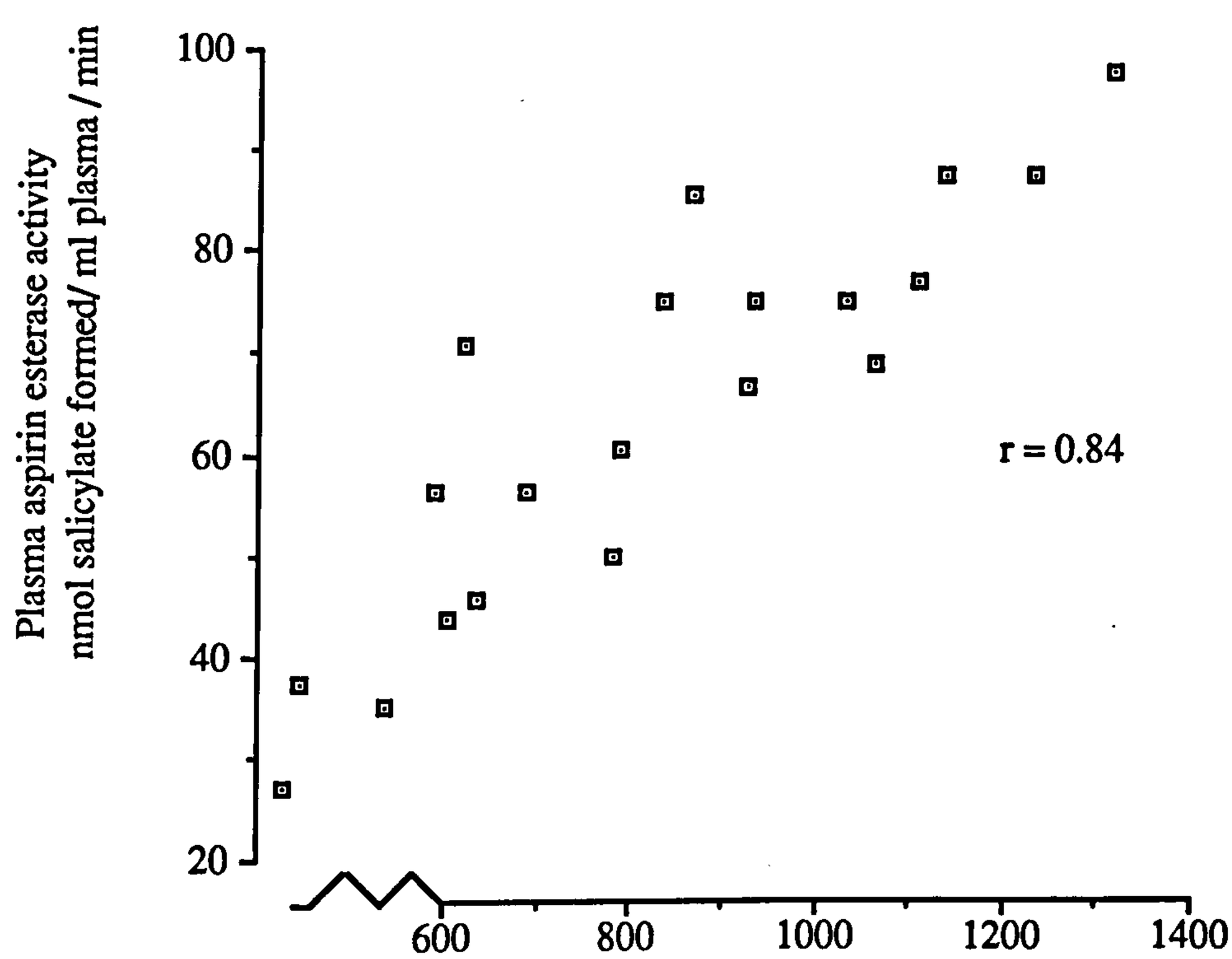


Figure 11.14

Title : The correlation between plasma aspirin esterase activity and cholinesterase activity in 20 frail elderly volunteers.

Ordinate : Aspirin esterase activity at 1 Mm aspirin concentration and at 37°C

Abscissa : Cholinesterase activity at 37°C

$p < 0.001$ correlation significantly different from zero

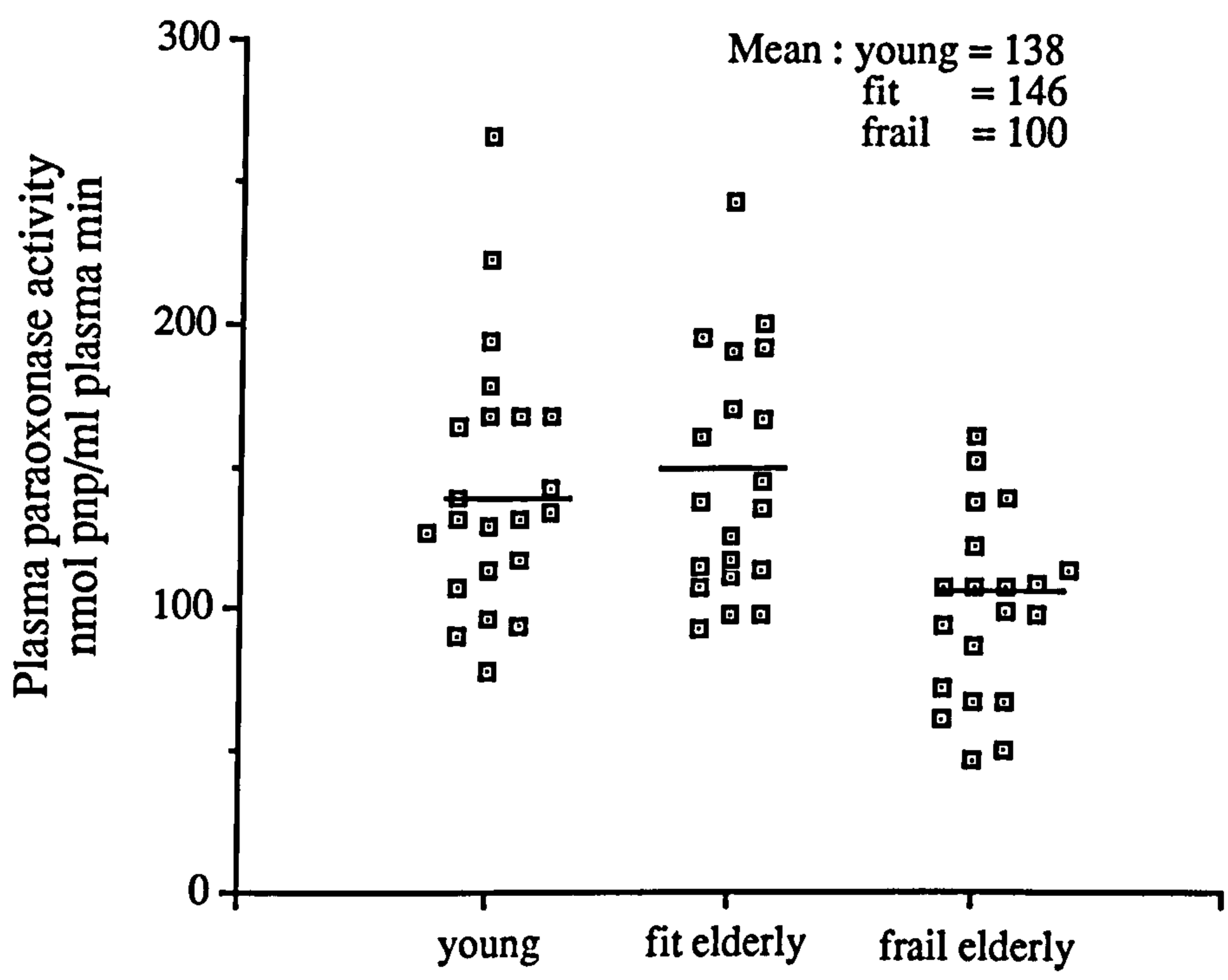


Figure: 11.15

Title: Plasma paraoxonase activity in young; fit and frail elderly

Ordinate : Plasma paraoxonase activity, nmol paranitrophenol (pnp) produced per ml plasma in a 1 min. period at 30 °C

Abscissa : Values in young; fit and frail elderly

and 11.16. In addition the frequency distributions for paraoxonase activity is plotted in Fig 11.17, 11.18 and 11.19. There is no evidence of bimodality in any of the three groups.

There was also a significant correlation between paraoxonase and phenylacetate esterase activity in the frail and fit elderly at $p < 0.001$ ($t = 5.1$ and $t = 6.3$ respectively) as shown in Table 11.2 and Figs 11.20 and 11.21, but not in the young ($r=0.34$, $t=1.6$)

Red Blood Cell Esterases

Figures 11.22 and 11.23 show the distribution of RBC esterase activity and RBC acetylcholinesterase activity in the three groups. There was no significant correlation between the intracellular RBC esterase and membrane bound acetylcholinesterase (Table 11.2). The mean number of red blood cells in the frail elderly individuals is $4.0 \times 10^{12} \pm 0.1/l$ (Table 11.2). Unfortunately, these were only obtained for the frail elderly subjects and so these values were compared with the usual reference values. The mean value for females was $3.95 \times 10^{12} \pm 0.1/l$ which was at the lower end of the normal range ($3.9 - 5.6 \times 10^{12}/l$). The mean value for the four male subjects was $4.0 \times 10^{12} \pm 0.3/l$ which is below the normal range for men ($4.5 - 6.5 \times 10^{12}/l$).

Figures 11.24 and 11.25 show a decline in red blood cell acetylcholinesterase and intracellular esterase activities with increased storage time, respectively. As a result both these assays were carried out within 24 hours of blood sample collection.

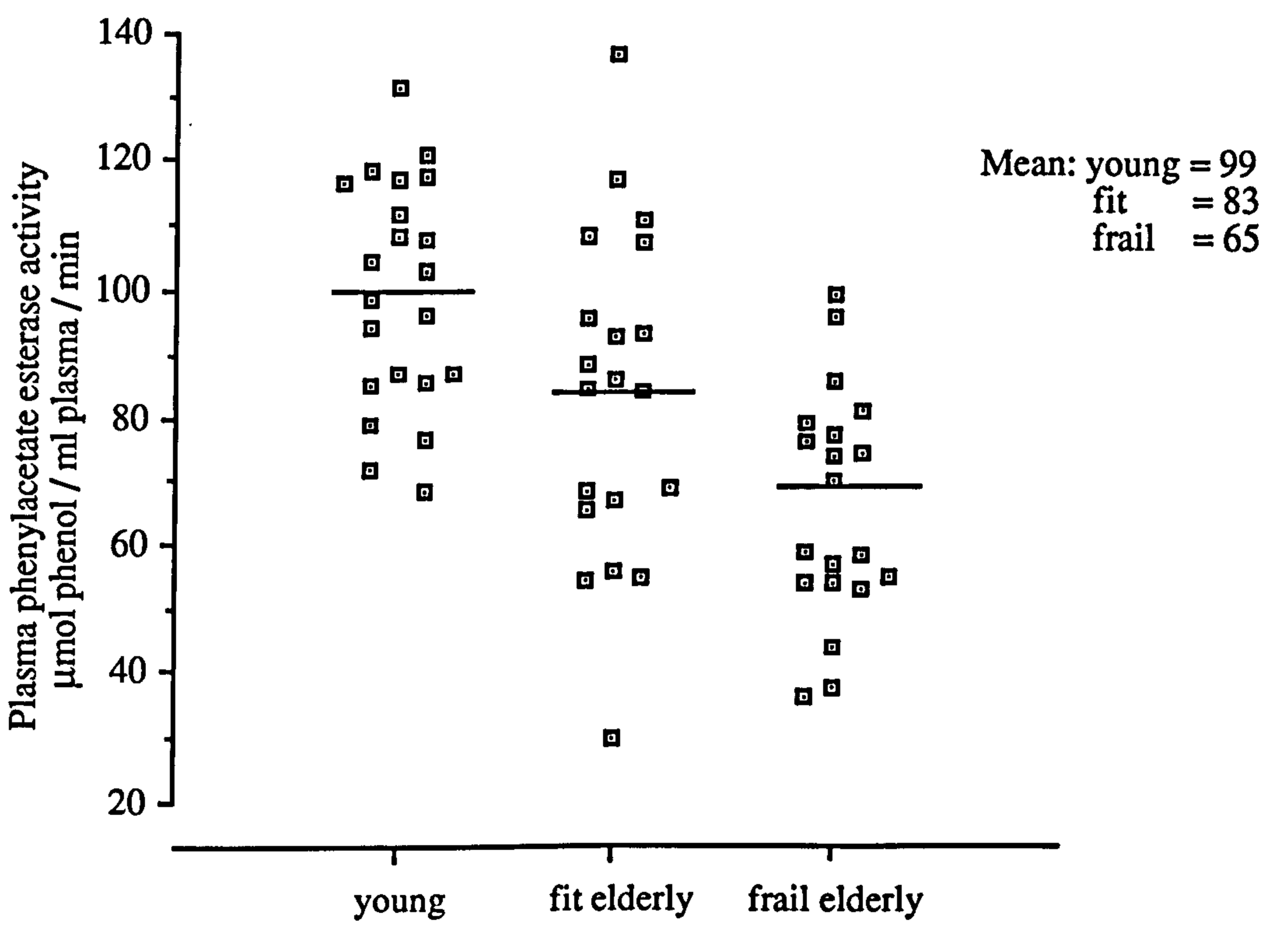


Figure 11.16

Title : Plasma phenylacetate esterase activity in young; fit and frail elderly.

Ordinate : Plasma phenylactate esterase activity, µmol phenol produced per ml plasma in a 1 min. time period.

Abscissa: Values in young; fit and frail elderly.

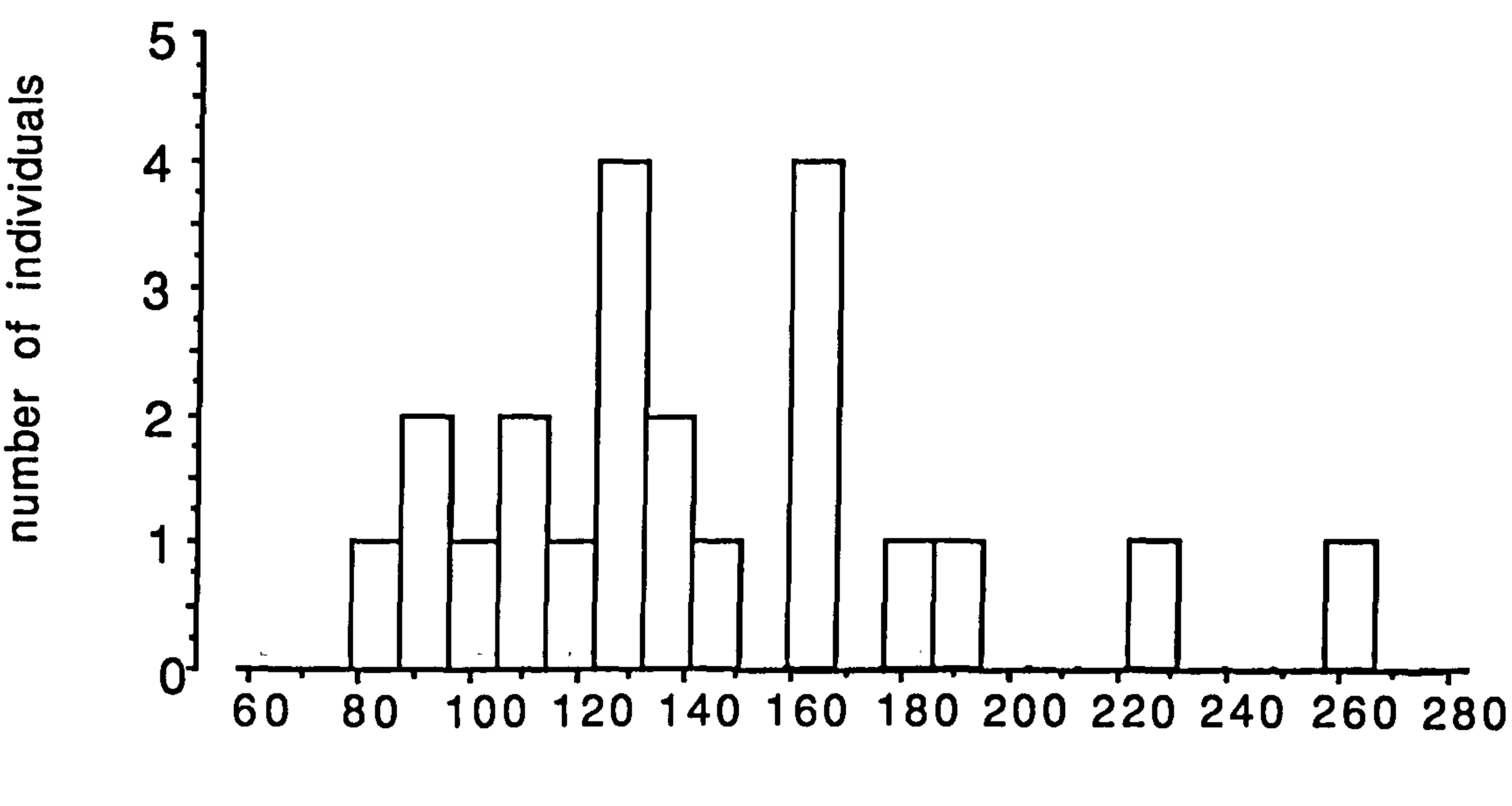


Figure 11.17

Title: The distribution of paraoxonase activity in frail elderly individuals (n=20)

Ordinate: number of individuals

Abscissa: plasma paraoxonase activity

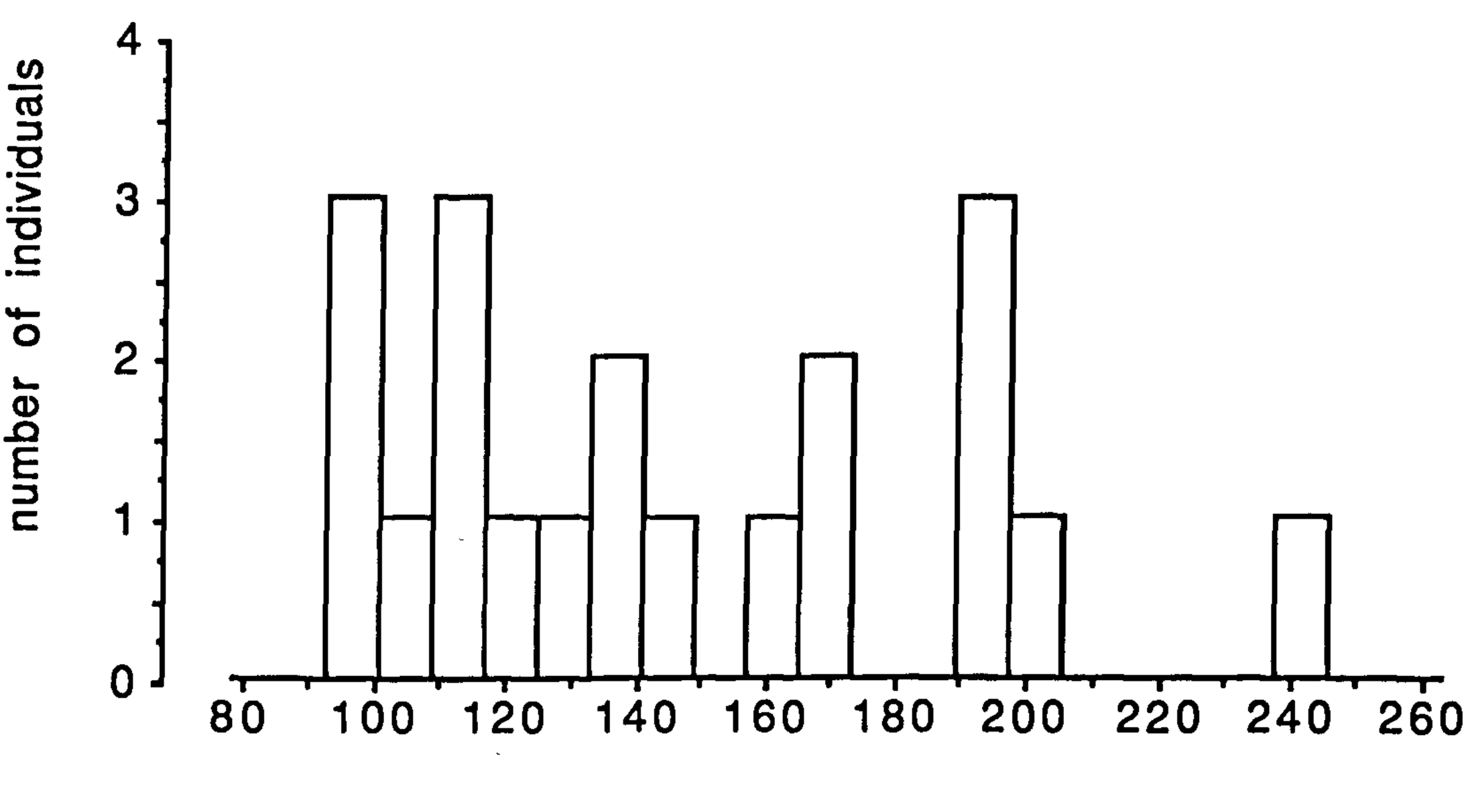


Figure 11.18

Title: The distribution of paraoxonase activity in fit elderly individuals (n=20)

Ordinate: number of individuals

Abscissa: plasma paraoxonase activity

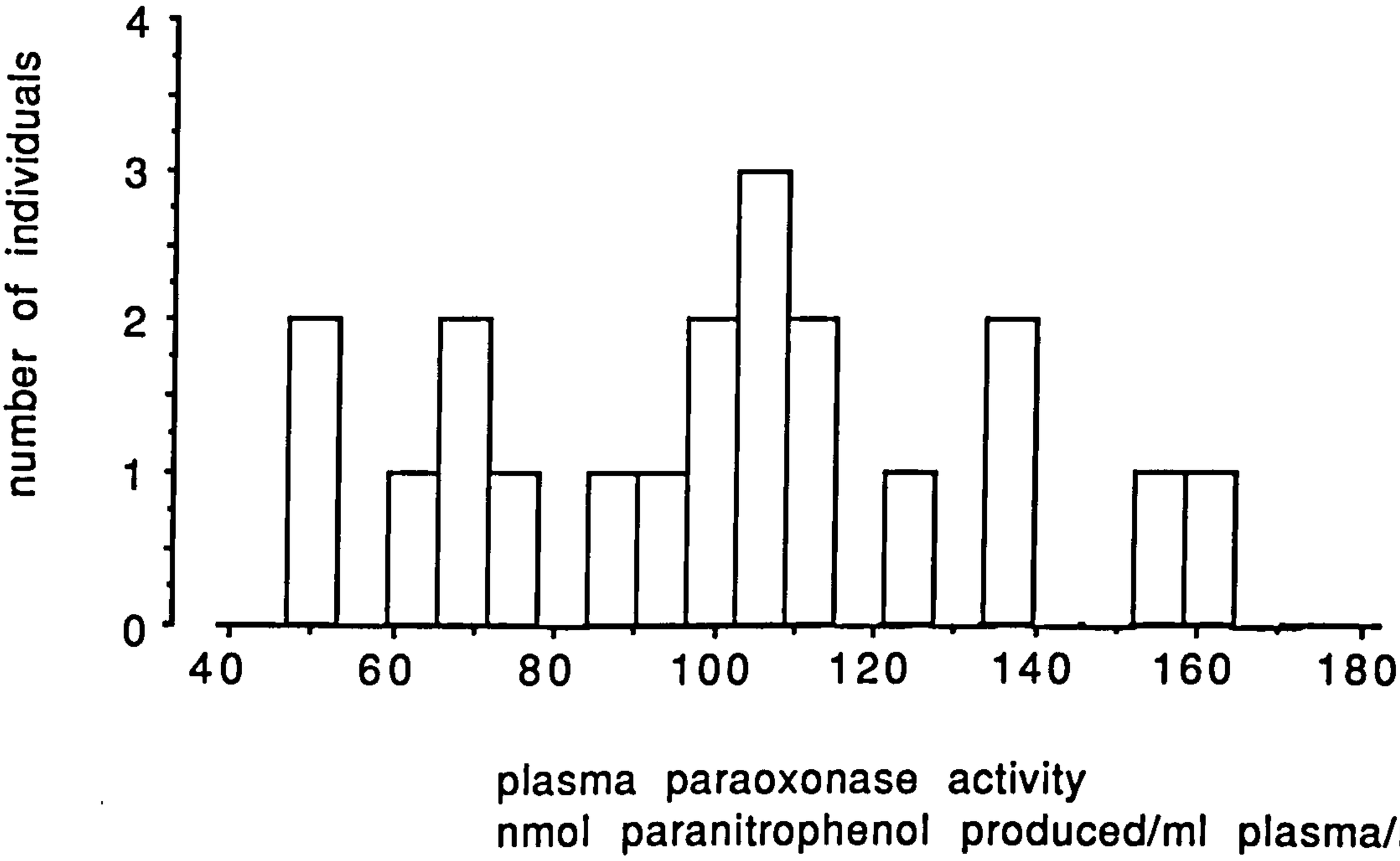


Figure 11.19

Title: The distribution of paraoxonase activity in young individuals (n=20)

Ordinate: number of individuals

Abscissa: plasma paraoxonase activity

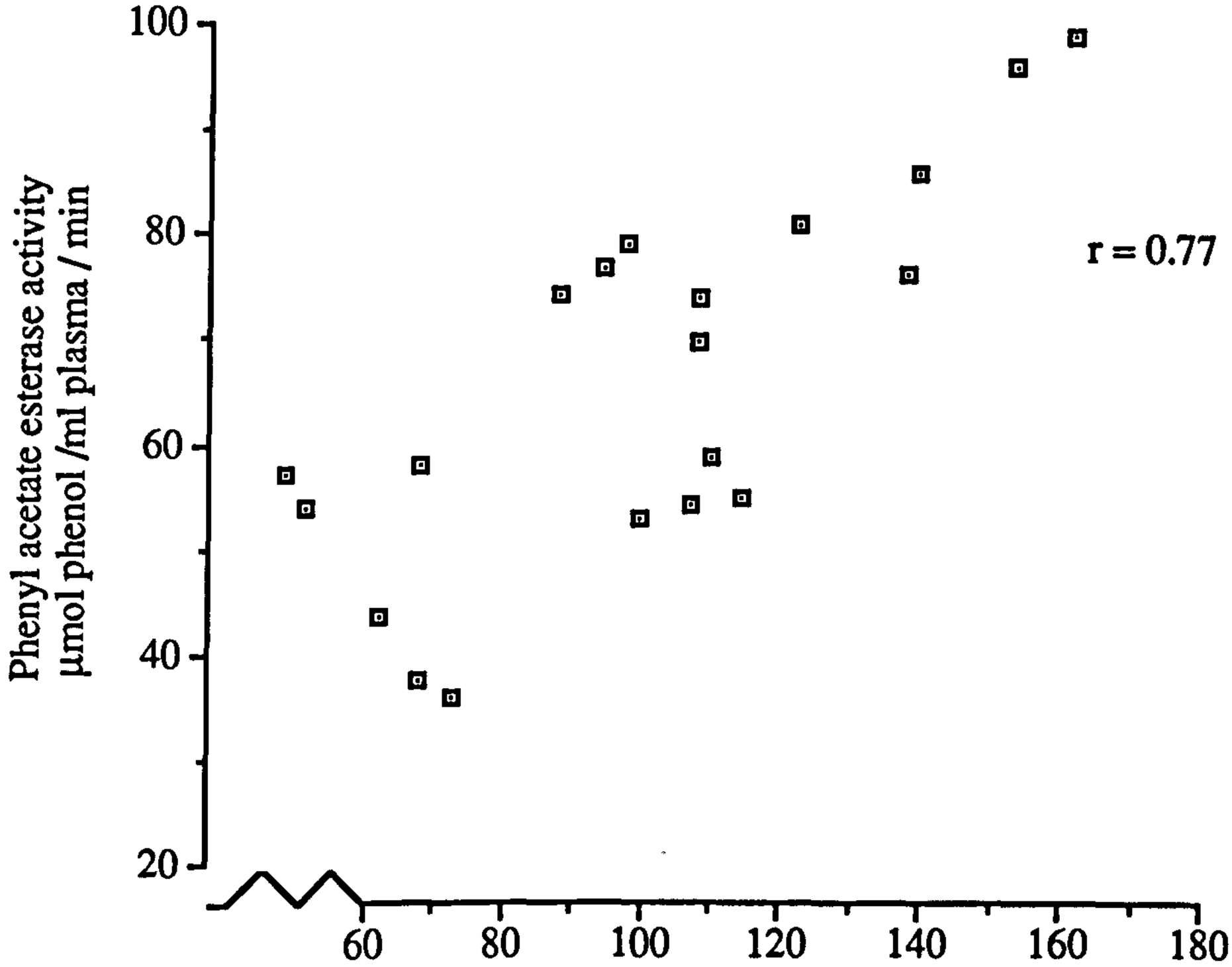


Figure 11.20

Title : The correlation between plasma phenylacetate esterase activity and paraoxonase activity in 20 frail elderly volunteers.

Ordinate : Plasma phenylacetate esterase activity at 30°C

Abscissa : Plasma paraoxonase activity at 30°C

$p < 0.001$ correlation significantly different from zero

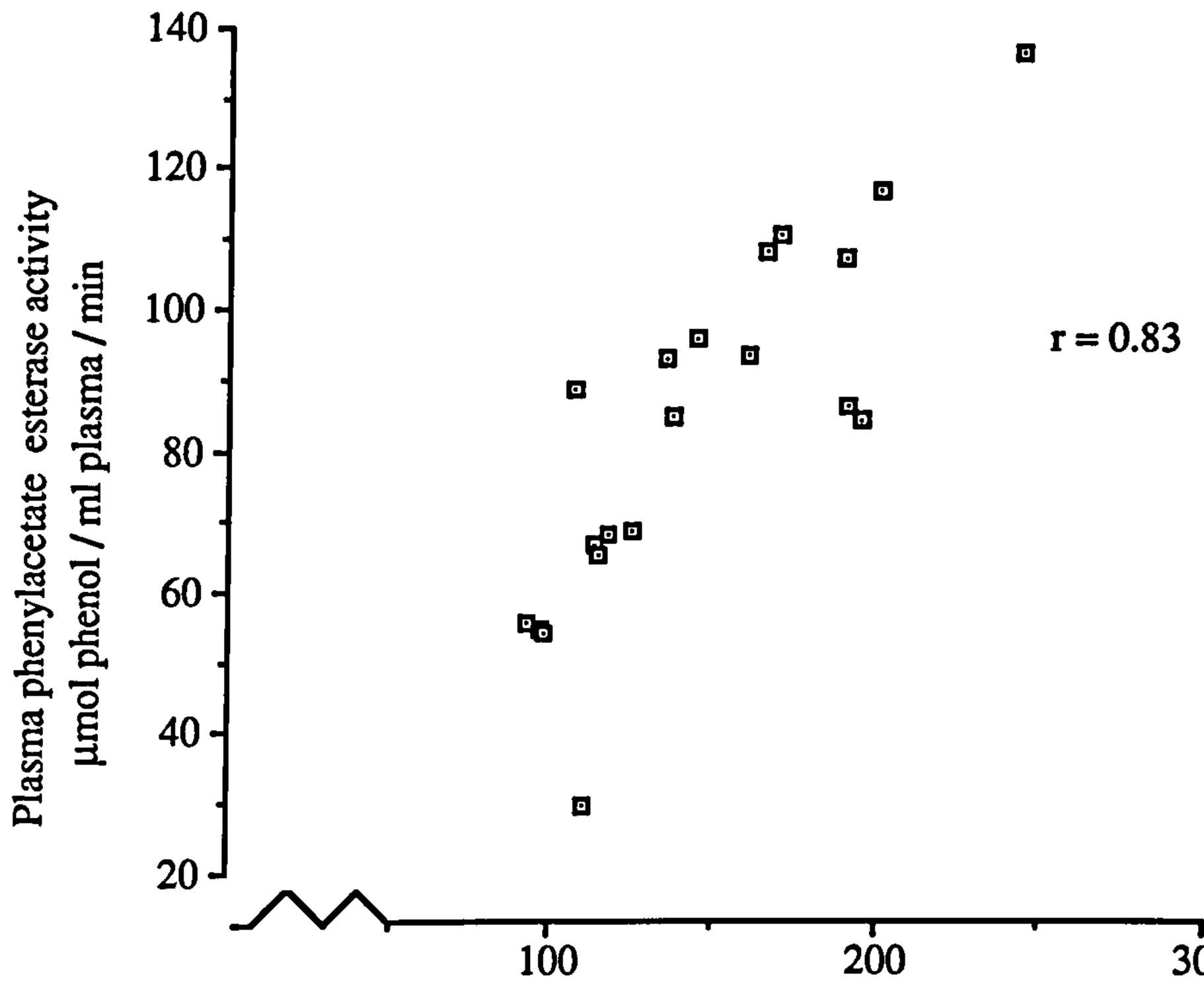


Figure 11.21

Title : The correlation between plasma phenylacetate esterase activity and paraoxonase activity in 20 fit elderly volunteers

Ordinate : Plasma phenylacetate esterase activity at 30°C

Abscissa : Plasma paraoxonase activity at 30°C

$p<0.001$ correlation significantly different from zero

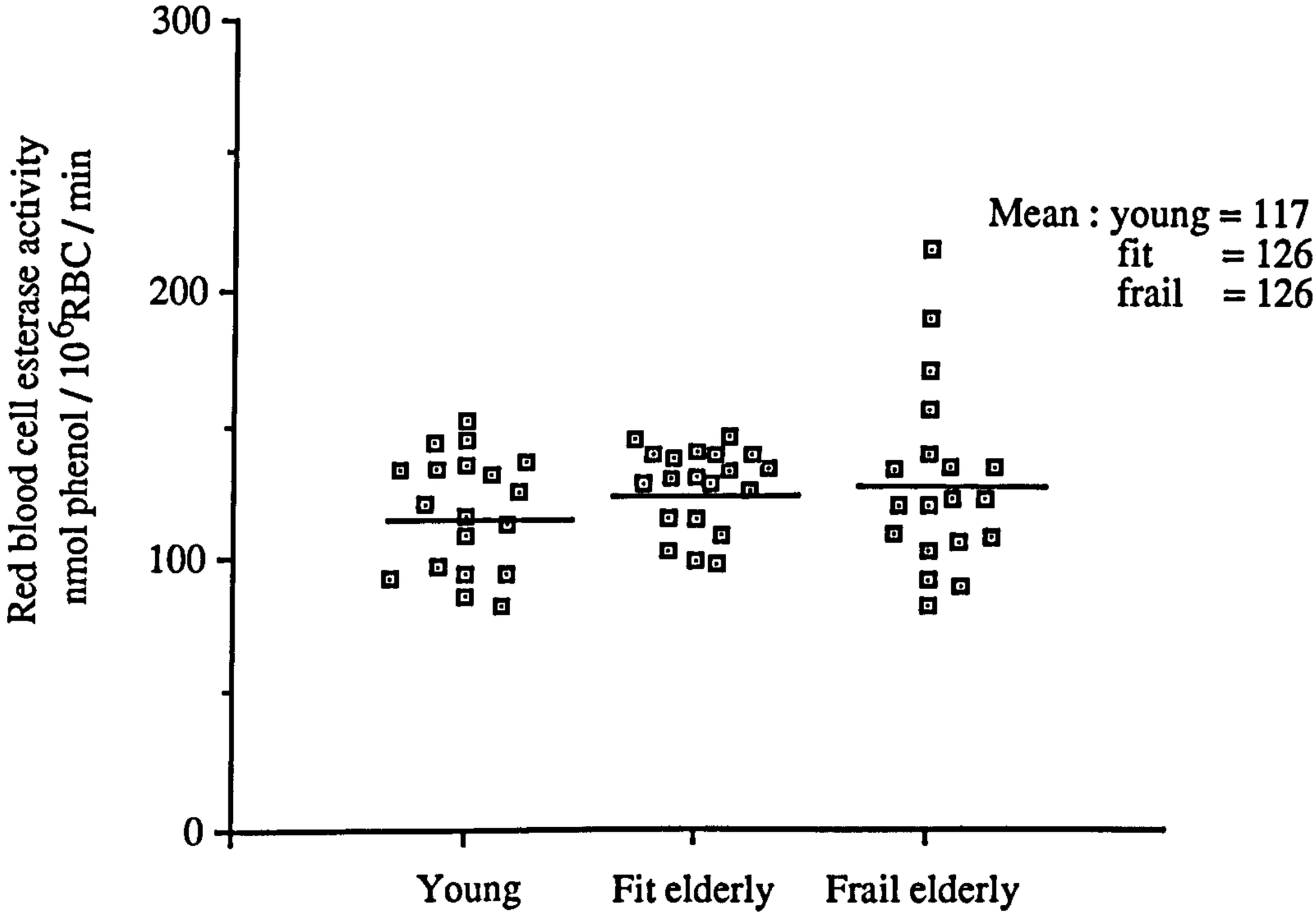


Figure 11.22

Title: Red blood cell esterase (RBC) activity in young; fit and frail elderly.

Ordinate : RBC esterase activity, nmol phenol produced by 10^6 RBC in a 1 min. time period at 37^oC

Abscissa : Values in young; fit and frail elderly

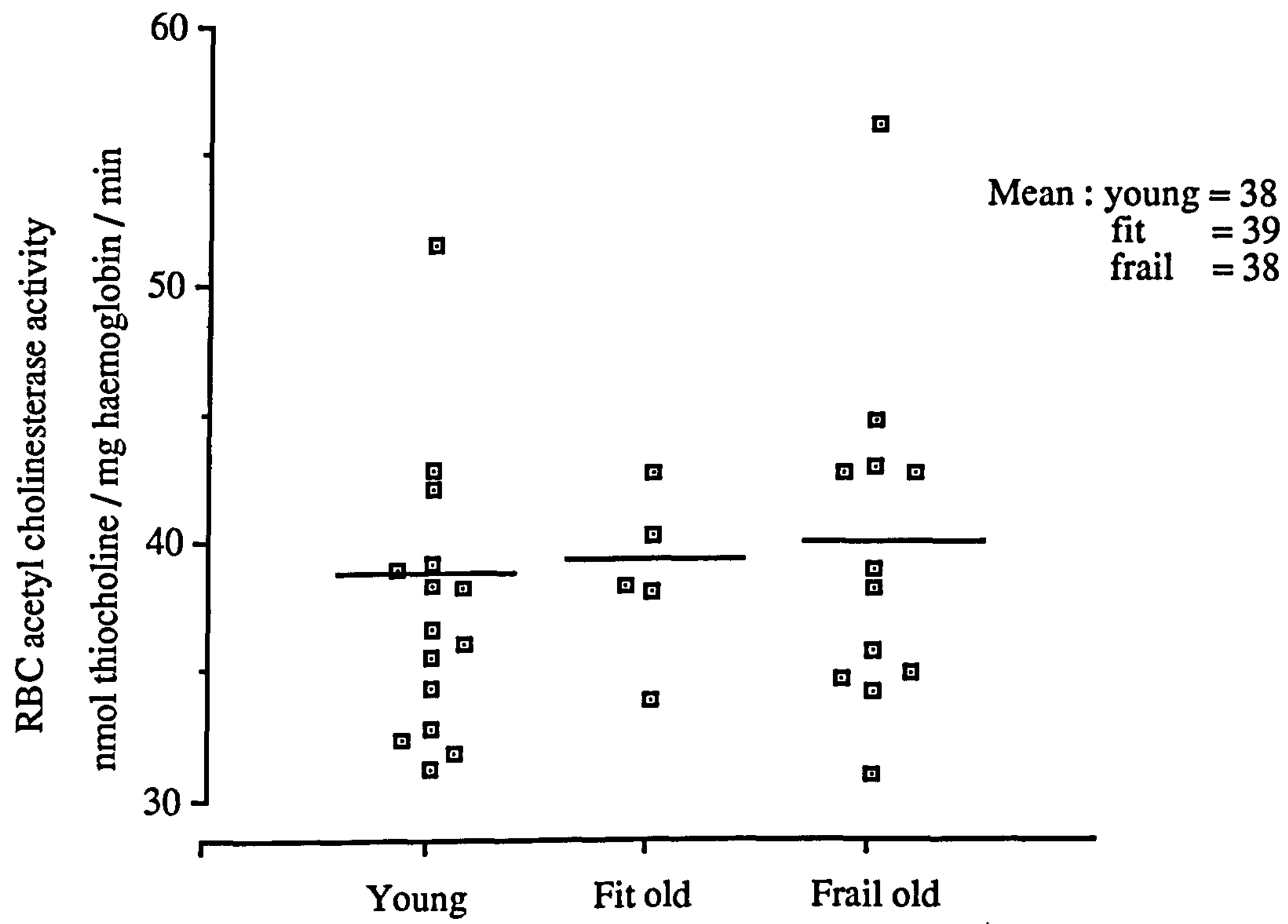


Figure 11.23

Title: Red blood cell (RBC) acetylcholinesterase in young; fit and frail elderly.

Ordinate : RBC acetylcholinesterase activity, nmol thiocholine produced per mg haemoglobin in a 1 min. time period at 30 C°

Abscissa : values in young ; fit and frail elderly

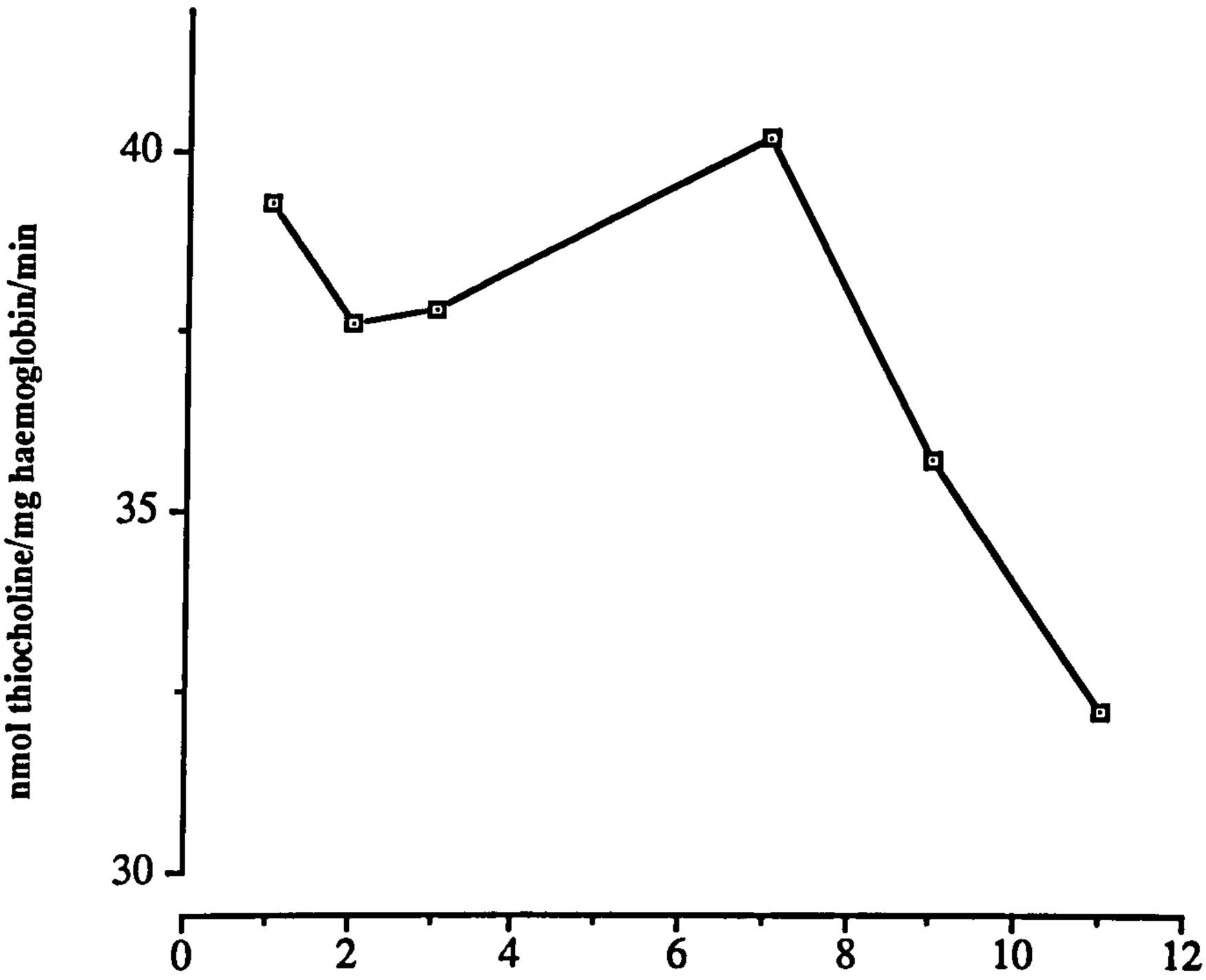


Figure 11.24

Title: The effect of storage time at -80°C on red blood cells prepared for the acetylcholinesterase assay: the reduction in enzyme activity

Ordinate: nmol of thiocholine produced per mg of haemoglobin over a 1 min. time period at 30°C.

Abscissa: number of days storage at -80°C.

The values are the mean of two determinations.

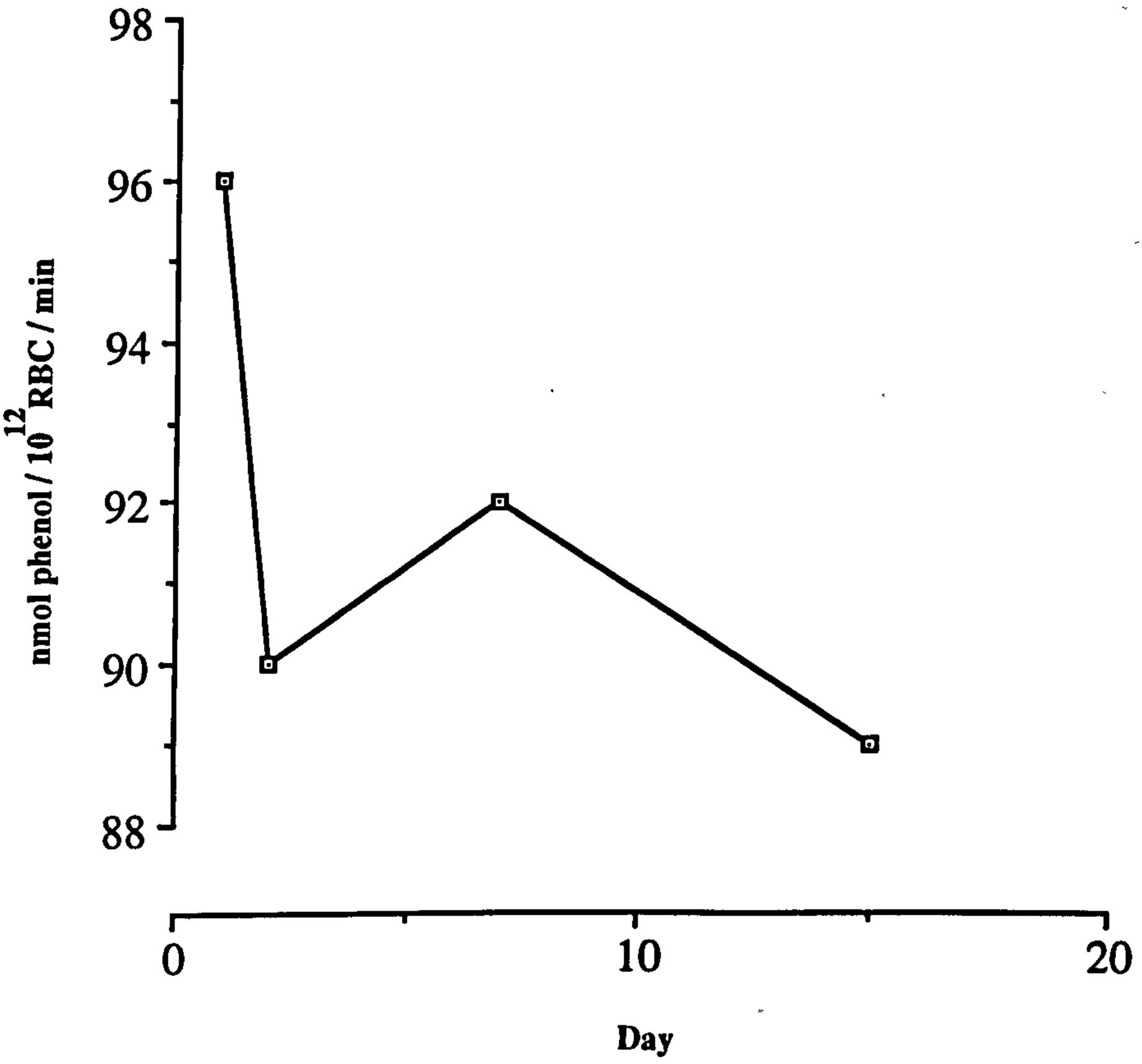


Figure 11.25

Title: The effect of storage time at -80°C on red blood cells prepared for the 'red blood cell esterase' assay: the reduction in enzyme activity

Ordinate: nmol of phenol produced by 10^{12} red blood cells over a 1 min. time period at 37°C

Abscissa: number of days storage at -80°C.

The values are the mean of two determinations.

11.8 Discussion

Plasma aspirin esterase, cholinesterase, paraoxonase and phenylacetate esterase (arylesterase) activities were all significantly reduced in the frail elderly. Williams et al (1989) also found this to be the case with plasma aspirin esterase, cholinesterase and phenylacetate esterase.

Playfer et al (1977) investigated plasma paraoxonase activities in old age and found no reduced activity. However the elderly population he used was a cross-sectional sample of people from nursing homes, long-stay geriatric hospitals and healthy elderly living independent lives at home.

Zech and Zurcher (1974) state that the average phosphoryl phophatase activity of older persons is lower but give no details of patient type, age etc.

My sample of frail elderly people had a mean age almost ten years above that of the fit elderly. This highlights the constant problem of finding suitable age-matched controls for studies involving frail elderly subjects.

Phenotyping of paraoxonase activity is carried out by means of frequency distributions resulting from qualitative properties of the enzyme such as stimulation by 1M sodium chloride (Eckerson et al, 1983a). Using small populations it isn't possible to identify the presence or absence of bimodality with certainty (Jackson et al, 1989). Since my samples were randomly selected on this basis, I assumed they were unimodally distributed. There was no evidence of bimodality when the activities were plotted as frequency distributions (Fig 11.17,11.18,11.19). The assay I used measured paraoxonase

activity in an alkaline medium, thus both the activities of EDTA-sensitive enzyme and EDTA-insensitive albumin are determined. The reduced paraoxonase activity in the frail elderly may, in part, be due to a reduced albumin content of the plasma.

Plasma paraoxonase and phenylacetate esterase activities were highly correlated in the fit and frail elderly individuals. La Du and Eckerson (1984) also found a high correlation between the activity of the two esterases and concluded that this would be expected if the two enzymes were identical. They explained the bimodality of paraoxonase and unimodality of arylesterase activity by suggesting that paraoxon was a 'discriminating' substrate and phenylacetate a 'nondiscriminating' substrate (Eckerson et al, 1983b).

However, this correlation was not repeated in the fit young, which, together with results in Chapter 12 suggests that the relationship between the two esterases may not be quite so simple. Mackness et al (1987a) differentiated between 'A'-esterase (paraoxonase) and arylesterase (phenylacetate esterase) activity in avian plasma. As a result the two enzymes are now classified separately (Mackness, 1989).

I obtained lower limits of activity for cholinesterase (480 - 1570) and aspirin esterase (66.6 - 112.4) than Williams et al (1989) (1523 - 1940 and 75.1 - 83.0 respectively), using similar methods, although I did have similar upper limits. These esterases, together with phenylacetate esterase (Takahashi et al, 1967) and paraoxonase (Playfer et al, 1977) are produced in the liver and are good peripheral markers of protein synthesis. Low activities may be due to reduced protein synthesis (Brown et al, 1981). This resulted in unforeseen problems

retaining patients recruited in the following study (Chapter 12).

There was no correlation between intracellular RBC esterase and AChE. Neither of these showed a reduced activity when expressed per 10^6 RBC. However the mean RBC number in the frail elderly was lower than the normal range which suggests that these enzyme activities may be reduced due to the increased tendency of these individuals to suffer from chronic anaemias (Weatherall, 1987).

CHAPTER 12

The Effect of Improved Nutrition on Plasma Esterase Activity in a Hospitalized Frail Elderly Population

Chapter 12

The Effect of Improved Nutrition in Plasma Esterase Activity in a Hospitalized Frail Elderly Population.

12.1 Introduction

The reasons for the reduced plasma esterase activities demonstrated in the frail elderly individual are probably multi-fold (Williams et al, 1989; Summerbell et al, 1990). They may be a general marker of declining protein synthesis in these individuals. Low activities of cholinesterase have been noted in individuals preceding death (Brown et al, 1981). Serum cholinesterase activity has been found to correlate significantly with mid-arm circumference (MAC) and triceps skinfold thickness (TSF) in men but its use for assessment of nutritional status remains speculative (DHSS 1979a). Some workers have observed increases in plasma esterase activities in humans as the result of improved diet (Waterlow, 1950; Burch et al, 1957). Others have noted diet-modifying effects on cholinesterase activities in the liver and plasma of rats (Kean et al, 1986; Osada et al, 1989). These have been discussed in greater depth in the General Introduction.

In studies of a similar population of frail elderly patients to those used in these studies, Lipski et al (in press) noted the energy intake of these individuals was only 65% of calculated daily requirements. This results in the predisposition of such a group to subclinical malnutrition. This may become evident as an increased tendency to suffer from infections, fracture events, cognitive impairment etc (Beaumont and James, 1985).

Background

Malnutrition occurs when an individual's average dietary intake falls below that required to maintain a positive energy balance (ie energy intake is equal to or exceeds energy expenditure); and to maintain tissue and organ integrity and function. Individuals vary in their requirements depending on their sex, age, stature and daily expenditure (ie occupation).

Malnourishment amongst elderly people in institutional care has been widely reported. (Exton-Smith et al, 1965; Davies, 1984). People suffering from severe mental or physical incapacity are most at risk since both affect the ability to obtain and prepare food. Table 12.1 shows the recommended daily intake of the major nutrients in the elderly . These are the results from a 1979 survey which have been superceded by a more recent report published in 1991 (DHSS, 1991). Nevertheless it is still useful as a summary and guideline of nutrient needs since the 1991 report is less general concerning the specific nutrient requirements of adults above 59 years old.

Poor nutrition is associated with general weakness and disability and may be a factor in the aetiology of dementia (Goodwin et al, 1983). Attempts have been made to improve the prognosis of dementia by dietary means. Lecithin in the diet is a source of choline, a precursor of brain acetylcholine. White et al (1977) showed a selective loss of cholinergic neurones in Alzheimer's disease. Etienne et al (1978) demonstrated significant increases in plasma choline in a group of seven elderly patients following a four week dietary regime supplemented with lecithin powder. There were no improvements in memory function or constructional ability in the treated group, however.

Table 12.1

Recommended Daily Amounts of Food Energy

and some Major Nutrients for the Elderly in the UK (DHSS 1979b)

Men age (years)	Energy	Protein	calcium	thiamine	riboflavin	nicotinic acid	folate	ascorbic acid	retinol	vit D	iron
	KJ	g	mg	mg	mg	mg	mg	mg	µg	µg	mg
65-74	10000	60	500	1	1.6	18	300	30	750	10	10
75+	9000	54	500	0.9	1.6	18	300	30	750	10	10
Women											
55-74	8000	47	500	0.8	1.3	15	300	30	750	10	10
75+	7000	42	500	0.7	1.3	15	300	30	750	10	10

In addition to cognitive impairment, there is evidence that poor nutrition may contribute to some of the alterations in drug metabolism in old age. Chronic vitamin C deficiency in the elderly is associated with impaired clearance by oxidation of the model drug antipyrine. Supplementation of the diet with vitamin C restored drug elimination rates to normal levels (Smithard and Langman, 1978; Ginter and Vejmolova, 1981)

Wynne et al (in press) showed a reduced elimination of conjugated paracetamol in institutionalized frail elderly. The formation of the glucuronide conjugate was particularly impaired and this may be due to a reduced carbohydrate and protein intake resulting in reduced availability of glucuronic acid (Sonne et al, 1989). Pantuck et al (1984) enhanced glucuronidation in humans by feeding volunteers on a diet rich in cruciferous vegetables for ten days. This was demonstrated by a reduction of paracetamol half-life from 2.5 hours to 2.4 hours which was significant at the 5% level, and an increased ratio in the plasma concentration of paracetamol glucuronide to paracetamol from one hour through to eleven hours. These vegetables are rich in indoles which have induced glucuronidation.

Primary causes of malnutrition in the elderly population include factors such as poverty, physical and mental disabilities and ignorance concerning adequate nutritional intake. Secondary factors are more exclusive to this group. They are also more diverse and difficult to quantitate and include oral problems, malabsorption, alcoholism and drug therapy (Lehmann, 1989).

12.1.2 Nutritional Status in the Institutionalized Elderly

This group of elderly people includes the 'frail' group of individuals referred to earlier. Any assessment of nutritional status in the elderly, and in subgroups of this population is very difficult due to a lack of anthropometric and biochemical reference limits.

As an individual ages he is increasingly susceptible to acute and chronic illness which can affect the appetite (both by psychological and physical means) and also the nitrogen balance (if calorie intake is too low protein is broken down for its calorific value). In the elderly a common psychological cause of a reduced appetite is endogenous depression (Lipschitz and Mitchell, 1984) and a multitude of other psychiatric disorders including paranoia may occur (Morley, 1990).

Physical loss of appetite can be due to oral or dental problems, oesophageal strictures and loss of ol factory sense (Cain and Stevens, 1989).

Less well defined is the effect of cognitive dysfunction in nutritional status. There is some controversy surrounding the cause/effect relationship between the two (Morgan et al, 1986; Goodwin, 1989). Kay et al (1980) found 22% of the 80+ years age group were moderately or severely demented. Some authors have suggested that dementia is associated with metabolic changes resulting in weight loss (Morgan and Hullin, 1982; Asplund et al, 1982; Abalan, 1984) or malabsorption (Burns et al, 1986).

Vir and Love (1979) found that the nutritional intake of institutionalized elderly in Belfast hospitals was poor compared with those in residential care.

Lipski et al (in press) compared the dietary intake and nutritional status of fit young and fit old (community based) and elderly hospitalized patients. This study was carried out in Newcastle District Hospitals and on similar groups of people as in this thesis and is therefore especially relevant. They found that the elderly long-stay patients were clinically malnourished and did not consume sufficient nutrients to meet basic metabolic needs. The energy intake for men and women was only 68% and 63% of daily requirements respectively.

Morgan et al (1986) compared calorie intake of fit active elderly with that of a group of women suffering from senile dementia in a long-stay geriatric hospital. Despite similar energy intakes the hospitalized elderly weighed, on average, 15 kg less as a group. However, the methods used for assessing dietary intake were different in hospital and community elderly (who assessed their own intake) and no data is published to substantiate these claims.

Burns et al (1989) carried out a six month study which compared the weights of hospitalized demented patients with community based demented patients and found that the former group suffered a continued weight loss over this period over this period of time whilst the latter did not, despite comparable diets. These are in contrast to the findings of Lipski et al (in press) and Vir and Love (1979) discussed earlier. The discrepancies may occur in the way in which dietary intake was assessed. Lipski et al (in press) used the validated diet history method in a structured interview with the elderly living at home. In order to assess the dietary intake of the hospitalized elderly a nurse completed a daily record of weighed food intake for seven days. Burns et al (1989) and Morgan et al (1986) used the weighed dietary intake on randomly selected individuals from each group for a period of 3 days and 5 days respectively as a means of

assessing food consumed over the whole period. Vir and Love (1979) also weighed the food intake for three days but on all the subjects under study. The number of individuals in each study was also quite variable. Burns et al (1989) concluded that it was not dementia *per se* which caused weight loss but "some factor associated with hospitalization". The valid comparability of hospitalized with community based subjects must be questioned, however. The authors do concede that the hospitalized group were more cognitively impaired,, had a longer mean duration of illness and were receiving more intensive drug therapy.

Singh et al (1988) suggested the weight loss may be due to the aimless repetitive movements many Alzheimer's patients carry out. However this would not explain the results of Burns et al (1989) since as the disease progresses the patient usually becomes less mobile. Thus a parallel increase in weight would be expected.

The heterogeneity of the elderly population cannot be emphasized enough. Consequently each patient should be treated as an individual. A detailed dietary assessment is really the only means of ensuring an adequate nutritional intake. This must include careful assessment of the possible effects on concurrent illness and drug therapy.

Some of the clinical signs of malnutrition are very similar to those associated with 'normal' ageing eg hair and skin changes, muscle wasting and mental confusion. For a patient who is in long-term care, therefore, serial assessments are a useful adjunct to the monitoring of nutritional status (Bowman and Rosenberg, 1982).

12.1.3 The Effect of Drug Therapy on Nutrition

The frail elderly population are often on more regular medication than the fit elderly which can be seen in Table 12.4. The elderly as a whole form the largest group of prescription and self-administered drug users. In 1985 the proportion of elderly people in the population was 18% and they received 39% of the prescription items dispensed (Cartwright, 1988).

Drugs can affect nutritional status in a variety of ways. Whether or not clinical malnutrition manifests depends largely on pre-existing deficiencies, doseage and duration of drug treatment.

Anorexia may be caused by drugs such as the phenothiazines which can cause somnelance and hence a disinterest in food. Digoxin has a low therapeutic index and toxicity is particularly a problem in the elderly who have a reduced renal clearance (Baskin and Kendrick, 1978).

Some drugs cause nutritional side effects which may go unnoticed in younger people. For example corticosteroids inhibit bone formation which may exacerbate pre-existing oste oporosis.

Hypokalaemia is associated with diuretic use resulting in muscle weakness and cardiac arrhythmias. Again, though common to all age groups, the elderly are most vulnerable (Rikans, 1986). This is related to a reduced potassium intake and diminished homeostatic control of blood pressure (Vestal, 1978).

Other nutritional effects include the magnesium deficiency related to diuretic and digoxin use (Roe, 1984) and salicylate inhibition of folate uptake by red cells (Munro et al, 1987).

12.1.4 Dietary Assessment

Biochemical Markers of Nutritional Status

Nitrogen Balance

Measuring nitrogen balance is a method whereby nitrogen (protein) intake is compared with nitrogen excretion. When the body is in a state of negative nitrogen balance there is a loss of nitrogen due to its use as an energy source instead of fat or glycogen whose supplies have been exhausted. This process cannot continue indefinitely. Eventually the formation of body tissue, enzymes and cellular components suffers and the organism dies.

Albumin Measurement

The usefulness of albumin measurements as a marker of nutritional state remains controversial. Lipschitz and Mitchell (1984) claimed that hypoalbuminaemia was an excellent indicator of protein - energy malnutrition (PEM) in the elderly. However, other authors (Friedman et al, 1985) and more recently O'Keefe and Dicker (1988) challenged this claim. They qualified their doubts by observing fluid retention and hence dilution of plasma albumin in patients with cardiac, renal or liver failure. In their studies they observed rises in albumin concentration in the absence of significant changes in dietary intake.

Albumin synthesis is sensitive to protein intake. This is based on the observation that prolonged starvation is associated with a gradual reduction in plasma albumin concentration (O'Keefe and Dicker, 1988). The decline is not large because skeletal muscle is used as a store to replace such visceral proteins in order to maintain oncotic pressure (Munro, 1964). However the elderly do appear to be more vulnerable to the immediate effects of a negative nitrogen balance due to a lack of adequate hormone response (namely increase cortisol and insulin ratio) which protects younger people (Lehmann, 1989).

Albumin is probably a useful screening test or a quantitative measure when used to monitor a treatment (Lehmann, 1989). However it is insufficiently specific since albumin levels are also affected by a number of other conditions such as oedema, infection, inflammation and open wounds (Friedman et al, 1985).

An acute fall in albumin concentration, especially in the elderly is often a sign of sudden clinical deterioration such as sepsis (O'Keefe, 1988; Kemm and Allcock, 1984).

Serum Transferrin

Serum transferrin (the major iron transport protein) is also used as a measure of protein visceral stores. However tissue iron stores increase with age and consequently serum transferrin levels are reduced. This means some healthy and adequately nourished elderly will have transferrin levels in the range that would normally indicate poor nutritional status (Lipschitz et al, 1981).

Haemoglobin Concentration

Anaemia was found in 98% of elderly undernourished patients in one study (Mitchell and Lipschitz, 1982a) but as a general rule it is non-specific and more likely to be due to a blood loss.

Immune Function

Changes also occur in immune function with age which bear a close resemblance to those seen in malnutrition, but, it is difficult to ascribe host defence abnormalities to the ageing process or nutritional status (Lipschitz and Mitchell, 1984). Lymphopaenia has been used as a useful but non-specific tool which to monitor the treatment progress (Lehmann, 1989).

Creatinine

The Creatinine - Height Index (CHI) is calculated by relating the 24 hour excretion of creatinine to height. This combines biochemical with anthropometric measurements and hence the drawbacks of both. There is an increased variability to both with increasing age (Mitchell and Lipschitz, 1982a). In addition, the accurate measurement of height in bed-ridden, stooped patients can be very difficult. Current reference ranges were obtained from a relatively small number of young males which means they are inappropriate for use in older subjects (Bristian et al, 1975).

Mitchell and Lipschitz (1982b) related creatinine excretion to total arm length (TAL) in an attempt to partly overcome this problem. This was found to be a

better indication of malnourishment in males and younger females. However, neither CHI nor TAL could distinguish between well and poorly nourished groups of elderly females. This was attributed to a possible exhaustive depletion of lean body mass in old females or it may relate to a lower creatinine output in response to protein deprivation by inactive elderly compared with physically active more muscular individuals (Uauy, 1978).

Ideally a protein marker of malnutrition should have a small body pool size, a rapid rate of synthesis, a reasonable distribution in the intravascular space and a short biologic half-life (Long, 1984). These optimal criteria ensure the protein is maximally responsive. Unfortunately it also means it is reponsive to other stimuli and does not only reflect changes in nutritional status.

12.1.5 Anthropometric Measurements

Anthropometric measurements are carried out in order to quantify the amount, composition or rate of tissue change which occurs when a person gains or loses weight (Heymsfield et al, 1984). Muscle and fat are usually of most interest. From these two measurements fat free mass (FFM) or lean body mass can be calculated.

The instruments used have changed little over the years and include scales, tape measures and hand held calipers. All these are non-invasive. Ideal weight measurements for height and frame size have been defined over the years, usually as the result of mortality statistics (Metropolitan Life Assurance Company of New York).

The first comprehensive data on upper arm tissue distribution over various age ranges (to74 years) is based on the Health and Nutrition Survey (HANES II, 1971-1974) reported by Bishop et al (1981). A set of reference values based on various individual studies is issued by British Indicators, the manufacturers of Harpenden calipers

Obviously calibration of any instruments used with appropriate standards is imperative. Just as important is skill and technique in order to achieve meaningful and repeatable results.

Hall et al (1989) issue guidelines for the correct measurement of height, weight and limb measurements. Adherence to these standards ensures comparable results between centres.

Skinfold Calipers

These are instruments which measure skin fold thickness by applying a pressure to the skin surface between two jaws. The applied pressure is important since it must remain constant throughout the range of measured skinfolds. This has been standardized at 10 gmm^{-2} (Brozek, 1951). The contact surface area has not been standardized in the same way but within a reasonable range of surface areas, it seems there is little influence on the skinfold thickness (Heymsfield et al 1984).

a) **Errors in Measurement**

These are due mainly to difficulties with technique and anatomical differences between individuals especially due to age.

b) Errors in Technique

It can be difficult to distinguish between fat and muscle. British Indicators issue guidelines as to the correct use of Harpenden Calipers. Measurements should be made at consistent 'anatomical landmarks' and the observer should be practised at finding these (Plate 12). A tape measure is often useful. Muscles should be relaxed and the calipers placed perpendicular to the fold. A minimum of two measurements should be taken, however too many repeated measurments at the same site can result in leakage of water from compressed fat cells and consecutive measurements becoming smaller.

c) Errors due to Anatomic Anomalies associated with Age

Skinfold calipers only measure subcutaneous fat. However, total body fat is distributed between subcutaneous and internal sites which do not change in proportion to one another (Allen et al, 1956). The relative distribution varies between individuals depending on sex, race and age (Heymsfield et al, 1984) and this must be taken into account when interpreting results.

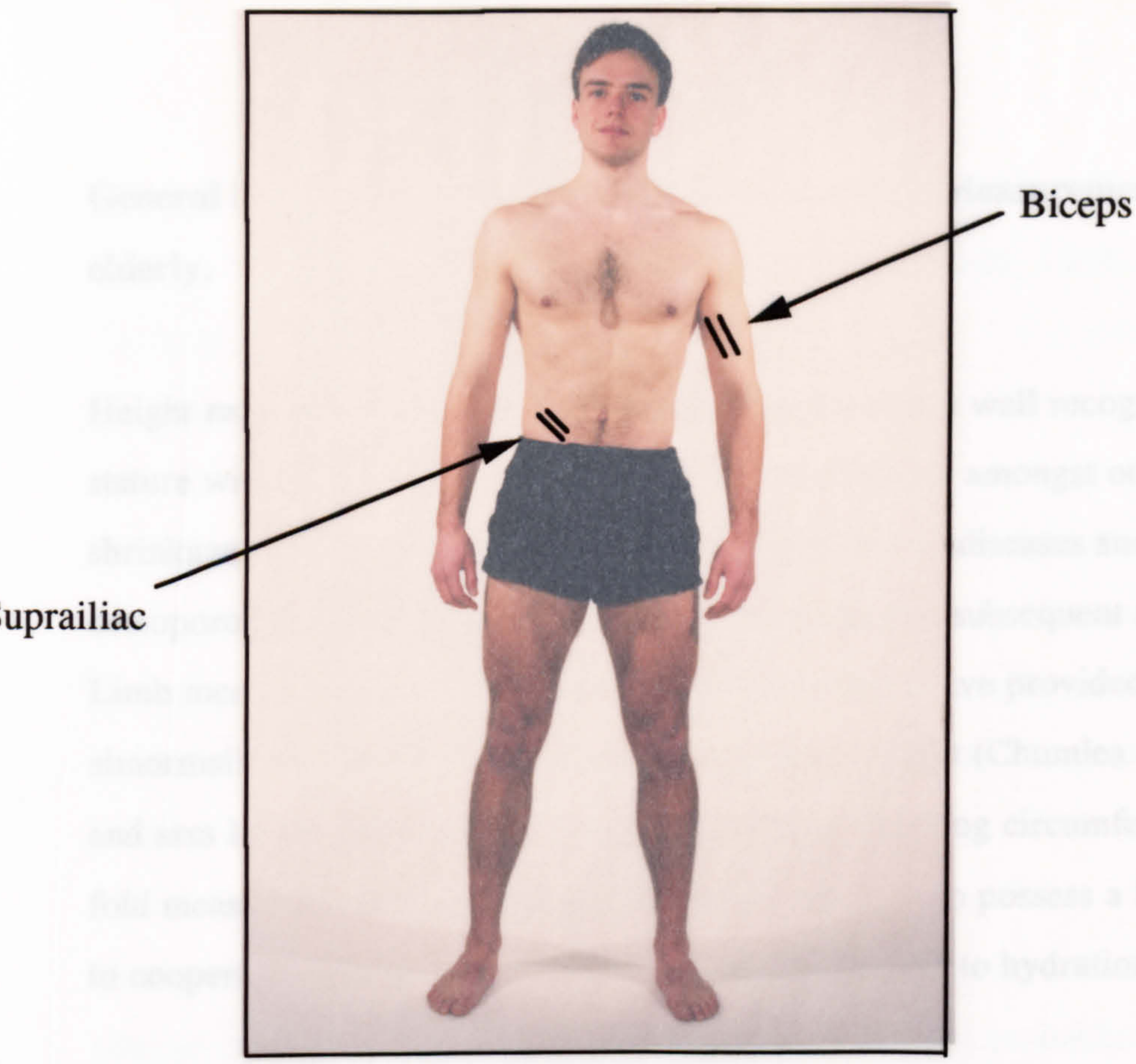
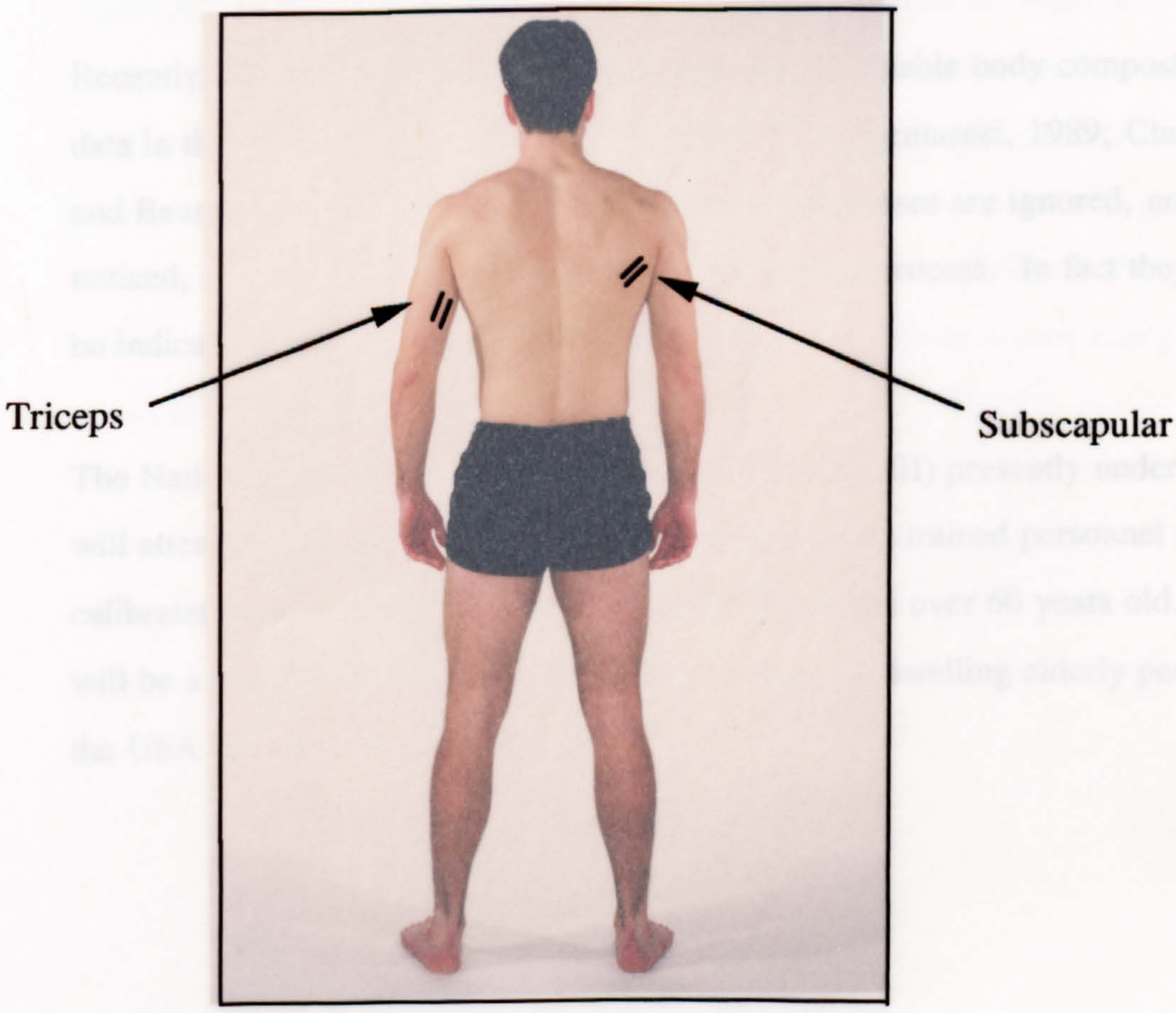


Plate 12: The Four Anatomical Sites of TSF Measurement



General Problems associated with Anthropometric Measurements in the elderly.

Height measurements are of limited use since there is a well recognized loss of stature with increasing age. This appears to be due to, amongst other things, shrinkage of the vertebrae and postural changes due to diseases such as osteoporosis resulting in increased fracture events and subsequent malformation. Limb measurement would appear to be a good alternative provided no gross abnormalities exist. Measurement includes knee height (Chumlea et al, 1985) and arm length (Mitchell and Lipschitz, 1982b). Making circumference and skin fold measurements is difficult in demented patients who possess a limited ability to cooperate. Weight measurements can also vary due to hydration status.

New Developments

Recently there has been concern about the need for reliable body compostition data in the elderly population over 75 years old (Kuczmarski, 1989; Chumlea and Baumgartner, 1989). Too often sudden weight losses are ignored, not even noticed, or regarded as the part of the normal ageing process. In fact they may be indicative of a protein-energy malnutrition.

The National Health and Nutrition Survey (NHANES III) presently underway will attempt to obtain accurate measurement data using trained personnel and calibrated equipment from approximately 5180 persons over 60 years old. These will be a representative sample of healthy community dwelling elderly people in the USA (Kuczmarski, 1989).

The data will not be available until 1994, when it is hoped it will serve as the baseline for longitudinal studies of diet and health (Woteki, 1988).

12.1.6 Nutritional Supplementation

Nutritional support may be offered to an elderly person as a highly concentrated food additive or substitute. There are a large number of products available including soluble powders which are mixed with foods, milky and fruit flavour drinks and savoury soups. The supplements vary in their composition, calories, protein, minerals and trace elements.

Ideally supplements should not suppress the appetite for the normal meals offered. For this reason, especially in hospital, they are probably best given at night-time when there is a longer meal-free period. A high nutrient density is preferable so the maximum energy content can be given in the minimum volume or mass (Elmstahl and Steen, 1987). A number of alternatives or flavours should also be offered. A study by Bolton et al (1990) compared short and long-term palatability of six commercial supplements in cancer patients. In general the milk-based products proved to be the most sustainable. 'Flavour fatigue' was recognized by Saukop and Calman (1979). This can by a very real problem especially in elderly patients.

12.2 Aims

The aim of this section work was to investigate the effect of improved nutrition on the activity of various blood esterases in hospitalized frail elderly individuals who exhibited a low body mass index (ratio of weight to height) and/or a poor nutritional intake (as determined from a record of food intake supervised by a state registered dietician). Anthropometric , physical and mental assessments were also made to determine those useful for monitoring changes in nutritional status.

12.3 Method

Frail elderly patients over 65 years old receiving long-term hospital care were recruited for the study by Dr H Wynne (Consultant Geriatrician). These patients had all been identified as having a reduced plasma esterase activity (one or more esterase measurement below the lowest value measured for the fit elderly group) in Chapter 11. Advice on the suitability of a patient regarding their dietary habits was sought from Catherine Hankey, a state registered hospital dietician. The patients were selected on the basis of a low body mass index which had to be visually judged due to the difficulty in obtaining an accurate measurement of stature (Chumlea and Baumgartner, 1989). They also exhibited a low Triceps Skinfold Thickness (TSF) as defined by McEvoy and James (1982). They used a fit elderly population in Newcastle upon Tyne as their reference range drawn from a similar socio-economic group as my subjects. In addition the subjects also showed a tendency towards a reduced plasma albumin concentration.

A single random number scale was used, allocating suitable individuals to the feed or control group on the basis of an odd or even number. A non-involved individual organized this procedure so that anyone involved in the selection process (myself, Dr Wynne and Miss Hankey) did not introduce bias.

A variety of supplements was offered to the feed group depending on individual preference, the details of which are shown in Table 12.2 . The control group continued to receive a hospital diet with no intervention.

Before the study period, a three day assessment was carried out for each patient. Anthropometric measurements were made which included weight on calibrated hospital scales in light indoor clothing. The scales were calibrated using 3x10 kg lead weight. TSF measurements were made in triplicate using Harpenden calipers on the right arm unless this was deformed in some way, in which case the left one was used. Readings were made at the mid-point between the acromion process and the olecranon process. Mid arm circumference was measured using a flexible tape measure at the same point.

A mental test score (Crichton Index) and physical assessment was also made (an example of some of the questions asked is shown in Fig. 12.1) .

Blood for esterase analysis was collected and treated as before (Chapter 11). A full biochemical and haematological profile was also obtained .

These measurements were all repeated at 4 weeks and 8 weeks.

Table 12.2

A Summary of the Composition of Supplements used

Supplement	Presentation	Constituents
Maxijul	Dry soluble powder. no flavour mix in with food	glucose polymer K+,Na+ 100g=360Cals
Buildup	Dry powder , mix with whole or skimmed milk, variety of sweet flavours	fat,carbohydrate, protein, vitamins and minerals. 325Cals/ serving (whole milk)
Fortisip	liquid milk based drink, variety of sweet and savoury flavours	carbohydrate, fat protein 300Cal/ serving
Provide	liquid water-based drink.Tropical fruit drink	high protein, low fat, carbohydrate, Na+,K+. 150 Cal/serving
Ensure	milk based drink. Sweet and savoury flavours	Carbohydrate, fat, protein, minerals, and vitamins. 253 Cals/serving

Figure 12.1

An Example of the Questions asked in the Crichton Royal Behavioural Scale

Questions answered by a nurse or careworker:

	DATE			
Communication				
Always clear, retains information	0	0	0	0
Can indicate needs, understands simple verbal directions, can deal with simple information	1	1	1	1
Understands simple verbal and nonverbal information, cannot indicate needs	2	2	2	2
Cannot understand verbal or nonverbal, cannot indicate needs information	3	3	3	3
No effective contact	4	4	4	4
Orientation				
Complete	0	0	0	0
Orientated in ward, identifies persons correctly	1	1	1	1
Misidentifies people and suuruondings but can find way around	2	2	2	2
Cannot find own bed or toilet unassisted	3	3	3	3
Lost	4	4	4	4
Abbreviated Mental Test Score (answered by patient: maximum score is 10)				
Age	1	1	1	1
Time (nearest hr	1	1	1	1
Year	1	1	1	1
Name of Place	1	1	1	1
Recognition of 2 persons	1	1	1	1
Birthday (date and month)	1	1	1	1
Date of World War 1	1	1	1	1
Queen's name	1	1	1	1
Counting backwards 20-1	1	1	1	1
Address (42, West Street)	1	1	1	1

12.3.1 Provision of Supplements

Maxijul was supplied as individual named and dated bags which contained 140 g of dry powder equivalent to 500 calories. Each bag contained the maximum amount of powder to be included in one days calorific intake. Unused powder was weighed and the amount included in the patient's food could be calculated.

Other supplements were offered twice daily. Most patients tolerated Build-up. Those who didn't were offered one of the alternatives until a preference was found.

12.3.2 Intra-Individual Variation in Plasma Esterase Activity

This study took place over a nine month time period. During this time there may be an intra-individual variation of plasma esterase activities due to environmental and biological factors other than dietary. The patient group which did not receive dietary supplementation during this time controlled for these factors. In addition, blood samples were taken from a young volunteer throughout the nine-month period and an intra-individual coefficient of variation was obtained, shown in Table 12.3.

Table 12.3

The Intra-individual Variation of Blood Esterases During a Nine Month Time Period

Assay	RBC Esterase	Plasma Aspirin Esterase	Cholin-esterase	Phenyl-acetate Esterase	Paraoxon-ase	RBC acetyl-cholin-esteras
CV% (n)	9.6 (4)	8(4)	8(5)	3(4)	11(4)	6(4)

n= number of samples taken over a nine month period

12.3.3 Collation of Dietary Data

When collecting a three day food intake record, it is necessary to standardize the food portion size. This was validated by weighing food portions in the main kitchen of the hospital. When the details, including food type (eg yoghurt, mashed potato, glass of lemonade) and portion size (weight, volume) are entered onto the computer a comprehensive estimation of the amount of macro - and micro - nutrients in the food is provided. These are summarized for a whole days' intake when the entry is complete.

The software package used was Salford University's Microdiet system. This uses 'The Composition of Foods' (McCance and Widdowson, 1960) as the basis of the nutritional data.

12.3.4 Statistics

ANOVA was used to compare the means of the three groups. For repeated measures on the same group, ANOVA for repeated measures was used (an equivalent of the paired t-test). An unpaired t-test was used to compare the mean value of the feed groups with the equivalent value in the control group (see Appendix III)

12.4 Results

Dietary

The clinical details of those patients who entered the study are shown in Table 12.4. They are the same patients recruited in Chapter 11. Table 12.5 shows the average daily intake of all major nutrients in the feed group (normal daily intake as hospital meals plus supplemental feeding) compared with the 'control' group (normal daily intake as hospital meals only). These amounts however are only an indication of the nutrients offered to the patients since unconsumed food was not collected. Figures 12.2 and 12.3 show a comparison of the nutrient values before and after supplementation in the 'feed' and control groups.

Anthropometric

There was a trend towards a weight gain in the feed group (mean weight $\pm$ SEM increased from 45.8 $\pm$ 3.5 kg to 47.1 $\pm$ 3.5 kg) whilst the 'control' group remained constant (38.0 $\pm$ 2.6 kg to 37.8 $\pm$ 3.0 kg. See Fig 12.4). This was not matched by a similar trend in TSF ('feed' mean $\pm$ SEM: 8.2 $\pm$ 1mm to 7.6 $\pm$ 1mm Fig 12.5) or MAC ('feed' mean $\pm$ SEM: 19.5 $\pm$ 1.2cm to 19.3 $\pm$ 1.3cm Fig 12.6)and consequently AMC. The TSF values are much lower than those obtained by McEvoy and James (1982) (Table 12.6). This emphasizes the importance of using a single trained observer. All the anthropometric data is summarized in Table 12.7.

Table 12.4

Volunteer Details

Volunteer (sex)	Age (years)	Drugs (if any)
Feed		
6(F)	88	docusate,bendrofluazide,chlormethiazole, morphine
8(F)	86	chlormethiazole
10(F)	88	paracetamol,codeine
11(F)	85	dothiepin,diazepam
14 (M)	86	haloperidol,ranitidine, paracetamol.senna
15(M)	90	paracetamol, senna
16(F)	91	dothiepin, paracetamol, temazepam
Control		
1(F)	93	dothiepin, codeine
5(F)	85	
7(F)	88	
12(F)	86	codeine, micralax enema
13(M)	70	
17(F)	94	digoxin, pericyclazine, chlormethiazole
20(F)	96	

Table 12.5

Comparison of Average Daily Intake between Frail Elderly Patients Receiving Supplements and a Control Group

Nutrient	Average Daily Intake±SEM Feed group (after supplements at 8 weeks)	Control group (no supplements at 8 weeks)
carbohydrate	164.7±14.4g*	105.9±6.8g
fat	67.2±6.7g	59.3±6.2g
protein	58.4±6.1g*	40.2±4.5g
fibre	11±1.4g	7.9±1.1g
calcium	1210±168.7mg*	612±59.7mg
vitamin D	4.5±0.9mg*	1.4±0.3mg
vitamin B_{12}	3.8+0.6mg	2.5+0.3mg
vitamin B_6	6.1±1.4µg	0.5±0.1µg
vitamin E	6.3±0.6µg*	2.9±1.3µg
iron	10.1±1.2µg	7.9±1.1µg
riboflavin	2.9±0.8µg	1.1+0.1µg
nicotinic acid	16.7±5.6µg*	6.3±0.3µg
retinol	289.6±31.3mg	311.1±37.8mg
biotin	43.7±6.4mg*	25.3±4.0mg
carotene	1456±24.6mg	973±380 mg

* significant at 95% level

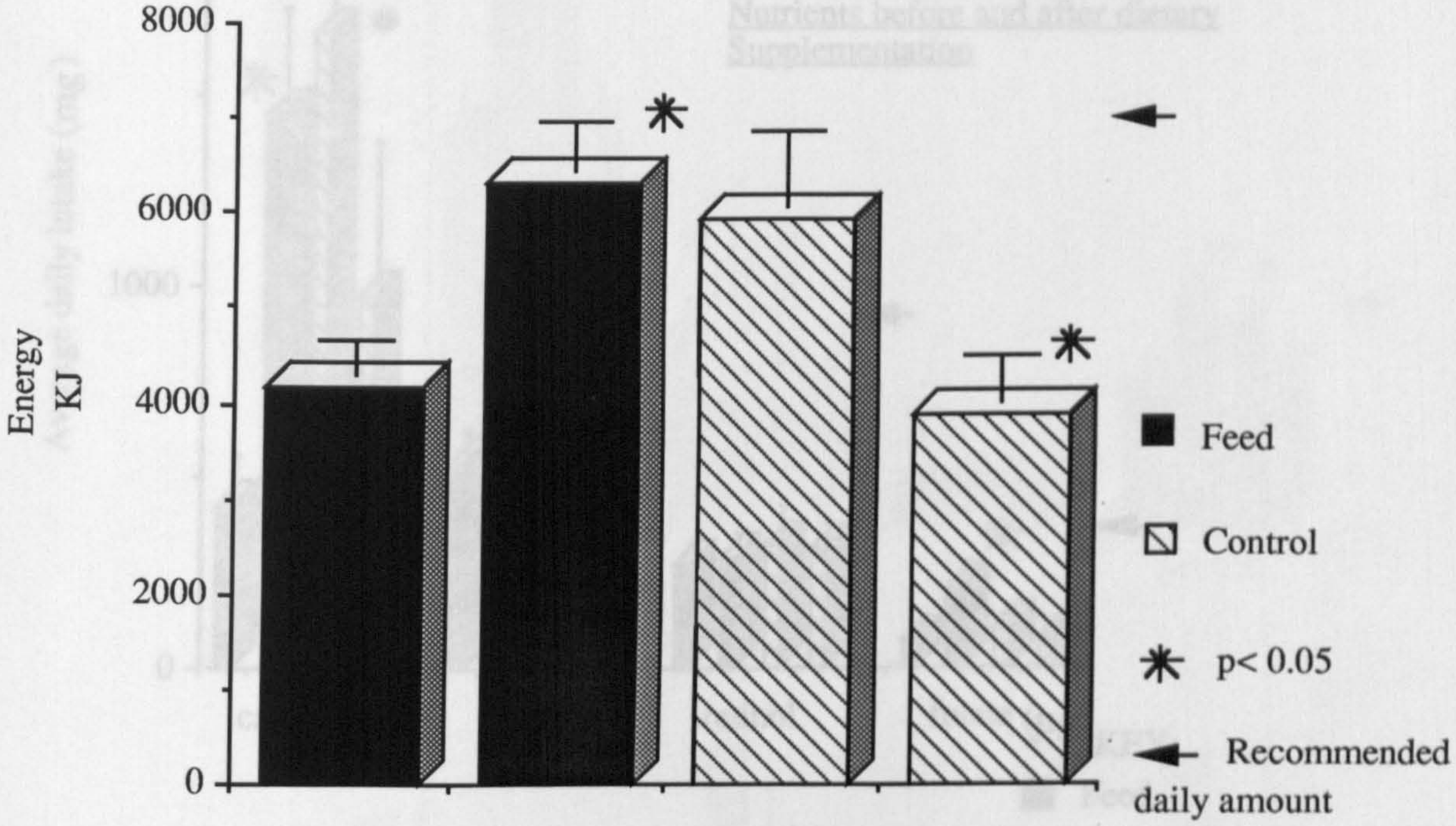


Figure 12.2

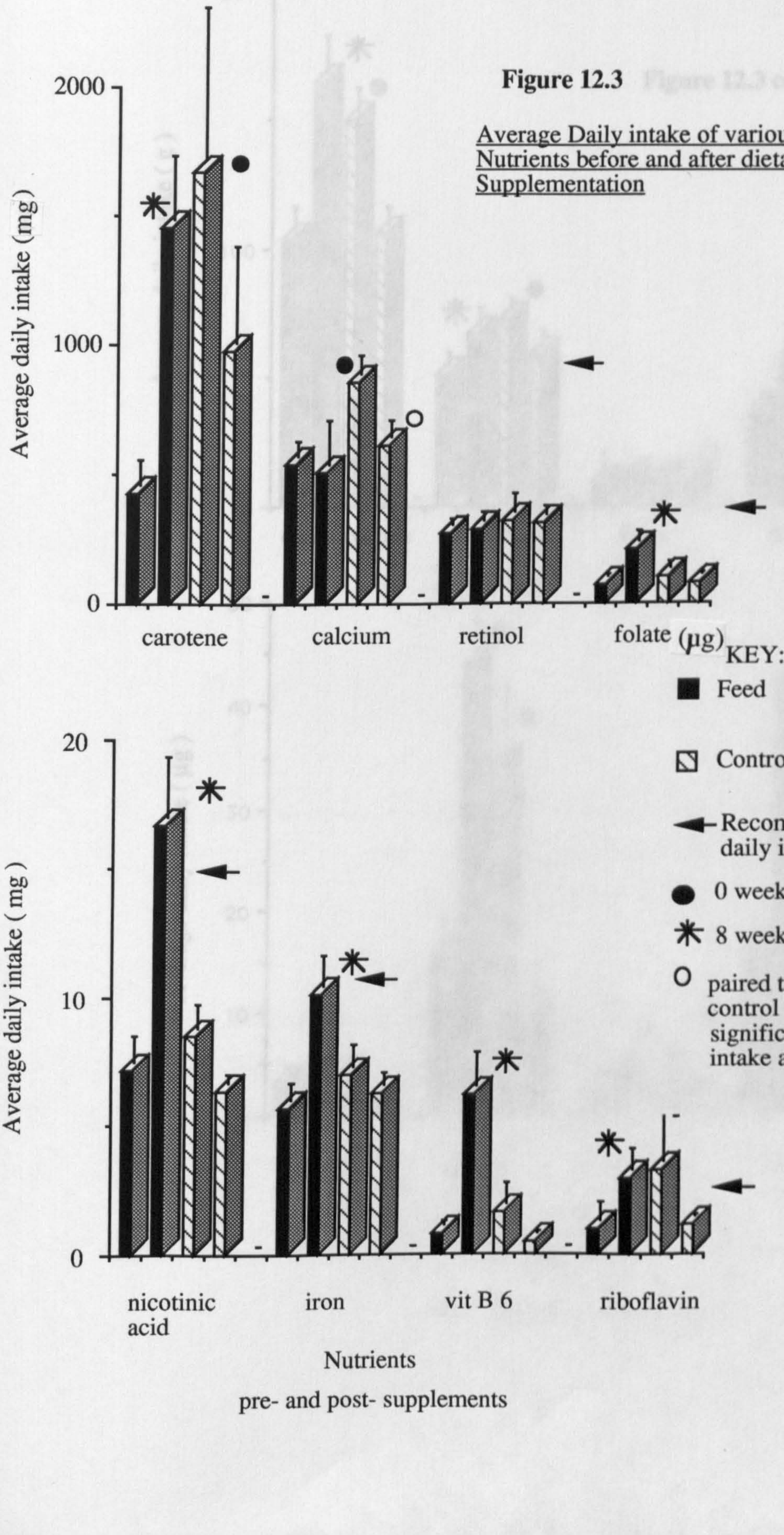
Title : Average daily energy intake before and after dietary supplements : comparison of feed and control groups.

Ordinate : Average daily intake ± SEM

Abscissa : Before and after 8 week study period

Figure 12.3

Average Daily intake of various Nutrients before and after dietary Supplementation



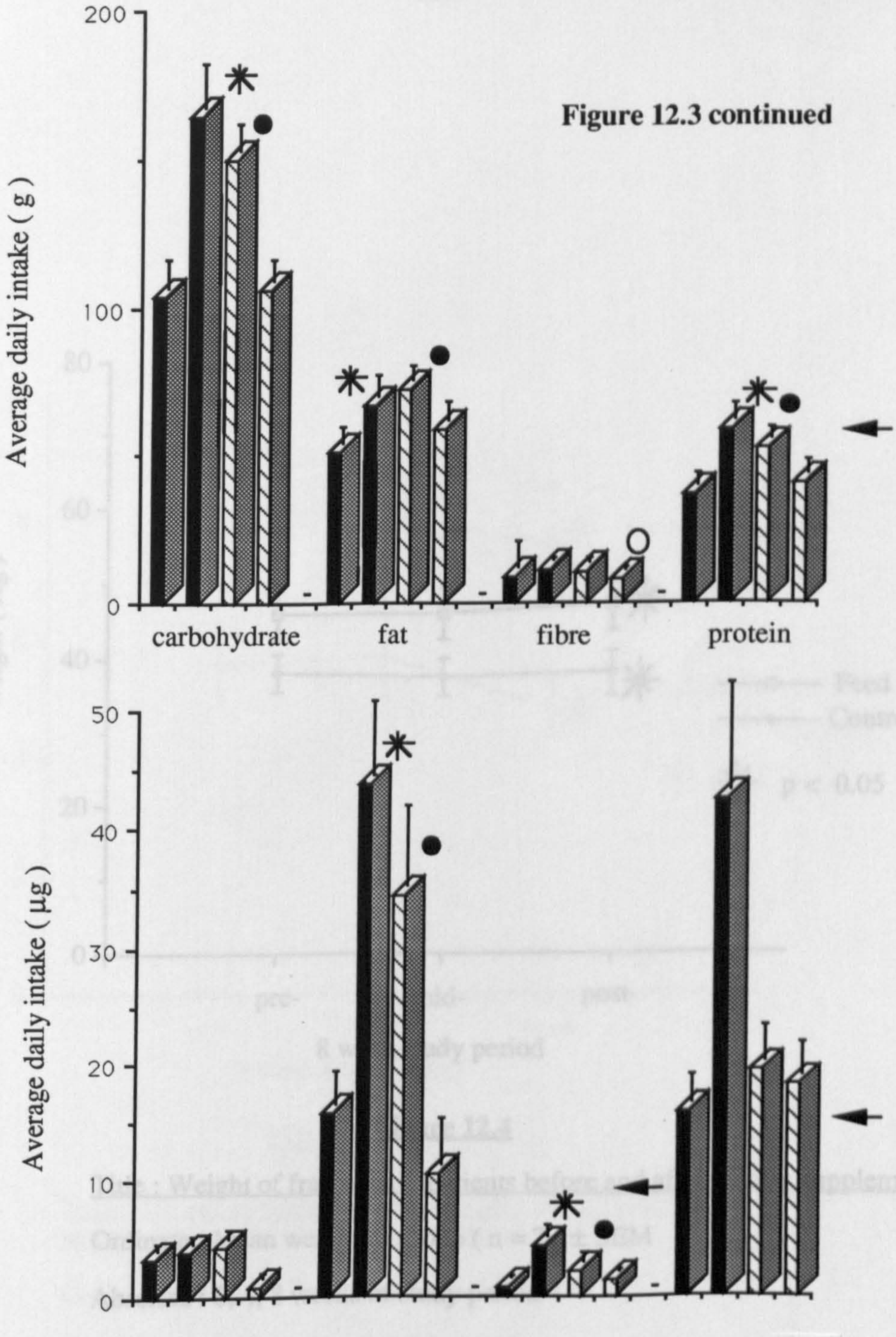


Figure 12.3 continued

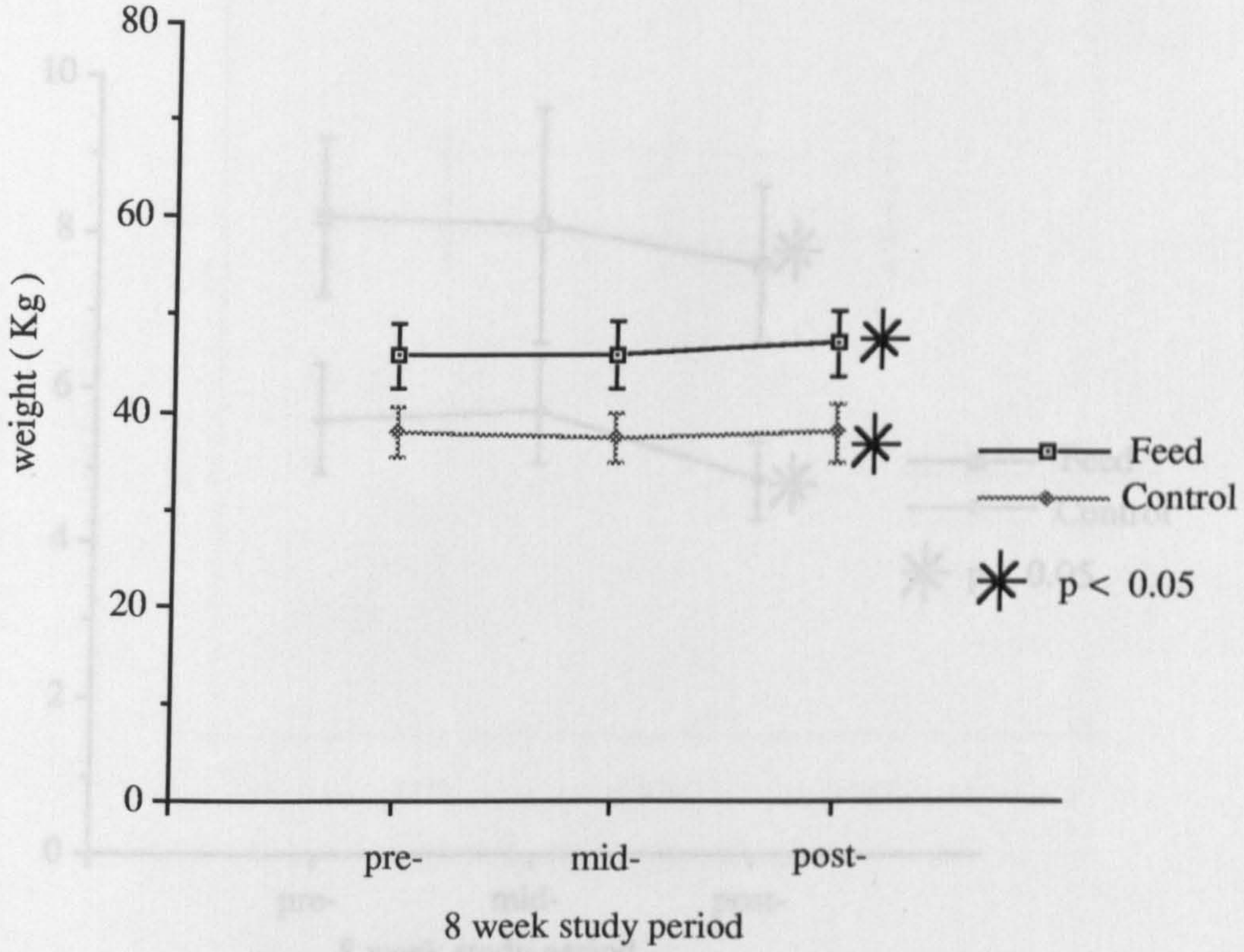


Figure 12.4

Title : Weight of frail elderly patients before and after dietary supplements

Ordinate : Mean weight of group (n = 7) ± SEM

Abscissa : 0, 4, 8 weeks of study period

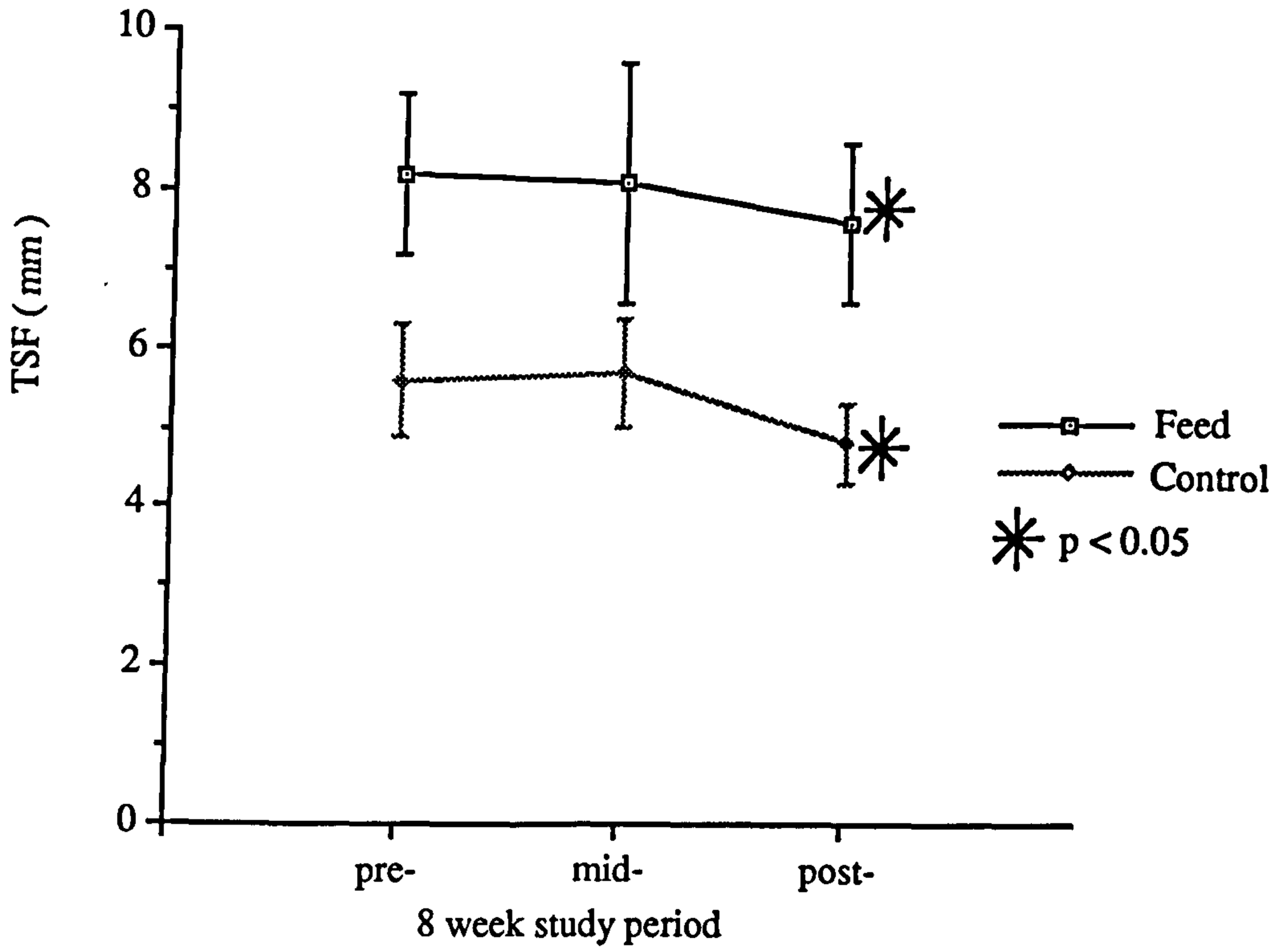


Figure 12.5

Title : Triceps skinfold thickness (TSF) in frail elderly patients before and after dietary supplements

Ordinate : Mean TSF of group (n = 7) ± SEM

Abscissa : 0, 4 , 8 weeks of study period

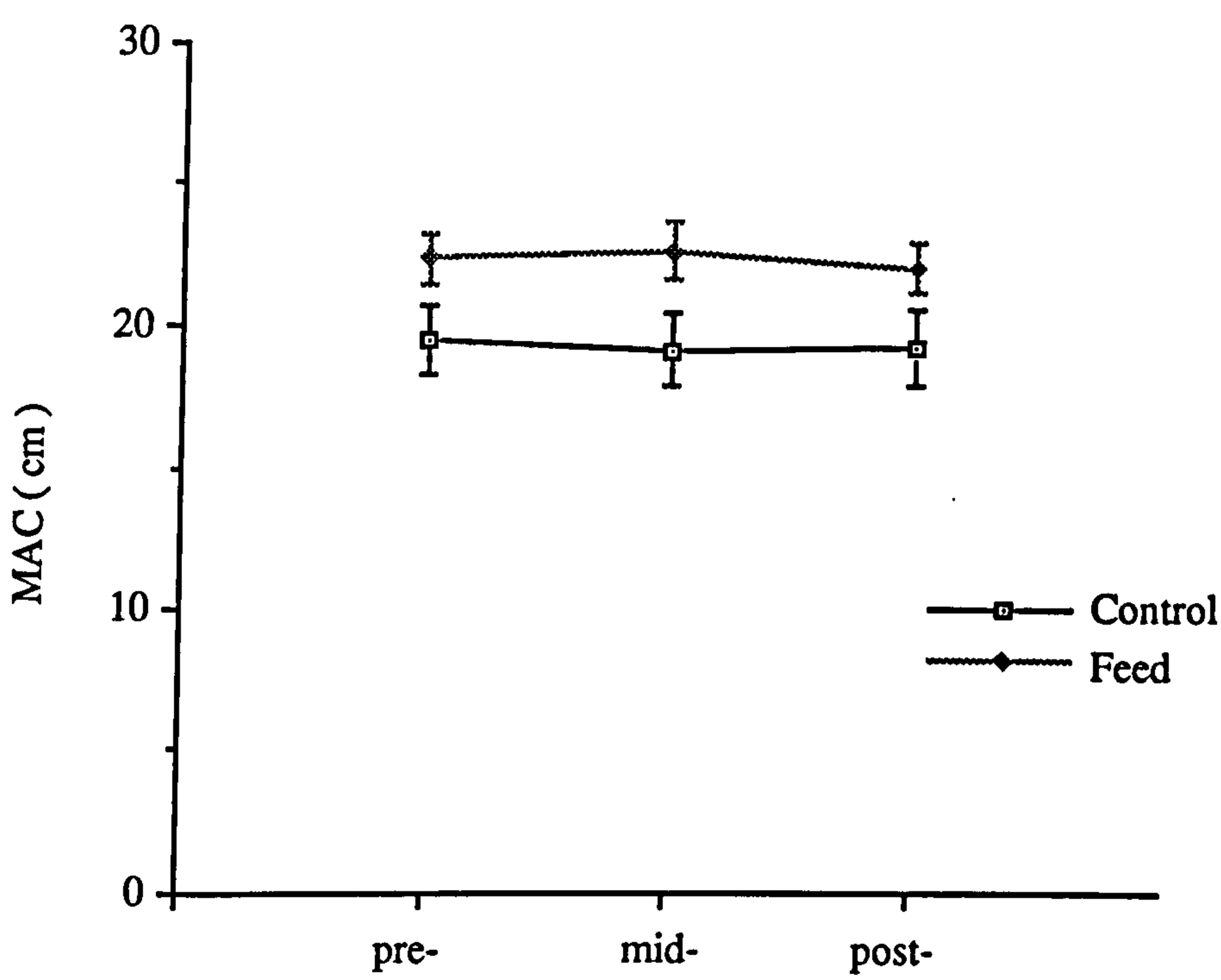


Figure 12.6

Title : Mid - arm circumference (MAC) in frail elderly patients before and after dietary supplements.

Ordinate : mean MAC of group (n = 7) ± SEM

Abscissa : 0, 4, 8 weeks

Table 12.6

Anthropometric Standards from a Fit Elderly Population
(Mc Evoy and James ,1982)

	MALE		FEMALE	
	Mean	S . D .	Mean	S . D .
TSF (mm)	13.38	5.0	19.8	5.0
MAC (cm)	29.1	2.7	27.7	2.7
*AMC (cm)	24.9	2.2	21.5	2.6

* AMC= Arm Muscle Circumference

where

AMC = MAC - (TSFx0.314)

Table 12.7

Anthropometric Measurements at 0 ,4 and 8 weeks

	weight (Kg)			TSF (mm)			MAC (cm)			AMC (cm)		
subject	pre	mid	post	pre	mid	post	pre	mid	post	pre	mid	post
FEED												
6	41.8	42	42.2	10.3	10	9.9	20.5	20.5	20	17.3	17.4	16.9
8	42.7	39	44.8	8	7.2	7.2	22.5	23	22	20	22.2	20.8
10	44.5	46.4	45.5	7.2	7.4	6.9	23	24.5	23	16.3	16.5	16.6
11	33.5	33.3	33.3	5.5	4.8	4.5	18	18	18	23.2	22.9	22.8
14	55.1	54.5	56.2	5.7	5.2	5.4	25	24.5	24.5	22.7	23.2	23
15	61	60.9	61.5	7.3	5.5	5.9	25	25	24.5	25	25	24.5
16	42.5	45.2	46	13	12.8	12.6	23	23.5	23	23	23.5	23
mean ±SEM	45.8±	45.9±	47.1±	8.2±	8.1±	7.6±	22.4±	22.7±	22.1±	19.9±	21.5±	21.1±
	3.5	3.5	3.5	1	1.5	1	0.9	1	0.9	0.9	1.1	1.1
CONTROL												
1	31.9	32.4	31.7	4.5	5	4	18	18	18.5	16.6	16.4	17.2
5	34.2	32.6	32.6	4.2	4	4	18.5	17.5	17.5	17.2	16.2	16.2
7	35	37.1	34.7	3.3	3.2	2.7	15.5	14.5	14.5	14.5	13.5	13.7
12	39.8	37.3	34.2	6.4	5.9	5.1	19.5	18	19	17.5	16.1	17.4
13	50	50.3	50.5	5.7	6.2	6.2	22	22	22	20.2	20.1	20.1
17	32.2	33.9	33.2	6.1	6.2	5.3	18	18	18.5	16.1	16.6	16.8
20	43.6	42.2	42.3	9.2	9.2	9.5	25	25	25	22.1	22.1	22
mean ±SEM	38.0±	37.3±	37.8±	5.6±	5.7+	4.8±	19.5±	19.2±	19.3±	17.7±	17.3±	17.6±
	2.6	2.7	3	0.7	0.7	0.5	1.2	1.3	1.3	0.9	1	0.9

Plasma Proteins

There was no significant change in albumin concentrations (mean albumin conc. g/l of feed group ± SEM: 33.7 ± 1.3 to 34.9 ± 2.4. Fig 12.7). The detailed esterase activities are shown in Table 12.8 and summarized in Table 12.9. There was a significant ($p < 0.05$; $F = 6.9$) increase in plasma cholinesterase activity in the feed group after 4 weeks (mean activity ± SEM for 'feed' increased from 944 ± 106 nmol benzoylcholine chloride hydrolysed/ml plasma/min to 1158 ± 140 nmol benzoylcholine chloride hydrolysed/ml plasma/min) but this decreased again after 8 weeks to 988 ± 140 nmol benzoylcholine chloride hydrolysed/ml plasma/min (Fig 12.8). Plasma aspirin esterase activity followed a similar profile of activity ('feed' mean plasma aspirin esterase activity ± SEM in nmol salicylate produced/ml plasma/min baseline value was 71.0 ± 8; at 4 weeks was 80 ± 7 and at 8 weeks was 71.9 ± 9. Fig 12.9) but did not show such a marked spread of activity.

Paraoxonase activity showed a trend towards increased activity at 8 weeks in the 'feed' group (mean paraoxonase activity ± SEM changed from 116.9 ± 9.5 nmol pnp/ml plasma/min at 0 weeks to 126.5 ± 5.3 at 4 weeks and 129.9 ± 10.5 at 8 weeks) and a decreased activity in the control group (99.4 ± 13.5 nmol pnp/ml plasma/min at 0 weeks to 106 ± 11.6 at 4 weeks and 94.2 ± 12.6 at 8 weeks. Fig 12.10). These two values were significantly different at 8 weeks ($p < 0.05$; $t = 2.3$).

Phenylacetate esterase activity showed a different pattern of activity, tending towards a reduced activity in both groups at 8 weeks. (In feed group mean value reduced by 3.1 nmol phenol/ml plasma/min and in control group it reduced by 5.9 nmol phenol/ml plasma/min. Fig 12.11).

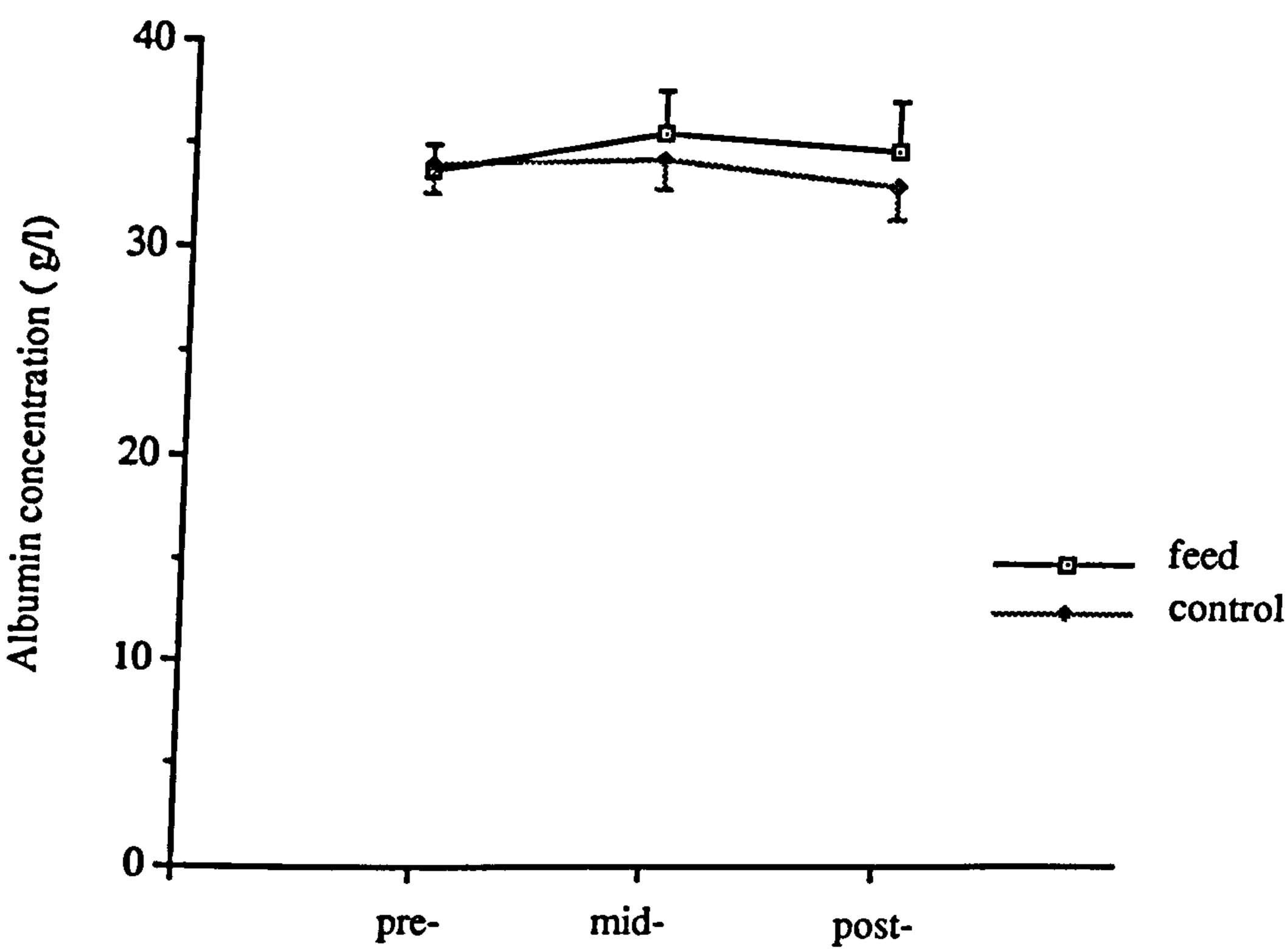


Figure 12.7

Title : Albumin concentration in frail elderly patients before and after dietary supplements.

Ordinate: Mean albumin concentration of group (n=7) ±SEM

Abscissa: 0,4,8 weeks of study period

Table 12.8			
	Esterase Measurements at 0, 4 and 8 weeks		
	of Study period in Feed and Control Groups		

Volunteer	Albumin	Aspirin esterase	Cholinesterase
	g/L	nmol salicylate	nmol benzoyl
		/ml plasma /min	choline chloride
			/ml plasma/min

FEED	pre	mid	post	pre	mid	post	pre	mid	post
6	29	28	24	56	50	31	685	654	485
8	37	42	39	87	104	85	1227	1480	1285
10	30	34	34	85	87	87	865	1373	1103
11	33	29	30	35	58	42	538	606	529
14	38	41	43	69	87	85	1061	1204	1150
15	34	38	37	67	87	75	923	1415	1120
16	35	37	37	98	87	93	1312	1373	1247
CONTROL									
1	34	33	33	60	63	56	788	868	879
5	39	39	27	75	63	25	1030	879	458
7	33	29	32	56	60	54	588	705	758
12	29	31	27	46	62	33	636	879	485
13	37	36	37	87	71	83	1135	986	1079
17	35	35	36	77	67	60	916	980	905
20	31	37	38	44	56	65	602	879	876

Table 12.8 continued

Esterase Measurements at 0, 4 and 8 weeks of Study Period in Feed and Control Groups

Volunteer	Phenylacetate esterase µmol phenol/ml plasma min			Paraoxonase nmol pnp/ ml plasma/ min			RBC Esterase nmol phenol/ 10^6 RBC/min			RBC AChE nmol thiocholine/ mg Hb/min		
FEED	pre	mid	post	pre	mid	post	pre	mid	post	pre	mid	post
6	81	75	58	121	108	110	110	111	108	34	44	43
8	99	117	98	161	148	167	214	198	229	35	36	33
10	74	81	73	87	133	143	122	149	114	38	45	39
11	70	64	56	107	133	97	82	106	112	43	41	37
14	76	79	79	137	134	164	134	134	129	22	23	23
15	53	78	70	99	118	119	92	125	110	29	22	22
16	54	59	58	107	114	110	89	115	91	22	26	27
CONTROL												
1	96	91	85	152	147	144	189	167	181	43	43	44
5	85	82	41	138	138	45	135	115	118	45	45	39
7	44	34	41	61	81	79	134	113	118	31	35	37
12	37	53	41	67	80	76	119	122	127	35	36	38
13	58	52	57	109	102	110	156	149	138	21	25	26
17	79	72	76	97	127	124	170	172	126	27	26	17
20	36	47	54	72	71	82	NA	119	126	NA	24	29

Table 12.9

A Summary of the Results in Table 12.8 Showing the Mean Values of Esterase Measurements.

Esterase	Feed Group Mean ±SEM			Control Group Mean ±SEM		
	pre-	mid-	post-	pre-	mid-	post-
[1]Aspirin Esterase	71±8	80±7	71±9	64±6	63±2	53±7
[2]Cholin-esterase	944±106	1158±140	988±140	841±92	882±35	777±87
[3]PhAc Esterase	73±7.2	79±7.1	70±5.6	62±9.3	62±7.8	56.3±6.8
[4]Paraox-onase	117±9.5	127±5.3	130±10.5	99±13.5	106±11.6	94±12.6
[5]RBC AChE	37±2.1 24±2.3	42±2.0 24±1.2	38±2.1 24±1.5	39+3.3 24±1.7	40±2.5 25±0.6	40+1.6 24±3.4
RBC Esterase	120±17	134±12	128±17	146±10	137±10	130±9
[6]Albumin conc.	34±1.3	36±2.1	35±2.4	34±1.3	34±1.3	33±1.6

[1] nmol salicylate/ ml plasma /min

[2] nmol benzoylcholine chloride hydrolysed /ml plasma /min

[3] μmol phenol /ml plasma /min

[4] nmol thiocholine/mg haemoglobin/min (two values due to non-comparable substrate)

[5] nmol phenol/10^6 RBC /min

[6] g/L

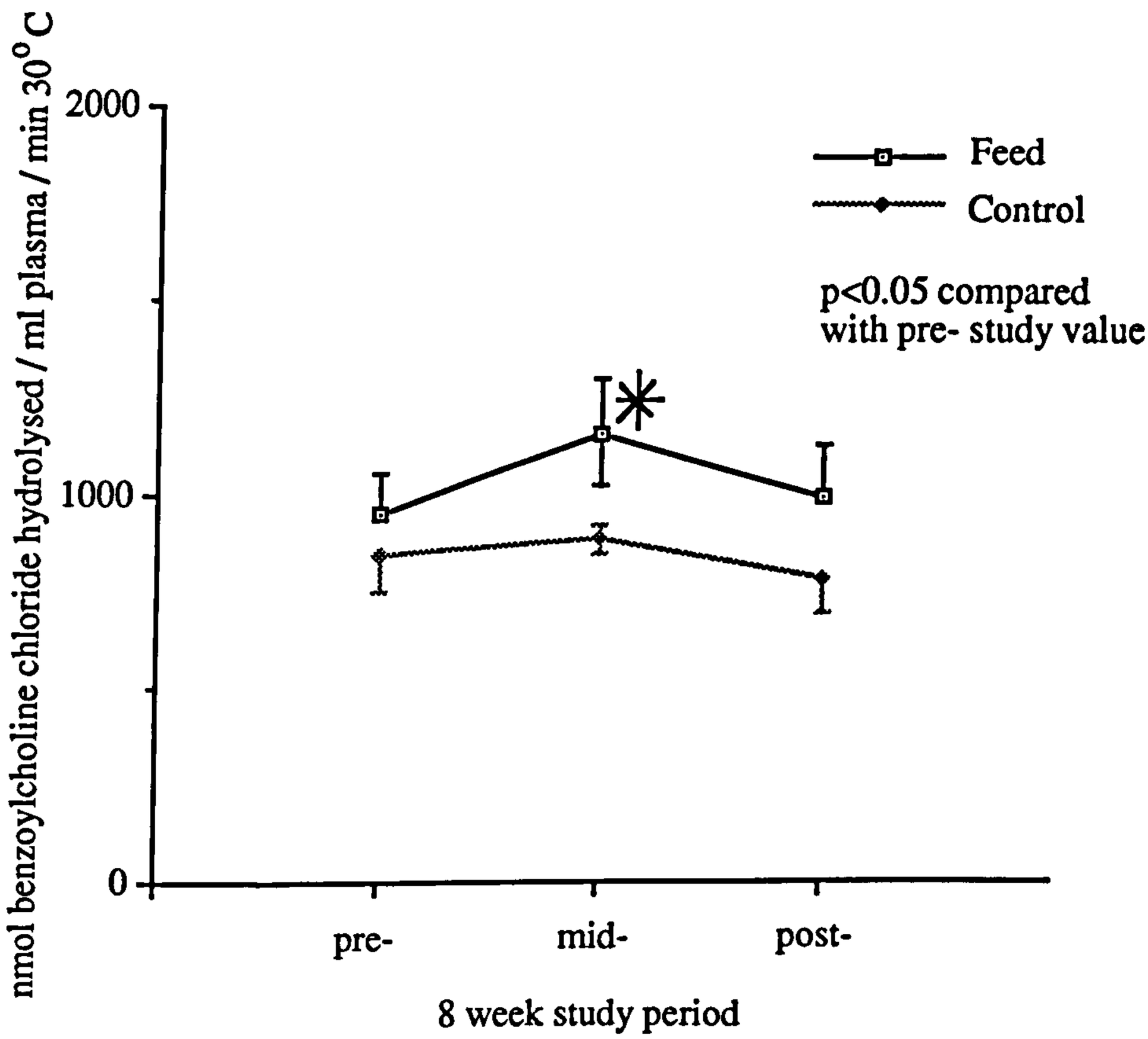


Figure 12.8

TItle : Plasma cholinesterase activity in frail elderly patients before and after dietary supplements.

Ordinate : Mean plasma cholinesterase activity of group (n=7) ± SEM (30^0 C)

Abscissa : 0, 4, 8 weeks of study period

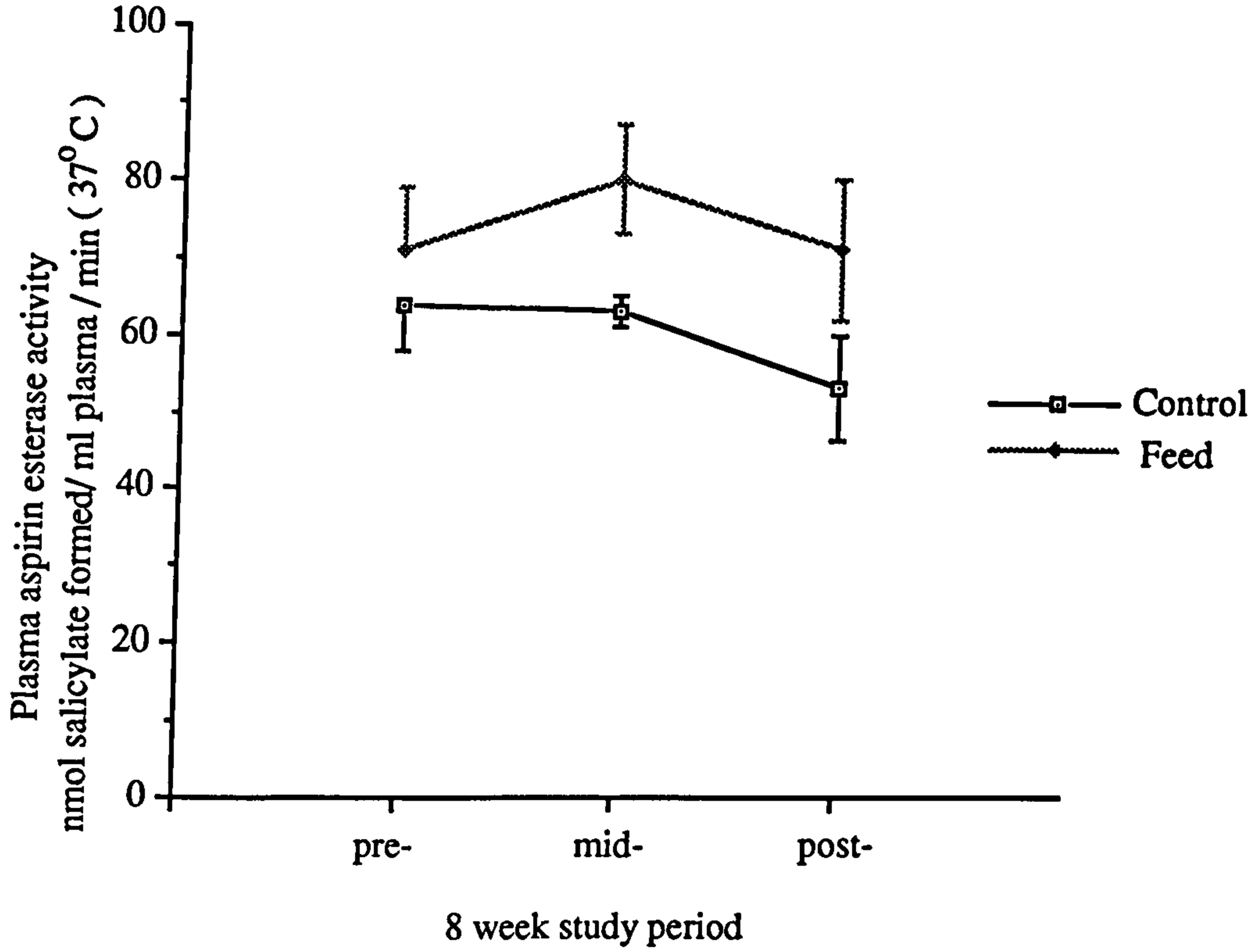


Figure 12.9

Title : Plasma aspirin esterase activity in frail elderly patients before and after dietary supplements.

Ordinate : Mean plasma aspirin esterase activity of group (n = 7) ± SEM (37^0 C)

Abscissa : 0, 4, 8 weeks of study period

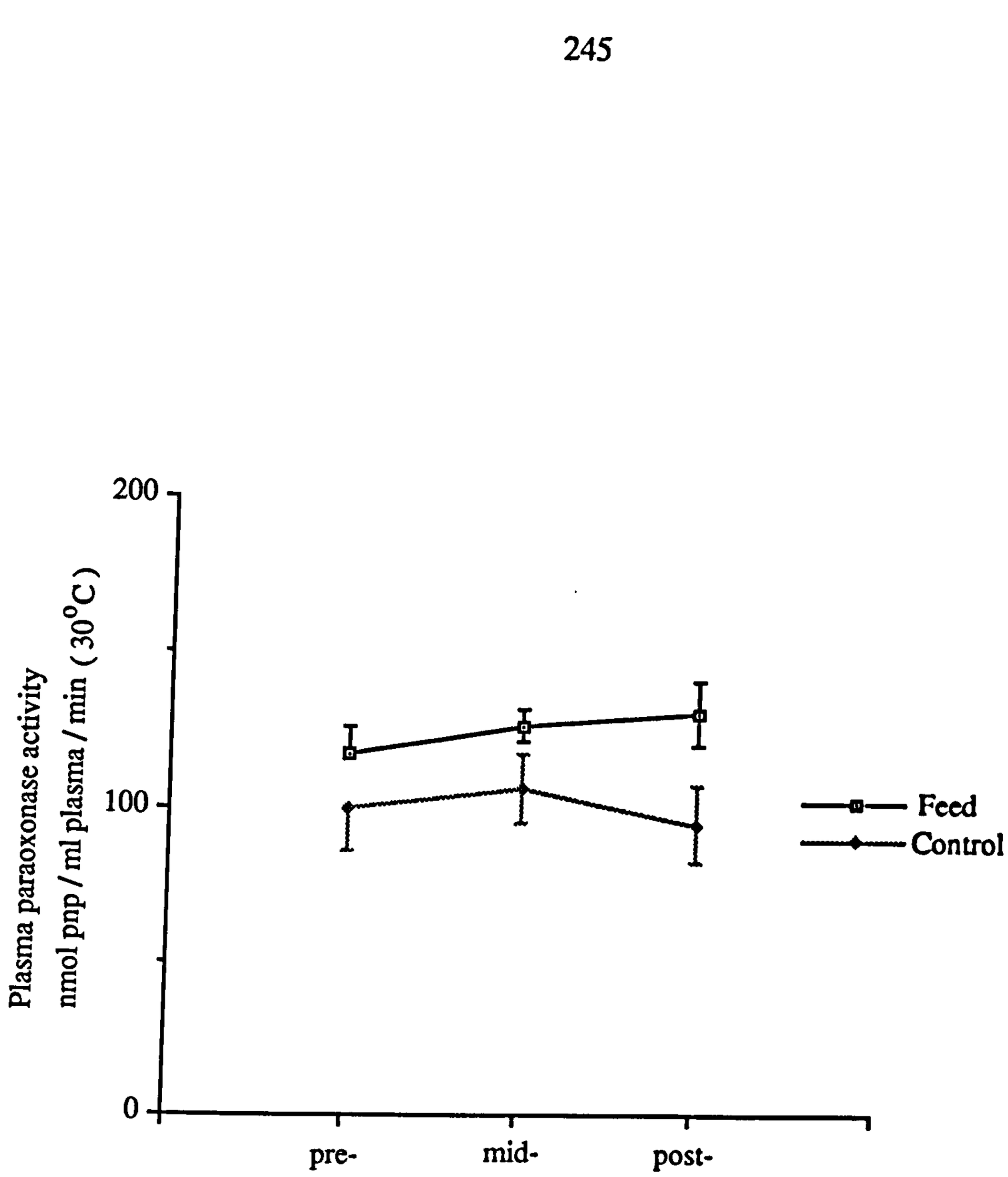


Figure 12.10

Title ; Plasma paraoxonase activity in frail elderly patients before and after dietary supplements.

Ordinate : Mean plasma paraoxonase activity of group (n = 7) ± SEM (30^0C)

Abscissa : 0, 4, 8 weeks of study period

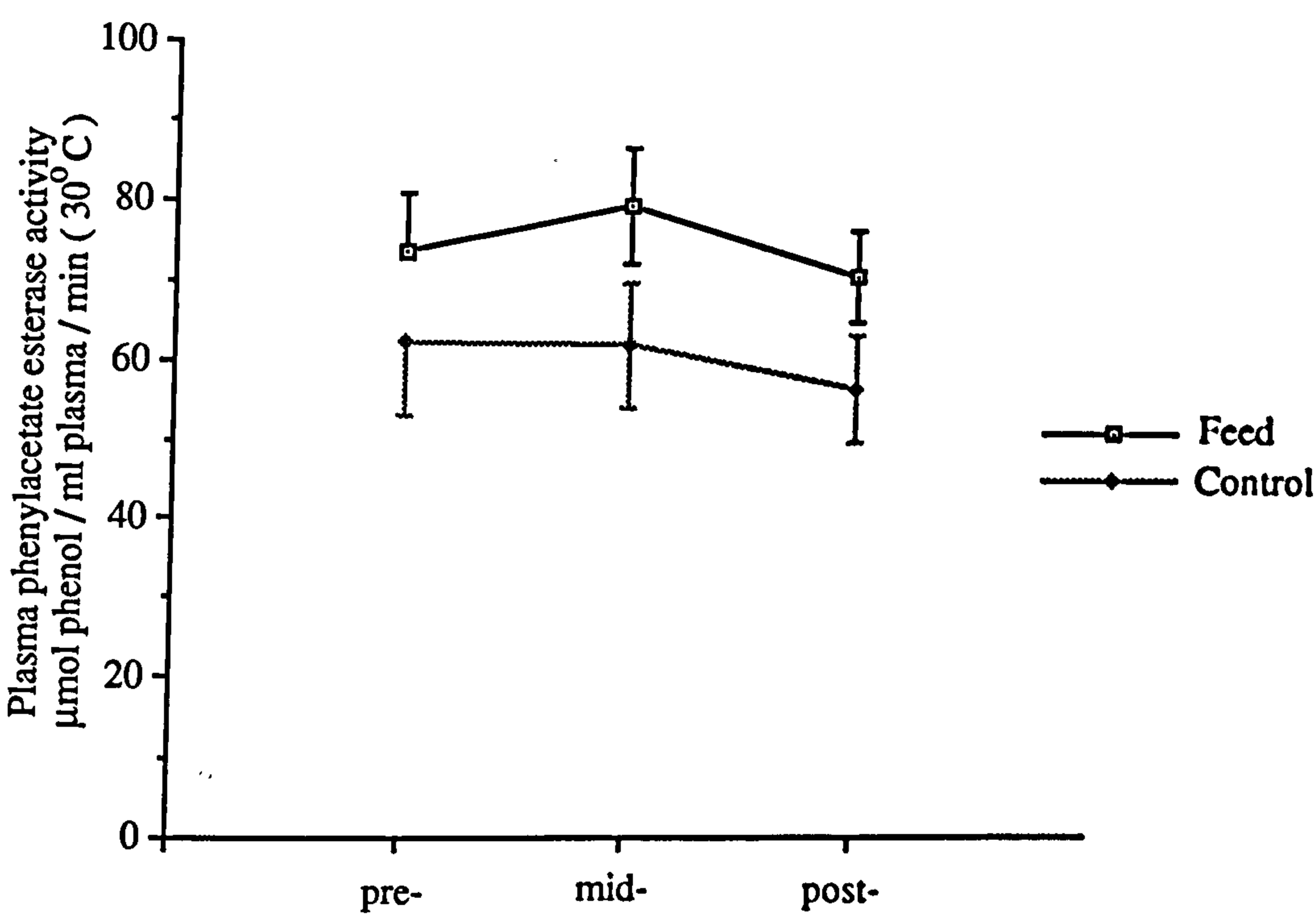


Figure 12.11

Title : Plasma phenylacetate esterase activity in frail elderly patients before and after dietary supplements

Ordinate : Mean phenylacetate esterase activity of group (n = 7) ± SEM (30^0 C)

Abscissa : 0, 4, 8 weeks of study period

Red Blood Cells

These results are shown in Fig 12.12, 12.13 and Table 12.8. There was no significant change in red blood cell esterase activity expressed as nmol phenol/106 RBC/min in the feed or control groups. The feed group did show a trend towards improved esterase activity (mean activity $\pm$ SEM at 0 weeks 120 $\pm$ 17; at 4 weeks 134 $\pm$ 12; at 8 weeks 128 $\pm$ 17) which was not apparent in the control group (146 $\pm$ 10 at 0 weeks; 137 $\pm$ 10 at 4 weeks; 130 $\pm$ 9 at 8 weeks). The number of red cells, however did not change, over this period (Table 12.10).

Due to two batches of non-comparable substrate, numbers were reduced in the measurement of RBC acetylcholinesterase. Nevertheless, no change in activity was apparent over the eight week period (Fig. 12.12).

Table 12.11 shows that there was no improvement in physical or mental functioning after 8 weeks.

Although randomly selected, the control group showed reduced anthropometric and esterase levels compared with the group selected for feeding. These differences were not statistically significant at week 0, although TSF and weight values did become statistically significant at $p < 0.05$ at weeks 8 ($t = 2.3$ and $t = 2.3$ respectively).

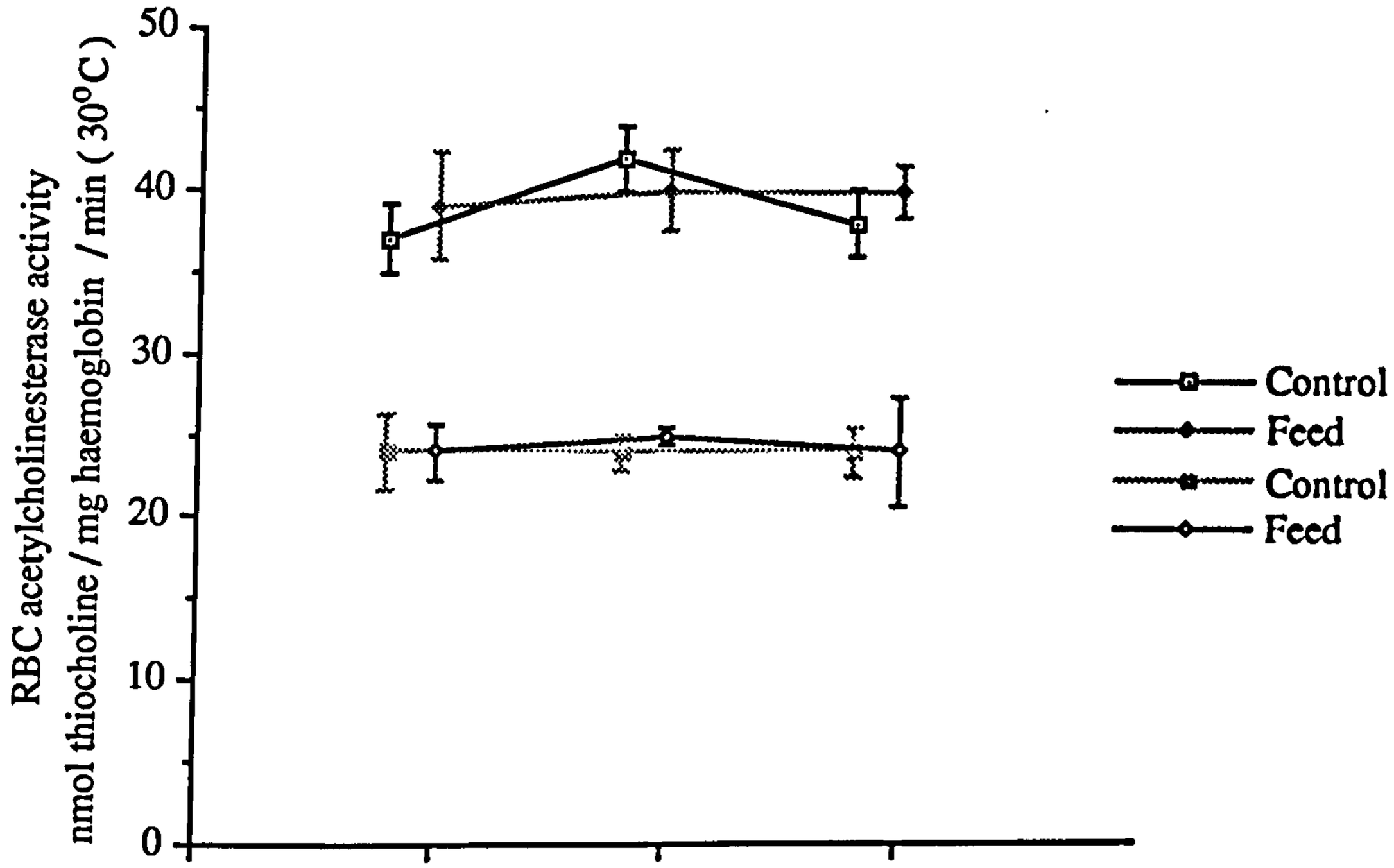


Figure 12.12

Title : Red blood cell (RBC) acetylcholinesterase activity in frail elderly patients before and after dietary supplements

Ordinate : Mean RBC acetylcholinesterase activity of group (n = 7) ± SEM (30^0 C)

Abscissa : 0, 4, 8 weeks of study period

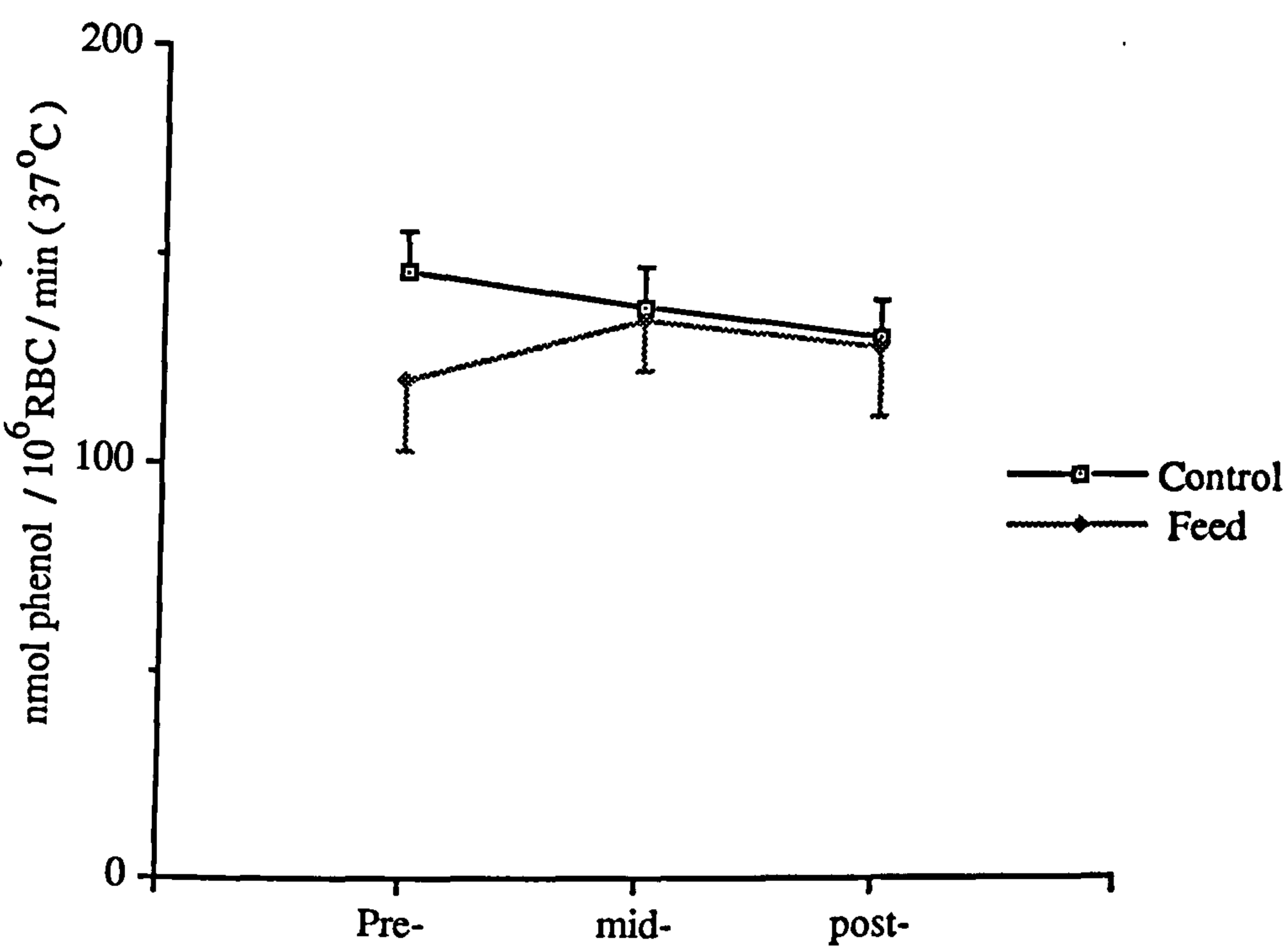


Figure 1213

Title: Red blood cell esterase (RBC) esterase activity in frail elderly patients before and after dietary supplements

Ordinate : Mean RBC esterase activity of group (n = 7) ± SEM(37^oC)

Abscissa : 0, 4, 8 weeks of study period

Table 12.10

RBC Numbers before and after Study Period in 'Feed' and 'Control' Groups

Volunteer	RBC x 10^{12} /l week of study	
Feed	0	8
6	4.14	4.17
8	4.43	4.71
10	3.84	2.88
11	3.15	3.15
14	3.50	3.22
15	4.43	4.32
16	4.64	4.69
Mean± SEM	4.0±0.2	3.9±0.3
Control		
1	4.26	4.33
5	3.83	4.81
7	3.75	4.25
12	4.07	4.07
13	4.9	4.79
17	3.50	3.69
20	3.63	4.25
Mean ±SEM	4.5±0.2	4.3±0.1

Table 12.11

Mental and Physical Assessment Results of Frail Elderly Patients.
A comparison of results before and after dietary supplements

Volunteer	Pre-study value		Post-study value	
Control	Mental	Physical	Mental	Physical
1	0	26	0	26
5	2	27	2	25
7	0	32	0	24
12	0	29	0	32
13	0	14	0	15
17	1	24	0	22
20	3	9	6	8
Mean ±SEM	1±0.5	23±2.9	1±0.8	22±2.8
Feed				
6	7	20	8	19
8	4	29	4	25
10	4	21	8	19
11	4	19	4	21
14	4	28	5	8
15	0	20	0	17
16	4	11	2	13
Mean ±SEM	4±0.7	20±2.1	4±1.0	17±1.9

Mental : maximum / minimum proficiency = 10/0

Intra-individual Variation of Blood Esterases

Over a nine month period there was considerable intra-individual variation in blood esterases measured (Table 12.3). The calculated coefficient of variation is a composite of intra-individual variation and interassay variation. The 'non-feed' group is used to control for the former. The latter is controlled for by using an interassay quality control which is plasma of a known activity. The CV for each assay is given in the methods.

12.5 Discussion

It was only possible to obtain an estimate of increased nutrient intake in the feed group based on the quantity of Maxijul used and drug record charts of Build-up accepted (but not necessarily all consumed by the patient). Using the mean weight gain of 1.3 kg; the value 0f 37 KJ/g fat (DHSS, 1979b) and the study period in days (56 days) it is possible to estimate the mean increase in KJ consumed per day. In this case the value is 859 KJ/day.

Banerjee et al (1978) found that supplemented patients in their study using Complan (a similar supplemental drink) were selectively reducing their intake of fat and carbohydrate.

McEvoy and James (1982a)also used Build-up in order to determine the feasibility of supplementation on an acute geriatric unit. They recorded a significant weight increase over a four week period which was due to an increase in fat rather than muscle mass. I observed a trend towards an increased weight gain over the 8 weeks but no similar trend in TSF or MAC. (In old age, fat is

centrally rather than peripherally sited (12.1.5). This discrepancy may be due to the difference in the elderly population I used compared with McEvoy and James (1982a) They used a population which may not have been clinically stable since they were in an acute rather than long-stay geriatric ward.

The frail elderly people in my study were taking more prescribed medication than the fit elderly and younger people. The effect of drugs on plasma esterase activities is largely unknown. Gupta et al (1979) investigated the effect of a limited range of drugs commonly ingested with aspirin including codeine, dextropropoxyphene, propranolol and cimetidine which all inhibited plasma aspirin esterase, and many others which had no effect. This is discussed in greater detail in Chapter 2.

Drugs may also result in malabsorption of nutrients (eg folate by sulphasalazine or calcium by tetracycline), utilization (eg folate uptake into red cells is inhibited by salicylates) or increased vitamin catabolism (eg of vitamin D by anticonvulsants). In addition microbial overgrowth of the bowel (McEvoy et al, 1983) and disease state (from overt malabsorption syndromes to diabetes) are more common in the elderly.

Albumin concentrations did not change. The relative merits of this protein as an indication of poor nutritional status has been discussed in 12.1.4.

No significant changes were seen in any of the blood esterase activities except for a rise in plasma cholinesterase activity at 4 weeks ($p < 0.05$). This esterase may be the most sensitive to changes in nutrient status since it has been closely related to lipid metabolism (5.4) and has an estimated half-life of 12 days. The

substrate is also very specific which may improve the sensitivity of the assay to dietary changes.

Paraoxonase activity is also measured using a specific substrate and again showed a trend towards increased activity in the 'feed' group. This profile was not followed by the arylesterase activity suggesting that the two esterases are not the same enzyme.

The RBC have a much longer life span (approximately 120 days) thus it was very unlikely any change in enzyme activities would occur over this time period. As discussed earlier, reduced RBC esterase activities may be a symptom of chronic subclinical anaemias rather than a reduced esterase level *per se*.

Mental and Physical Assessments.

Many of the people in the study were mentally and/or physically restricted. No significant change, as measured by the Crichton Index was detected after 8 weeks of supplemental feeding. Goodwin et al (1983) found a significant association between low blood levels of vitamin C, B12, folate and riboflavin and poor scores of cognitive function in a group of healthy elderly. Other authors have also found links between vitamin depletion and mental functioning (Strachen and Henderson, 1965, 1967) but these do not appear to be reversible with vitamin supplementation. (Beaumont and James, 1985). Tucker et al (1990) correlated electroencephalographic (EEG) indices with cognitive performance and nutrient status. It is not possible to decide whether these relationships reflect cause or effect.

12.5.1 Problems Encountered During the Study.

As with many clinical studies there were a number of problems due to the numbers of people involved.

Nursing Staff

This study depended quite heavily on the goodwill of nursing staff at a long stay geriatric hospital. A constant presence was not maintained at the hospital, although regular visits were made by clinicians and the dietician. I also made daily visits during this period to renew supplies and give encouragement. It was emphasized to staff during this time of the importance of maintained use of the supplements. Despite this, use of the Maxijul was especially sparodic depending on the duty staff. Initial problems centred around the practice of mixing the Maxijul powder with food or drink in front of the patient with subsequent refusal of both. This made the nurses reluctant to use it.

Patients

Maxijul was well tolerated by most patients when it was included in their diet. Some more cognitively astute patients claimed they could taste it, which was particularly a problem if they saw the nurse mix it with their food.

Build-up was also very well tolerated. Finding a supplement for those who didn't like it was difficult for two reasons. Firstly, most supplements are milk based so the choice was restricted. Secondly these people tended to be naturally more wary of 'new' foodstuffs.

Patient Failure to Complete Study

This occurred for two reasons. Either the patient was discharged to a nursing home (although remaining clinically stable) or there was a rapid deterioration in their health and they died suddenly. This study took place over the winter months which may have augmented this problem.

Some of the patients who entered this study had very low plasma esterase activities. As discussed in Chapter 12, some of these were below the range of Williams et al (1989). This may have been an indication of declining protein synthesis and hence liver function since some of the patients with the lowest values failed to complete the study (compare Tables 11.2 and 11.3 with Table 12.4).

Anthropometric Measurements

Some of the problems encountered were purely of a practical nature. The calibration of hospital scales was imperative, as, they varied as much as $\pm$ 1 kg from day to day.

Use of the Harpenden calipers was restricted by the inability of most people to cooperate due to physical or mental impairment. Repeated measurements were not possible because of a tendency for the reading to reduce as water is squeezed from the cells.

12.5.2 The Power of the Study

The power of a study is the chance of detecting a difference between samples. This depends on the difference between the sample means and on σ^2/n.

Obviously, these aren't known before a study has been performed. The investigator must therefore decide on a threshold value of $\mu 1 - \mu 2 / \sigma$; such that if two groups of patients (in my case 'feed' and 'control') differed by at least this value then this would represent an important clinical finding.

eg Consider Table 12.3 where the combined intra-and inter assay coefficient of variation for the cholinesterase assay is approximately 8% and consider the mean 8 week cholinesterase activity level in the 'feed' group from Table 12.9. (988 ± 104 (SEM) nmol benzoylcholine chloride hydrolysed/ml plasma/min).

Sample size n

$$n = 2S^2 D^{-2} (E B + 1.96)^2$$

where, for a chosen power of 80% and 5% significance level

S = estimate of population standard deviation

D = difference between the means

EB = 0.84

Thus: estimated S = 300

I would like to detect a D = 200

estimated n in this case would be:

$$n = \frac{2 \times 300^2 \times 1 \quad (0.84 + 1.96)^2}{200^2}$$

$$= 35.28 \simeq 35$$

I would require two sample sizes of 35 individuals in order to achieve 80% power to detect a change of 200 nmol benzoylcholine chloride hydrolysed/ml plasma/min at the 5% level of significance.

In order to achieve these numbers the study would have to extend over a longer period of time, probably in more than one geriatric unit and using more resources. I believe that if suitable elderly people were identified on admission to such units and special attention was paid at the outset to their dietary needs, significant improvements and sustenance in enzyme activities and anthropometric measurements would be seen.

CHAPTER 13

General Discussion and Summary

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General Discussion and Summary

The first section of work deals with the enzyme kinetics governing aspirin (acetylsalicylic acid) hydrolysis in the plasma which is thought to be due to cholinesterase and albumin. It was postulated that the lower plasma aspirin esterase demonstrated in the frail elderly by Williams et al (1989) was due to either a reduced cholinesterase activity or a reduced albumin concentration. I postulated that if the former were true ,it could be due to one or both of the following reasons: the cholinesterase enzyme may be present in a reduced quantity in the frail elderly , or the active sites of the enzyme may be altered in some way so as to reduce the affinity of the enzyme for substrate molecules.

Kinetic analysis using whole plasma as the source of enzyme suggested that the first hypothesis appeared to be the case . However , the Km value obtained was probably a composite of that for cholinesterase and albumin which was not evident as a bi- exponential Eadie Hofstee plot.

Thus in Chapter 10 , purification of whole plasma in DEAE Sephacel ion exchange gel was used to remove the albumin component so that a true Km value for the cholinesterase contribution towards aspirin esterase could be obtained. These values were very similar to those obtained using plasma, and there was no significant difference between the young; fit and frail elderly. In addition the Km value for commercial cholinesterase (as a standard) was very similar and comparable with that obtained by Valentino et al (1981).

Plasma aspirin esterase, cholinesterase and phenylacetate esterase have already been demonstrated to exhibit a reduced activity in the frail elderly (Williams et al, 1989).

In Chapter 11 I confirmed these findings and extended the hypothesis to paraoxonase and the red blood cell intracellular esterase and acetylcholinesterase. Other workers investigating the effect of age on paraoxonase activity had produced conflicting results (Zech and Zurcher , 1974; Playfer et al ,1977) but neither had considered the effect of frailty and age. I found that paraoxonase activity was significantly reduced in the frail elderly. This may be expected by virtue of the fact that phenylacetate esterase was also significantly reduced and the two are thought to be the same enzyme by some workers (Eckerson et al, 1983b).

The red blood cell esterases were not significantly reduced *per se* but the elderly (and especially the frail elderly) showed an increased tendency towards anaemias and thus also have lowered red blood cell esterase activities.

It is interesting, in the light of work using rodents (Kutty et al, 1981; Osada et al,1989) to consider the influence of diet on esterase acitvity in humans. Waterlow (1950) showed that the plasma cholinesterase activity in malnourished infants could be improved by increasing their nutritional intake. In Chapter 12 I postulated that the same may be true in the frail elderly who are often demented and show early signs of malnourishment which may become more acute during illness (Chandra, 1990).

I offered supplemental feeding to randomly selected group of frail elderly

individuals who had demonstrated a trend towards low blood esterase activity and poor nutritional status. Because of the small groups (due to a high proportion of individuals who failed to complete the study) it was not possible to make firm conclusions. A small increase in weight and a tendency towards improvement in some esterases was demonstrated. This led me to conclude that a larger study in which such frail elderly patients are identified on admission to hospital, and dietary intervention is given a high priority would be worth considering.

Unfortunately, due the nature of the individuals I dealt with in this thesis ie frail elderly, studies with large numbers were difficult to achieve. This was particularly the case with the last study which extended over several months from the moment of recruitment to final completion. Any future follow-up work would have to be extended over a longer period of time in order to achieve significant results.

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Appendix

Appendix I

Materials

All the general reagents used in the following experiments were of analytical grade or better. Those used for gel electrophoresis were electrophoretically pure and or filtered through Whatman no. 4 filters.

Specific Specialist Reagents

Electran molecular weight markers	B D H
Chemicals,Poole,	Dorset.
D E A E - Sephacel Ion Exchange Gel	Pharmacia FineChemicals Uppsala, Sweden.
Human Albumin 45%	Blood Products Lab., Therapeutics Division Elstree, Herts.
Human Cholinesterase Enzyme	Sigma Chemical Co. St. Louis, MO, USA
Phenyl Valerate (purity confirmed by n.m.r.) in double distilled Di- methylformamide	A gift from Mrs EMutch synthesized by the method of Johnson (1977)
Aspirin (acetylsalicylic acid) MW=138.1	Sigma Chemical Co. St. Louis, MO, USA

Dietary Supplements

Build up	Nestle Health Care St. George's House Croydon Surrey
Maxijul	SHS Ltd 38, Queensland St. Liverpool
Provide	Fresenius Ltd 6/8 Christleton Court Stuart Road Cheshire
Ensure	Abbott laboratories Ltd Abbott House Moorbridge road Maidenhead Berks

Equipment

Phillips Pye Unicam pu 8800 uv/vis spectrophotometer used for end point assays and those incubations at 37oC.

Kontron Uvikon 930 spectrophotometer used for incubations at 30oC.

Pharmacia XK 26 glass column.

Gilson fraction collector 202

Biorad power supply 500/200

Gel electrophoresis slab diffusion destainer Biorad 222

Biorad vertical electrophoresis unit and casting kit.

Appendix II

Enzyme Kinetics

The distinguishing feature of enzyme-catalysed reactions from a chemical reaction is that they show saturation. At low substrate concentrations nearly all enzyme catalysed reactions show first-order dependence which ultimately becomes zero-order. Michaelis and Menten (1913) proposed a mechanism for this reaction which is shown as

$$E + A \underset{}{\overset{Ko}{=}} EA \dashrightarrow E + P$$

where A is substrate; E is enzyme; EA is enzyme - substrate complex and P is the product.

Briggs and Haldane (1925) extended this theory by postulating a steady-state situation in which the binding of substrate was assumed to be reversible but not necessarily at equilibrium during the reaction.

This resulted in the fundamental equation of enzyme kinetics: the Michaelis-Menten equation

$$V = \frac{Vmax\ (S)}{Km + (S)} \qquad (1)$$

V = reaction rate; (S) is the substrate concentration; Vmax is the maximum velocity of the reaction at saturating substrate concentrations and Km is the

Michaelis-Menten constant for the reaction.

When V = 1/2 Vmax

$$\frac{Vmax}{2} = \frac{Vmax\ (S)}{Km + (S)} \qquad (2)$$

and

$$\frac{1}{2} = \frac{(S)}{Km + (S)} \qquad (3)$$

therefore

$$Km + (S) = 2\,(S) \qquad (4)$$

$$Km = (S) \qquad (5)$$

At half the maximum velocity Km is equal to the substrate concentration. Km is the value which reflects the affinity of an enzyme for a substrate, where affinity is proportional to the reciprocal of the constant.

Eadie-Hofstee Analysis

This equation is derived from linear transformation of the original Michaelis -Menten formula. Although not entirely free from distortion it is less affected than the double-reciprocal plot (Lineweaver - Burke).

$$V = Vmax - Km \frac{V}{S} \qquad (6)$$

Thus when V is plotted against V/S, a straight line with a negative slope, Km, and y-axis intercept of Vmax is obtained.

The Eadie - Hofstee plot is a useful means of identifying a reaction which may exhibit biphasic enzyme kinetics. Such a reaction has two Km and two Vmax values:

$$V = (Vmax1 - Km1 \frac{V}{S}) + (Vmax2 - Km2 \frac{V}{S}) + \ldots\ldots\ldots \qquad (7)$$

Km1 and Vmax1 are the apparent Km and Vmax for the high affinity component and can be calculated by least squares regression analysis of the slope of the terminal phase and its y-axis intercept respectively. Extrapolation of this line to the y-axis produced an estimate of the contribution of the high affinity component of the reaction to the low affinity component. This value is substracted from each data point on the first phase of the Eadie - Hofstee plot to obtain a second straight line from which the kinetic parameters of the low affinity component (Km2 and Vmax2) can be calculated.

The direct-linear plot

This has been advocated as the least biased estimate of Vmax and Km when there is only one enzyme (Eisenthal and Cornish-Bowden, 1974).

$$\frac{Vmax}{V} - \frac{Km}{(S)} = 1 \qquad (8)$$

Each observation is plotted as a straight line rather than a point. If Vmax and Km are treated as variables and V and (S) as constants, this reaction defines a straight-line with intercepts V on the Vmax axis and - (S) on the Km axis. Thus all possible pairs of values for Km and V which satisfy this observation are shown. A second line drawn in the same way does likewise. The point of intersection of all the observation lines provides the coordinates (Km, Vmax) which satisfies all the observations .

In practice, the lines fail to intersect at exactly one point due to experimental error. Each intersection point is marked on the Km and Vmax axis and the median of these values is taken to be the best estimate of each parameter.

Appendix III

Statistical Tests

Where the average of a group of variables is given, it is expressed as the arithmetic mean $\pm$ the standard error of the mean (SEM). The SEM relates to the precision of the sample mean as an estimate of the population mean, and is especially relevant when one mean is compared with another.

The studies undertaken involve the statistical testing of a formulated hypo thesis of no difference is made. Statistical manipulation allows the estimation how far above or below zero a difference or relationship can be expected to lie due to random sampling error.

A 'significant' difference between the means of two or more groups exists when the probability that the observed difference is due to chance is less than a certain value. For example if two means differ at the 5% level of significance ie $p < 0.05$, this means that 95 times out of 100 a difference of this size will not be due to chance alone.

The term of 'not significant' does not necessarily imply no true difference exists, merely that there is greater than, say, a 5% chance that the observed differences are due to chance.

The tests used in the previous studies were one-way Analysis of Variance (ANOVA) and ANOVA for repeated measures. The former is an equivalent of the unpaired t-test and the latter is an equivalent of the paired t test. Both are used to test the level of significance of the difference between two or more samples if a difference exists, the Scheffe f-test identifies which of the means actually differ.

The significance of a correlation between two values was tested for using the formula below. The quantity 't' is a Students' t with n-2 degrees of freedom and is referred to in the usual tables.

$$t = \frac{r\,(n-2)^{1/2}}{(1-r^2)^{1/2}}$$

r = correlation coefficient

n = number of paired data points.

Stat works 512 on a Mackintosh computer was used for all statistical calculations.